Assessment of the Autonomic Nervous System through the study of cardiovascular autonomic reflexes and their association with inflammation in three clinical settings.

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Copyright Statement

Abstract:

Heart Rate Variability describes the beat-to-beat variation in heart rate arising from activity of the sympathetic and parasympathetic branches of the Autonomic Nervous System (ANS). Reduced ANS tone measured by reduced heart rate variability (HRV) is a powerful predictor of adverse diagnosis in patients and is associated with increased mortality. Published research suggests that inflammation has a deleterious effect on Autonomic Nervous System tone.

This study aimed to: establish if mild inflammatory conditions are associated with changes in autonomic tone as defined by heart rate variability studies in the following conditions:

a. Influenza vaccination
b. Reduction in oesophageal inflammation
c. Reduction in weight

The aim of the first study was to assess the link between inflammation resulting from the influenza vaccination and the associated changes on heart rate variability. 71 healthy volunteers opting to have a routine influenza vaccination were investigated for potential changes in cardiovascular autonomic tone associated with the temporary inflammatory effects of an Influenza vaccination. A number of temporal and frequency domain parameters of heart rate and breathing were assessed 2-5 days prior to vaccination and 1-4 days post vaccination. A sub-group of 15 volunteers who reported significant symptomatic reaction to the vaccination for at least 24 hours following vaccination displayed a statistically significant ($p=<0.02$) reduction in five of the six HRV parameters obtained during metronome-guided breathing. There was no evidence of significant reduction in autonomic tone following vaccination in the full sample of 71 volunteers.

The aim of the second study was to establish whether inflammation resulting from erosive or non-erosive oesophagitis caused by gastro-oesophageal reflux disease had any association with changes in heart rate variability. 12 volunteers with non-erosive oesophageal reflux disease (NERD) and 8 with erosive oesophageal reflux disease

(ERD) were investigated for HRV after initial diagnosis under gastroscopy. HRV assessment was repeated following 8 weeks of treatment with a proton-pump inhibitor (PPI). Initial reflux symptoms and response to PPI treatment were assessed using the GERD Impact Scale questionnaire. All participants had effective symptom response to treatment and there was no significant difference in symptoms score between NERD and ERD groups. There was a small but statistically significant increase in HRV detected following PPI treatment in the ERD group ($p=0.05$).

The aim of the third study was to assess the link between obesity / pro-inflammatory adiposity, weight loss and the associated changes in heart rate variability. 38 clinically obese volunteers (BMI 30-39) with a family history of diabetes were reviewed for HRV prior to and following a lifestyle intervention designed to reduce body weight and BMI. Volunteers underwent repeated HRV studies after 4 months and 8 months of treatment. Volunteers on average achieved a weight loss of 11.5% (±6.0). There were statistically significant changes in HRV parameters in sub-group A (BMI $\geq$36) and correlation of biochemical measures with weight loss. These results further elucidate the effect of mild inflammatory triggers on autonomic tone as measured by HRV. These effects and their significance are discussed in detail in this document. The significance of the 'cholinergic anti-inflammatory pathway' is discussed with respect to the inflammatory conditions investigated. Suggestions for further work are proposed.

In conclusion it is entirely possible to measure subtle changes in heart rate variability associated with mild inflammation and that on the evidence presented here these changes in heart rate variability are hypothesised to be reversible.

My original contribution to knowledge is:

1. Changes in heart rate variability are associated with low grade inflammation resulting from the Influenza vaccination, erosive oesophagitis and increased adiposity.
2. Measurement of subtle changes in autonomic tone, associated with inflammatory challenges is possible and concurs with other published research.

3. The level of HRV attenuation does appear to be linked to those with a higher level of inflammation. In each study the most significant results came from subgroups of volunteers either demonstrating: a higher level of symptom severity, erosive oesophagitis or were in a subgroup of participants with the highest BMI / adipose tissue.
4. In the early stages of reduced heart rate variability we see that concurrent reduction in inflammation is associated with an increase in autonomic tone.

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List of Abbreviations

ANS	Autonomic nervous system
A	Average amplitude of beat to beat changes in heart rate elicited by forced breathing
B-B	Beat to beat
BMI	Body Mass Index
BP	Blood pressure
BPM	Beats per minute
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CORR	Heart rate breathing (chest wall movement) correlation
CRP	C-Reactive protein
ECG	Electrocardiogram
E:I ratio	Expiration to inspiration ratio
ERD	Erosive Reflux Disease
FFT	Fast Fourier Transform
FCORR	Correlation between Fast Fourier Transforms of chest wall movement and heart rate
GLP-1	Glucagon like pepitide-1
GORD	Gastro-oesophageal reflux disease
HF	High frequency
HmwA/L	High molecular weight adiponectin / leptin
HR	Heart Rate
HRV	Heart rate variability

IGT	Impaired Glucose Tolerance
IL6	Interleukin 6
LED	Light Emitting Diode
LF	Low frequency
LPP	Integral of the section of the FFT heart rate curve corresponding to the breathing rate
NERD	Non Erosive Reflux Disease
OGD	Oesophago-gastroduodenoscopy
OGTT	Oral Glucose Tolerance Test
PPG	Photoplethysmography
PPI	Proton Pump Inhibitor
PSD	Power spectral density
PTT	Pulse Transit Time
RDSU	Research, Development and Statistics Unit
R-R Interval	R wave to R wave timing
RSA	Respiratory sinus arrhythmia
SD	Standard deviation of heart rate
SDNN	Standard deviation of N-N interval
SNS	Sympathetic Autonomic nervous System
TA/L Ratio	Total adiponectin to leptin ratio
TLOSR	Transient Lower Oesophageal Sphincter Relaxations
TSP	Total spectral power
VM	Valsalva Manoeuvre
WHO	World Health Organisation

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The support extended to me by my husband Mark, my daughter Lily, my family, friends, and colleagues over the last six years has been invaluable. I thank you all.

Chapter 1: Introduction

1.1 Overview

Autonomic function testing is a routine clinical assessment performed by the Medical Physics Department at Poole Hospital. The assessment involves measurement of frequency and temporal parameters during a series of non-invasive bedside provocations. A full description of the assessment of autonomic function is detailed in Chapter 2.

The autonomic function assessment technique implemented at Poole Hospital has been refined over many years and incorporates a range of different measures designed to test the dual branches of the autonomic nervous system. Although the autonomic nervous system is a hugely complex system governing many important systems within the body, we can infer from the control over cardiovascular heart rate variability, the overall "health" of the autonomic nervous system by using a relatively simple set of heart rate measures.

Vasallo and Allen (1997) published research relating to a small group of patients with acute pneumonia. Their findings showed that autonomic function was reduced temporarily due to the inflammatory effect from pneumonia. This temporary reduction in autonomic tone improved after six weeks and further still at six months in conjunction with an improvement in the patient's overall health state. Previous research has documented the effects of inflammation on autonomic tone; these papers are referenced in the following chapters.

This PhD research incorporates three different projects with one common theme: the effect of inflammation on the autonomic nervous system. The first project addresses the effect of the Influenza vaccination on autonomic tone. Most published research in this area has looked at inflammatory blood markers after vaccination. We decided that although relatively subtle, the influenza vaccination offered the opportunity to look at the effect of low-grade inflammation on the autonomic nervous system prior to, and two days following vaccination. The second area of research addresses the effect of gastro-oesophageal reflux disease and erosive and non-erosive oesophagitis on autonomic tone before and after an eight-week healing dose of proton pump inhibitor

(PPI) therapy. The third area of research involves a cohort of clinically obese non-diabetic volunteers with a strong family history of diabetes undertaking an intensive eight-month weight loss, exercise and lifestyle programme with autonomic function testing, physiological measurement and blood testing.

These three pieces of research related through one common theme of the "subtle effect of inflammation on autonomic tone" would offer the opportunity to refine the current autonomic measurement technique. The following document aims to discuss this research in detail.

1.2 Scientific Background

The nervous system comprises the central nervous system (CNS) incorporating the brain and spinal cord and the peripheral nervous system composed of the efferent and afferent nerve pathways that communicate between the CNS and the body's organ systems (Appenzeller and Oribe 1997). The autonomic nervous system is part of the peripheral nervous system and is responsible for regulating involuntary body functions. The CNS transmits signals to muscles and glands via the efferent division of the autonomic nervous system. The efferent branch of the autonomic nervous system is responsible for controlling many important functions within the human body, including innervation of smooth muscle, cardiac muscle and exocrine and endocrine glands (Sherwood 2010).

Functionally the ANS operates without conscious control; it is governed by the hypothalamus and medulla oblongata (Furness 2006). Dysfunction of the ANS is associated with an increase in morbidity and mortality (Vinik 2012 and O'Brien et al 1991). Autonomic dysfunction and its clinical manifestations are associated with a range of peripheral and central nervous system disorders; these have been classified in the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology Consensus Statement of 1996. A general attenuation in autonomic control is associated with an increase in age, but is especially prevalent in those with long term diabetes mellitus and cardiovascular disease (Kempler et al 2002).

The Hoorn Study has also shown that reduced autonomic function is associated with a higher rate of mortality, particularly when associated with other conditions such as diabetes (Gerritsen et al 2001).

Autonomic dysfunction is difficult to measure objectively due to the dual innervation of the two branches of the ANS. Despite this, routine non-invasive assessment of autonomic function is taking place in the clinical setting; many of the methods implemented are based on the standard Ewing tests developed in the 1970's (Ravits 1997). Many tests now exist to determine the degree of loss of autonomic function; these include the tilt table test and the sudomotor tests for sweating. The most commonly used and most reliable measures of autonomic function assess cardiovascular autonomic control, looking at heart rate variability (Stein et al l994). HRV is derived from tests such as the forced breathing and the Valsalva manoeuvre. These tests will be discussed in more detail in the following chapters.

Heart rate variability can be assessed using an electrocardiogram (ECG), which records cardiac-induced skin potentials at the body's surface (Laizzo 2009). Five easily recognisable features of the ECG trace are known by the letters P-QRS-T, the Q, R and S waves known as the QRS complex; indicate ventricular depolarisation (Laizzo 2009). The timing of the QRS complex over many heartbeats is easily measured by identifying the peak of the R wave, which is the most distinctive feature of the complex. Fluctuations in the inter-beat intervals can be used to assess autonomic control over cardiovascular function, which can manifest in changes in the variability of the R-R interval sequence compared with normal controls (Ravits 1997).

Haemodynamic measures such as heart rate and blood pressure vary on a beat to beat basis (Akselrod et al 1981). The moment to moment changes in heart rate vary in response to many factors apart from those governed by the autonomic nervous system. These include exercise, emotional state and activities such as eating, drinking and nicotine (Low 1993).

Heart rate variability is reduced in patients with stable coronary heart disease (CHD) (Gerritsen et al 2001), and it is suggested that this reduction in HRV is present before a patient becomes symptomatic (Liao et al 2002). Hayano et al (1990) found evidence to suggest that there is a correlation between reduction in HRV and severity of CHD determined by angiography. Although HRV is decreased in CHD the exact mechanisms are not known (Gerritsen 2001). Increased risk of death in cardiac patients is thought to be associated with the increased risk of life threatening arrhythmia (Bigger et al 1992). Huikuri et al (1999) found that impaired autonomic function is also associated with non-fatal cardiovascular events in the general population therefore other mechanisms must be involved.

Using spectral analysis of autonomic function a decrease in HRV has been observed in patients following acute myocardial infarction (Bigger et al 1991). This reduction in HRV appears to be a transient occurrence. There is evidence that HRV partially recovers following the event, but remains at a lower level than healthy controls (Bigger et al 1991). Decreased HRV is also associated with congestive heart failure (Brouwer et al 1996), hypertension (Huikuri et al 1996), neurological conditions such as Parkinson's disease (Korpelainen et al 1996) chronic renal disease (Friedman 1995) and following pneumonia (Vassallo and Allen 1997).

For over a century it has been widely recognised that vagal modulation of the sinus node activity occurs at respiratory frequency (Sin et al 2012), meaning that changes in heart rate variability are linked to respiratory pattern. In light of this finding, assessment of cardiovascular autonomic function through the measurement of R-R interval variability with deep breathing has increasingly been used as an index for autonomic control (Malliani and Montano 2002). Power spectral analysis of regulated forced breathing in time with a metronome indicates high frequency (HF) power to be confined mainly to a narrow peak at the respiratory frequency (Bloomfield et al 2001).

This PhD study used both temporal and spectral analysis of forced breathing as a method to assess autonomic dysfunction during two standardised breathing cycles, (6

and 10 breaths per minute), of two minutes duration per cycle, while simultaneously recording chest wall movement. A number of other parameters were also simultaneously recorded; these included heart rate variability measures during maximal handgrip (see section 2.2.12), Valsalva Manoeuvre (see section 2.2.9) and lying to standing (see section 2.2.10), and other measures including Photoplethysmography (see section 2.2.18), beat to beat finger blood pressure (see section 2.2.14), and pupilometry, (see section 2.2.15).

We previously developed a technique for short-term measurement of chest plethysmography using a pressure cuff worn around the chest to infer first approximation of breathing by measuring chest wall movement during metronome-guided breathing and measurement of heart rate for the assessment of cardio-ventilatory coupling (Perring and Jones, 2003). We established that in asymptomatic Type one diabetics, while the amplitude of heart rate variation falls with age in normal volunteers, the correlation between heart rate and chest wall movement is stable with age in the absence of diabetic autonomic neuropathy. We indicated that correlation between heart rate and chest wall movement is a better indicator of diabetic autonomic attenuation than measurement of heart rate alone. We established that a breathing rate of 6 breaths per minute (0.1 Hz) gave optimum amplitude of heart rate variation and optimum correlation between heart rate and chest wall movement.

1.3 Rationale

The inflammatory response is both deleterious and protective within the body often at the same time, causing cell death and tissue damage while protecting against trauma and infection. The inflammatory response is initially governed by the afferent arm of the autonomic nervous system, activating an opposing motor response in the vagus that suppresses cytokine production. Detection of injury and infection activates a cholinergic anti-inflammatory pathway (Tracey 2007). Inflammation is known to significantly affect autonomic function and research suggests it may be more involved in disease processes (such as cancer, atherosclerosis and asthma) than previously

thought (Krishnamoorthy and Honn 2006). A host of research has been published reporting a link between inflammatory disease states (such as rheumatoid arthritis (Pontet et al 2003), diabetes mellitus, and other autoimmune disorders) and autonomic impairment (Toussirot et al 1993). In most clinical conditions characterized by an increase of inflammatory markers (for example in diabetes or acute coronary syndromes), a reduction in heart-rate variability parameters is consistently observed in high risk patients, strengthening the connection between inflammation and autonomic dysfunction (Lombardi 2004).

1.4 Aims of the Research

The main aims of this research are to:

1. Establish if mild inflammatory conditions (elicited by the conditions listed below) are associated with changes in autonomic tone as defined by heart rate variability studies:
    a. Influenza vaccination
    b. Reduction in oesophageal inflammation
    c. Reduction in weight

2. Further refine our age-matched normal ranges of autonomic heart rate variation based on metronome guided breathing and simultaneous chest plethysmography.

3. Establish the reproducibility of measures of heart rate variability.

4. Further illuminate the mechanism of ANS disruption by inflammatory processes.

1.5 Defining terminology used in this document

Many different terms are used throughout the literature to describe a reduction in the performance of the autonomic nervous system. The following terms have been used interchangeably in this document:

- Autonomic dysfunction
- Dysautonomia
- Cardiac autonomic neuropathy
- Decreased / attenuated autonomic tone
- Autonomic dysregulation
- Autonomic failure
- Decreased or attenuated heart rate variability

Chapter 2: Review of Literature

2.1 The Autonomic Nervous System

2.1.1 Anatomy of the Autonomic Nervous System (ANS)

The ANS performs vital regulatory functions, maintaining internal physiological homeostasis independent of volitional activity (Laizzo 2009). The ANS is predominantly reflexive by nature; it controls multiple organ systems including cardio-respiratory, gastrointestinal, sexual function, thermo-regulation, and genito-urinary (Guyton 2006). These systems are for the most part independent of conscious control, but can be affected by heightened emotional stress such as fear.

Unlike skeletal muscles, organs innervated by the ANS would often still function with limited or no nerve supply, for example the heart still beats when removed from the body (Tortora and Derrickson 2011 and Mathias and Bannister 2006). In the case of heart rate control, for example, the ANS does not initiate each heart contraction, but instead is involved in the 'fine-tuning' of heart rate, in order to respond to the demands of the body.

The ANS is anatomically and functionally divided into two distinct divisions (see Figure 1). The sympathetic (thoracolumbar) and parasympathetic (craniosacral) pathways (Shields 1993), which often function in an antagonistic way, are then further divided into the pre and post-ganglionic tracts. The pathways arising from the cranial and sacral area of the spinal cord carry the parasympathetic fibres; these have long cholinergic pre-ganglionic fibres and short cholinergic postganglionic fibres (Laizzo 2009). Those from the thoracic and lumbar region carry the sympathetic fibres (Loewy and Spyer 1990); these have short cholinergic preganglionic fibres and long adrenergic postganglionic fibres.

Parasympathetic and sympathetic preganglionic fibres release the same neurotransmitter, acetylcholine, but the postganglionic fibres release two different neurotransmitters. Parasympathetic postganglionic fibres release acetylcholine, and most sympathetic postganglionic fibres release noradrenaline (Sherwood 2010).

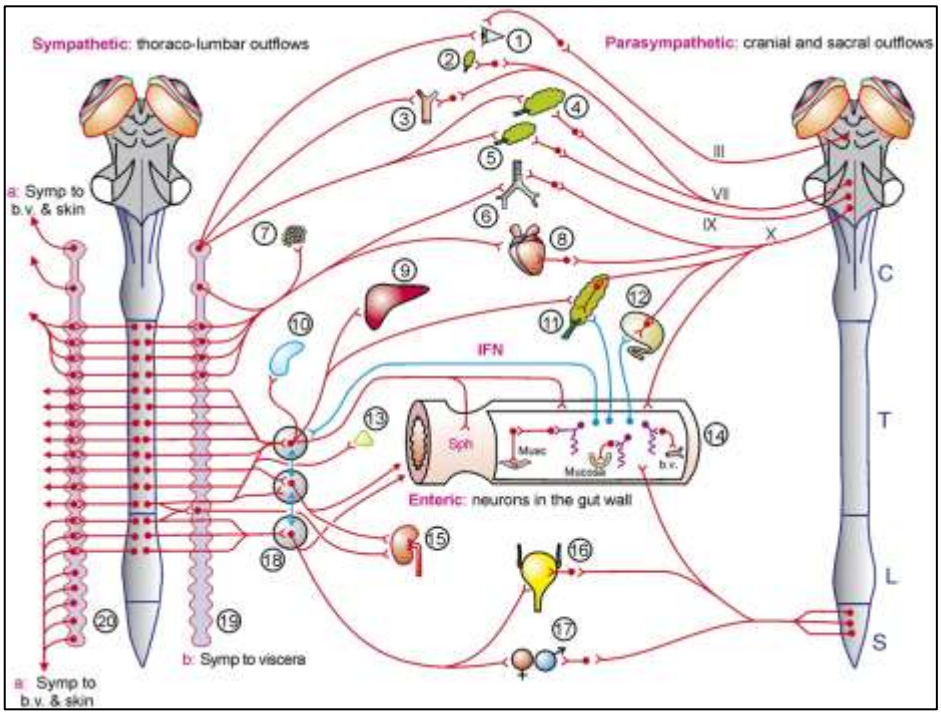


Figure 1. Branches of the autonomic nervous system cited in: Furness J (2006)

The organisation of the autonomic nervous system: Peripheral connections. Autonomic Neuroscience: Basic and Clinical 130, 1-5 Key: 1 Eye, 2 lacrimal glands, 3 intracranial arteries, 4,5 salivary glands, 6 airways, 7 brown fat, 8 heart, 9 liver, 10 spleen, 11 pancreas, 12 gallbladder, 13 adrenal glands, 14 tubular GI tract, 15 kidney, 16 bladder, 17 genital organs, 18 prevertebral ganglia and plexus, 19, 20 sympathetic chains.

The central autonomic system is composed of several interconnected areas in the brainstem and spinal cord. It is responsible for tonic, reflex, and adaptive control of autonomic function (Benarroch 1997). The structures forming the central autonomic network are distributed at the level of the cerebral cortex, hypothalamus, midbrain, pons, and medulla (Loewy 1990).

The hypothalamus is one of the most important areas of the brain, which is connected to both branches of the ANS (Sandroni 1998, Vinik 2012). The anterior and medial portion controls parasympathetic division and the posterior and lateral portions control sympathetic activity. The hypothalamus is the main control and integration centre of the ANS (Tortora 2011).

The peripheral part of the sympathetic autonomic nervous system passes through anatomically complex ganglia leading to various organs of the body including the eyes, lungs, heart, stomach, pancreas, liver, the intestine, kidneys, and sex organs (Furness 2006). The majority of the action of the parasympathetic nervous system in contrast is delivered by the vagus nerve, innervating the lungs, heart, intestine, pancreas, spleen, gallbladder, liver, kidneys, bladder and sex organs (Laizzo 2009).

Physiologically the body responds differently to sympathetic and parasympathetic stimulation. Sympathetic stimulation generally causes excitatory responses such as those experienced in "fight or flight response," increased heart rate (HR), blood pressure (BP) and sweating. The pupils dilate, blood flow to the kidneys and gut constrict and the liver releases glucose (Sherwood 2010). Sympathetic stimulation is more widespread and longer lasting than parasympathetic responses (Tortora 2011). Parasympathetic innervation generally has the opposite effect conserving and restoring energy; most of the responses are related to digestion, absorption of food and elimination of waste (Sherwood 2010). The parasympathetic branch is also responsible for decreasing heart rate, bronchoconstriction and constriction of the pupils. Both systems have associated sensory fibres that send feedback information into the central nervous system regarding the functional condition of the target tissues, and despite common misconceptions that the two systems are entirely antagonistic, they work together to control visceral organs and vasculature (Furness 2006).

2.1.1.1 Efferent and Afferent Pathways

The ANS is predominantly an efferent system transmitting impulses away from the Central Nervous System (CNS) to peripheral organ systems (Freeman 2006). The efferent systems are neural (autonomic nervous system) and hormonal (neuroendocrine) (Mathias and Bannister 2006). Its effects include control of heart rate and force of contraction, constriction and dilatation of blood vessels, contraction and relaxation of smooth muscle in various organs, pupillary size and secretions from exocrine and endocrine glands. *Autonomic nerves constitute all of the efferent fibres, which leave the CNS, except for those, which innervate skeletal muscle* (Freeman 2006).

"The efferent limb of neuronal autonomic reflexes consists of specific preganglionic nerves that synapse in autonomic ganglia, with postganglionic fibres. These postganglionic fibres mediate the desired response at the effector organ. The efferent limbs of these reflexes may also involve the somatic nervous system (e.g. coughing and vomiting). Simple reflexes are completed entirely within the organ concerned, whereas more complex reflexes are controlled by the higher autonomic centres in the CNS, principally the hypothalamus" (Pratt et al 2005).

There are some afferent autonomic fibres which transmit information from the periphery back to the CNS which are concerned with the mediation of visceral sensation and the regulation of vasomotor and respiratory reflexes, for example the baroreceptors and chemoreceptors in the carotid sinus and aortic arch which are important in the control of heart rate, blood pressure and respiratory activity (Freeman 2006). These afferent fibres are usually carried to the CNS by major autonomic nerves such as the vagus, splanchnic or pelvic nerves, with some afferent pain fibres from blood vessels carried by somatic nerves (Pratt et al 2005). Autonomic and endocrine regulation occurs in the hypothalamus, spinal cord, and brain stem. Overlap within the brain occurs not only between areas involved in autonomic and endocrine outputs but also between these and regions controlling the somatomotor system.

2.1.1.2 Enteric Nervous System

A third division of the autonomic nervous system is called the enteric nervous system, which consists of a collection of neurons embedded in the wall of the entire gastrointestinal tract (Furness 2006). The vagal nerve innervates parasympathetic fibres covering the length of the gastrointestinal tract apart from the last half of the large intestine, which is governed by the sacral spinal cord (Tortora 2011). Over the last thirty years there has been an increase in research into this complex area. Two interconnected networks of neurones are located in the two main plexus, the myenteric plexus of Auerbach and the submucosal plexus of Meissner (Tortora 2011). Both plexus contain afferent and efferent neurones, which bring about reflex changes in gut motility via excitatory or inhibitory action on the smooth muscle and also the regulation of secretion and absorption by mucosal cells (Mathias 2006). The extrinsic autonomic nervous system is semi-autonomous and can continue to carry out its motor function without intervention from the central nervous system (Mathias 2006). Extrinsic innervation consists of parasympathetic vagal and sacral nerves S2-S4 and thoracolumbar sympathetic nerves T5-L3. The interactions between the sympathetic, parasympathetic, brain and enteric nervous system integrate activity in the different regions of the gut and coordinate activity between the gut and other organs.

Gastrointestinal disorders may be caused by a myopathic or neuropathic process such as muscular dystrophy (myopathic) or Hirschsprung's or Chaga's disease (neuropathic). Central nervous system disorders associated with gastrointestinal dysmotility include Parkinsonism and Shy-Drager syndrome (Mathias 2006).

2.1.2 Cardiovascular Autonomic Control

Fluctuations in heart rate and blood pressure reflect the dynamic response of the cardiovascular control system to physiological changes. Heart rate is controlled by membrane processes of the sino-atrial node, which is governed by innervations from both divisions of the autonomic nervous system (Crick et al 2000). The main periodic

fluctuations are respiratory sinus arrhythmia, baroreflex, and thermoregulation related variability of heart rate. The most obvious fluctuation is in response to respiration where the heart rate increases during inspiration and slows down during expiration. The magnitude of the response depends on the rate and depth of respiration (Brown and Bolton 2002). An inverse relationship between breathing rate and frequency power has previously been reported (Brown and Bolton 2002). Beat to beat variation in heart rate or heart rate variability (HRV) is an important indicator of physiological resiliency and behavioural flexibility, reflecting the body's ability to adapt effectively to stress. The normal variability in heart rate is due to the synergistic action of the two divisions of the autonomic nervous system either accelerating or decelerating heart rate depending on the predominance of one system over the other. Heart rate demonstrates the net effect of the parasympathetic system (vagal tone will dominate resting heart rate) and the sympathetic system (will increases heart rate) in healthy individuals. At rest both divisions of the ANS are active with the parasympathetic or the vagal division dominant.

The best-known cardiovascular negative feedback mechanism is that of the baroreceptor arc (Tortora 2011). The baroreceptors, which are located in the carotid sinuses and aortic arch, are sensitive to pressure and are referred to as high-pressure sensors. The baroreceptors function by transmitting signals through the vagal portion of the autonomic nervous system to the arterial system to enhance vasodilation and decrease blood pressure in response to an increase in arterial pressure. This occurs reflexively when the baroreceptors are stretched by an increase in blood pressure (Guyton 2006).

2.1.3 General Features of Autonomic Dysfunction

In autonomic dysfunction the particular functions affected differ from patient to patient. Generally patients are elderly, and men are more likely to be affected than women (Bannister 1988). The most dramatic symptom and most common reason for seeking medical advice is postural dizziness or orthostatic hypotension (Khurana 2002) due to loss of predominantly sympathetically mediated reflexes with severity ranging from

light-headedness to syncope on standing. This can be judged from the duration of standing time to the onset of orthostatic symptoms (Khurana 1988 and 2002).

A generalised loss of sweating in the lower extremities may also present with heat intolerance, as control of thermoregulation is lost. This is thought to be due to damage to the sympathetic pathway resulting in sudomotor and vasomotor abnormalities (Clarke et al 1979). Salivation is another important secretomotor function that affects taste perception, oral lubrication, and antimicrobial activity. If a patient has to drink whilst eating to aid deglutition, it would suggest a loss of more than 50% salivary function (Wolff 1995).

Disturbance in micturition is a common early symptom in both genders. Bladder symptoms are a combination of increased urgency, frequency, and nocturia due to uninhibited detrusor activity or incontinence due to sphincter weakness. A loss of sensation for bladder fullness and urine retention is often also experienced (Low 1997). Bowel control is often compromised causing gastrointestinal motility disorders. This is caused by damage to the sympathetic and parasympathetic nervous system (Clarke et al 1979) causing patients to experience chronic constipation, nocturnal diarrhoea, or faecal incontinence.

2.1.4 Factors Affecting Autonomic Function

Factors affecting autonomic function are age, gender, level of exercise, high body mass index (Vinik and Erbas 2001) and medications (Sandroni 1998). An almost linear relationship can be seen between increased age and decreased respiratory sinus arrhythmia from age 18-80 years (Fagard 2001 and Schwartz et al 1991). It is known that sympathetic activity increases progressively with aging (Thijssen et al 2006 and Yamada et al 1989). During tests of autonomic function in healthy volunteers Braune et al (1996) and Low et al (1997) found that for both the Valsalva Manoeuvre and Forced Breathing, heart rate variability was reduced with age. Rodrigues (2010) found these

changes however, were not associated with gender, Moodithaya and Avadhany (2012) and Low et al (1997) found that during forced breathing there was no discernible difference for gender but during the Valsalva manoeuvre they found that heart rate responsiveness was reduced in women. Vinik and Erbas (2001) also agree that HRV is reduced in women. Sloan et al (2009) documented the effect of exercise on cardiac autonomic function. De Meersman (1993) compared a group of healthy male runners with an age matched sedentary group of males and Goldsmith et al (1992) compared a group of healthy endurance trained men with a group of age matched untrained men. Both studies found that the men who regularly perform physical exercise had significantly higher heart rate variability. They also concluded that long term aerobic activity promotes heart rate variability. Levy et al (1992) concluded from a similar study the decline seen in parasympathetic activity associated with an increase in age is partially reversed with intensive exercise activity and that strenuous activity improves heart rate variability. Lucini et al (2002) also demonstrated that active training promotes increases in R-R variance and the gain of overall spontaneous baroreflex.

Medication is known to have an effect on studies of heart rate variability. Beta-adrenergic blocking agents increase heart rate variability (Pagani et al 1986) by simultaneously decreasing adrenergic activity and increasing vagal activity (Singer et al 1995). Anti-arrhythmia drugs depress the parasympathetic nervous system and decrease heart rate variability (Anderson 1994).

2.2 Evaluation of Autonomic Function Testing

2.2.1 Assessment of Autonomic Function

A complete assessment of the autonomic nervous system is highly complex due to the number of functions it performs. Therefore there is no single test that can provide global assessment of autonomic function (Sandroni 1998). Testing usually involves a variety of procedures, which are directed towards cardiovascular autonomic function.

This is important since postural hypotension is often a presenting symptom (Ravits 1997).

2.2.2 Calculating Heart Rate Variability Using Electrocardiography

Heart rate is measured in beats per minute. During heart rate variability assessment it is important that the time interval between successive heart beats is accurately measured. Commonly the interval between the ECG R-waves is used. Accurate wave identification can be difficult if there is any artefact present on the ECG trace such as signal interference or poor electrode contact. R-wave identification using a rolling correlation coefficient technique as described by Buttfield and Bolton (2005) uses an archetype signal to identify the QRS pulse on an ECG trace (see figure 2). This technique has been proven to accurately identify each heartbeat, improving the accuracy of the R-R time interval and is reliable even with noise interference or baseline shift of the ECG signal. This technique has been adapted for use with other physiological signals, including the automated detection of oesophageal peristaltic waves (Perring and Jones 2009).

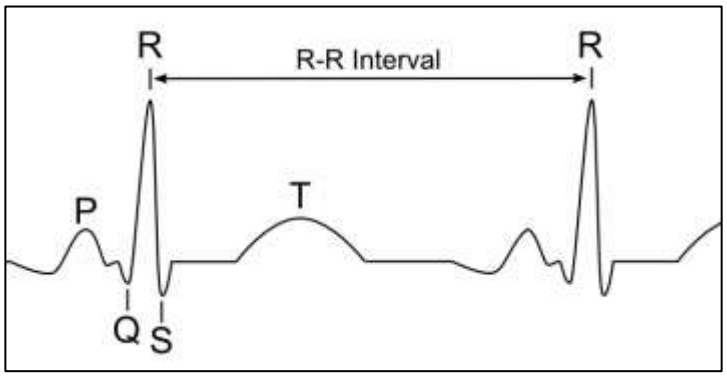


Figure 2 QRS complex, showing R-R interval

The variations in heart rate during autonomic function testing are small, relative to the average heart rate. This means that accurate timing between heart beats is imperative to ensure that the differences in time between successive beats are related to physiological changes and not measurement inaccuracy.

2.2.3 Bedside Testing of Autonomic Function

Autonomic function can be measured non-invasively at the bedside using relatively simple provocations. Ewing et al (1973) began using a battery of tests to aid in the interpretation of symptoms experienced by diabetics (Ravits 1997). These tests have evolved and can now lead to more accurate diagnosis of autonomic neuropathy. Because of the involvement of the sympathetic and parasympathetic nervous system some of the tests involved in the assessment elicit more prominent responses from one branch of the ANS than the other. Increased heart rate and reduced heart rate variability in response to deep breathing are primary indicators of parasympathetic dysfunction (Shields 2009). Tests such as heart rate and blood pressure responses to standing and handgrip are generally accepted as measures of sympathetic activity (Vinik and Erbas 2001). Most of the autonomic tests are continuous and not dichotomous therefore one abnormal result is not indicative of dysfunction. Hence a battery of tests is required for complete assessment (Weissler 2002). In 1996 a consensus report by the Therapeutics and Technology Subcommittee of the American Academy of Neurology (Low 1996) recommended the following tests:

2.2.4 Tests of Cardiovagal Function

- Heart rate response to deep breathing (Forced breathing)
- Valsalva ratio
- Heart rate response to standing

2.2.5 Tests of Adrenergic Function

- Beat to beat blood pressure recording of the Valsalva manoeuvre
- Blood pressure and heart rate response to standing

2.2.6 Tests of Sudomotor Function

- Thermoregulatory sweat test
- Quantitative sudomotor axon reflex test
- Sympathetic skin response

- Sweat imprint method

The two most important measures commonly included in a battery of tests designed to assess autonomic function are the "gold standard" Ewing tests (Ewing et al 1985), the response to forced breathing and Valsalva Manoeuvre. These methods have been cited in many papers (Low 1996, Ravits 1997, Gerritsen et al 2001) including those evaluating cardiac autonomic function in diabetic populations (Ravits 1997 and Braune et al 1996). Vinik and Erbas (2001) state that forced breathing is a more sensitive measure of parasympathetic attenuation and more useful in detecting early autonomic dysfunction and the Valsalva manoeuvre is more useful for sequential long term evaluation of severe autonomic dysfunction. Vinik and Erbas (2001) described tests of cardiovascular reflexes such as forced breathing assessment as simple, sensitive, non-invasive and reproducible method of assessing cardio-vagal tone.

2.2.7 Heart Rate Response to Forced Breathing

Wheeler and Watkins introduced the heart rate response to deep breathing (forced breathing) test in 1973 (Shields 2009), since then it has become a well-established method for assessing cardio-vagal dysfunction particularly in disorders such as diabetic autonomic neuropathy, uremic neuropathy and in multi system atrophy and other neurodegenerative disorders (Shields 2009). Heart rate response to forced breathing is a cyclical shortening and lengthening of the R-R interval (see figure 2) corresponding to the inspiratory and expiratory phases of the respiratory cycle i.e. increasing and decreasing heart rate (Galletly and Larsen 2001 and Sin et al 2012). The timing of the interaction between breathing and heart rate is governed by the respiratory sinus arrhythmia (RSA), which is discussed in 2.2.8 (Eckberg 1983).

2.2.8 Respiratory Sinus Arrhythmia (RSA)

Respiratory sinus arrhythmia is achieved through a complex mechanism of the medulla oblongata in association with the nucleus of the solitary tract (Khurana 1999).

Pulmonary stretch receptors generate afferent nerve impulses that are carried via the vagal nerve and interact with the medulla (Tortora 2011). The combination of cardioventilatory coupling and RSA forms a complex feedback system. During cardioventilatory coupling a heartbeat triggers inspiratory onset (Galletly and Larsen 2001) and in RSA the breathing cycle modulates heart rate (Hirsch 1981). Although coupling primarily influences inspiratory timing, it also affects heart rate variability through determining the onset of vagal modulation by RSA. During a coupled or cardiac initiated breath, inspiration will occur at a fixed interval after an ECG wave. The relationship between the timing of the R wave and onset of inspiration is complex. As breathing influences HRV through modulation of RSA it is therefore expected that cardioventilatory coupling will contribute to the pattern of HRV (Galletly and Larsen 1999).

2.2.9 Valsalva Manoeuvre

The Valsalva Manoeuvre (VM) is performed by blowing through a disposable mouthpiece, which is attached to an aneroid manometer. After inspiration the subject is asked to expire forcefully for approximately 10 seconds. The pressure attained should reach 30-50 mmHg (Hamilton et al 1936 and Vinik and Erbas 2001). Within the tubing there is a fixed leak forcing the subject to maintain an open glottis (Schuster et al 2002). The expiration is terminated quickly and the subject resumes breathing at their normal resting rate. Instantaneous heart rate (R-wave pulse) is again monitored using a three-lead electrocardiograph.

The Valsalva Manoeuvre produces a significant increase in intra-thoracic pressure, which in turn elicits a cardiovascular response. This response has been divided into four phases (Hamilton et al 1936), (see Figure 3).

1) On forced expiration there is an initial acute rise in both systolic and diastolic blood pressure and a variable decrease in heart rate. This is due to increased

intra-thoracic pressure blocking venous inflow and compressing the aorta, forcing the blood to the periphery.

2) During the continued forced expiration, mean blood pressure and pulse pressure decreases. This hypotension is a result of impaired venous return, which stimulates the sympathetic nervous system via baroreceptors. The blood pressure then quickly levels and returns to a steady state. This is followed by a rise in heart rate.
3) On termination of forced expiration intra-thoracic pressure drops, relieving compression from the aorta. An abrupt decline in blood pressure to normal or below normal pressure occurs. This is followed by a small increase in heart rate.
4) A marked increase in blood pressure and pulse pressure occurs which exceeds normal levels; this is due to normal cardiac output meeting a constricted peripheral arteriolar bed. As aortic pressure rises, baroreceptors stimulate a reflex bradycardia to below basal levels (Benarroch et al 1993).

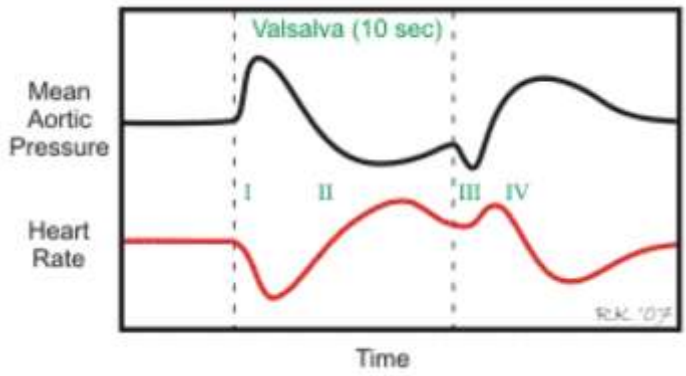


Figure 3 Heart rate during the Valsalva Manoeuvre.

The four phases of cardiovascular response to the Valsalva Manoeuvre Cited in Klabunde RE (2007) Haemodynamics of a Valsalva Maneuver//cvphysiology.com/ haemodynamics/H014.htm

The Valsalva manoeuvre is often employed as a measure of vagal activity. The Valsalva ratio (VR) is calculated by the longest beat-to-beat R-R interval immediately after forced expiration divided by the shortest R-R interval during the forced expiration. The normal value for Valsalva ratio is >1.21 (Bannister 1988).

Analysis of continuous blood pressure and heart rate during the Valsalva Manoeuvre can give valuable information regarding the performance of the baroreflex. During the Valsalva Manoeuvre a rise in heart rate in response to a rise in intra-thoracic pressure when the blood pressure falls suggests that both efferent and afferent pathways are intact (Mathias and Bannister 2006).

2.2.10 Heart Rate Response to a Change in Posture (Lying to Standing)

Normally a small to moderate rise in heart rate can be measured during postural change, not usually exceeding 110 beats per minute. In autonomic dysfunction or autonomic failure a substantial fall in blood pressure without a rapid change in heart rate is indicative of a baroreflex abnormality. The degree of postural hypotension is dependent on a number of factors as described by Mathias (1995) below:

- Speed of positional change
- Time of day
- Prolonged recumbence
- Warm environment
- Raising intra thoracic pressure (coughing, defecation)
- Food and alcohol ingestion
- Physical exertion
- Manoeuvres and positions
- Drugs with vasoactive properties

2.2.11 Measurement of Heart Rate Response to Standing

One of the most common methods of assessing and quantifying heart rate changes in response to postural change is the 15:30 ratio. On standing, the heart rate increases and the time intervals between the beats get smaller. This peaks on the fifteenth beat following standing. This is followed by a slowing of the heart rate and is measured on

the thirtieth beat. The ratio of the longest to shortest interval should be >1.04 (Mathias and Bannister 2006).

2.2.12 Response to Handgrip

Isometric exercise in the form of a sustained maximal handgrip for 10 seconds measured using a dynamometer (Takai grip strength meter) gives comparable results to those of the tilt table test (Khurana 1996). Heart rate and blood pressure changes in response to a sustained handgrip can also be measured over a period of 3-5 minutes at 30% of the maximal grip (Mathias and Bannister 2006).

2.2.13 Analysis of Handgrip Response

Maximum heart rate measured in beats per minute (bpm) during the handgrip – Minimum heart rate (bpm) divided by the average heart rate (bpm) (maximum HR + minimum HR)/2. A ratio of 1.1 or higher indicates normal autonomic responses (Bannister 1988).

During a sustained 3 minute isometric handgrip, muscle contraction causes an increase in diastolic blood pressures and heart rate. A rise of 16 mmHg or more in diastolic blood pressure is considered normal, less than 10mmHg is considered a marker for autonomic dysfunction (Appenzeller and Oribe 1997 and Vinik and Erbas 2001).

2.2.14 Baroreflex

The baroreflex is a complex system involving stretch receptors in the carotid arterial walls (carotid sinus) and the aorta (aortic-arch baroreceptors) (Laizzo 2009). It serves to maintain a constant blood pressure to the brain and other organs in the body. Baroreflex is important in the maintenance of short term blood pressure and moment to moment heart rate variability (Vallais et al 2010). Changes in mean arterial blood

pressure and pulse pressure trigger a reflex response. A rise in arterial blood pressure will initiate the parasympathetic branch of the autonomic nervous system, which will in turn decrease heart rate, cardiac contractility, vascular resistance, and venous return. A drop in pressure will activate the sympathetic branch and will increase all of the above systems (Tortora 2011). Baroreflex provides powerful beat-to-beat negative feedback regulation of arterial blood pressure that minimizes short-term fluctuations in pressure (Sherwood 2010).

Respiration can have a significant effect on the influence of the baroreflex (Tiinanen et al 2008). Inspiration decreases and expiration increases the cardiac vagal responses to baroreflex activation (Eckberg et al 1980 and Fisher et al 2010). A change in the breathing pattern, such as during the fixed two minute forced breathing period used in this study, may itself affect respiratory sinus arrhythmia, independent of the arterial pressure changes accompanying respiration (Calabrese et al 2000).

2.2.15 Pupil Response to Dark (Pupillometry)

The pupil regulates the amount of light transmitted into the retina; the size of the pupil is governed by the muscles of the iris. The smooth muscle of the iris is innervated entirely by the autonomic nervous system (Appenzeller and Oribe1997). The normal pupil is 2.5-5.5mm. It dilates and contracts in response to changes in light and with respiration (Appenzeller and Oribe 1997). Pupil size variability has been used to estimate autonomic function (Ravits 1997). Changes in pupil diameter are controlled by the dilator and sphincter muscles that are influenced by activity in the sympathetic and parasympathetic branches of the nervous system. Increased sympathetic activity increases the activity of the dilator muscle, prompting dilation, whereas inhibition of parasympathetic activity lessens constriction of the sphincter muscle, which also results in dilation. Thus, increases in pupillary diameter can be mediated by activity in either division of the autonomic nervous system (Steinhauer et al 2004).

Sympathetic dysfunction causes contraction of the pupil and attenuation of the startle reflex in the dark (Lowenstein 1950). Parasympathetic dysfunction causes dilation of

the pupil in the dark, attenuation of the light response and pupillotonia (Bremner and Smith 2006). The normal pupil is continually changing in size and shows irregular movements. During the normal waking state the sympathetic and parasympathetic branches are tonically active. They also mediate reflexes depending in part on emotion and ambient lighting. Darkness increases sympathetic tone and produces pupillodilation. Increased light produces increased parasympathetic tone and therefore pupillo-constriction (Reeves and Svenson 2008). The size of the pupil diminishes with advancing age and is significantly affected by disease such as diabetes mellitus.

2.2.16 Heart Rate Variability Analysis

Measurement of beat to beat variation in heart rate and / or blood pressure has become an important method in the assessment of autonomic function (Gerritsen et al 2001). "*Variable data such as heart rate can be described not only as a function of time, but as the sum of elementary oscillatory components, defined by their frequency and amplitude*", i.e. as a function of frequency (Malliani 1999). Heart rate variability analysis is usually based on time or frequency domain.

2.2.17 Frequency Domain Measures of Heart Rate Variability

Various spectral density analyses provide the basic information about how variance distributes as a function of frequency (power spectrum) (Task Force 1996). The methods for the calculation of power spectral density are classified as nonparametric e.g. the autoregressive model approach, or parametric e.g. FFT. Nonparametric methods have the advantage of having simple algorithms and high processing speed. Parametric methods are easier to process and give an accurate estimation of power spectral density on a small sample size, making no assumption about the data.

The two branches of the autonomic nervous system responsible for controlling heart rate are active within different frequency ranges. The power spectrum is commonly divided into four frequency bands, these are: ultra-low frequency (ULF) (<0.003Hz); very low frequency (VLF) (0.003 – 0.04Hz), low frequency (LF) (0.04 – 0.15Hz)

reflecting baroreflex feedback loops on both sympathetic and vagal activity and high frequency (HF) (0.15 – 0.40Hz) where the major contributor is the effect of the respiratory phase on efferent vagal activity (Task Force 1996). The power of the different components along with the total power is expressed in absolute units (s^2). Low and High frequency components can also be expressed as normalised units.

The association between spectral peaks of HRV and the two branches of autonomic activity is complex, and interpretation of changes in HRV parameters is not straightforward. The High Frequency peak has been shown to correlate with vagal activity (Akselrod 1995). The Low Frequency peak has more complex relations with the ANS because it is affected by both the sympathetic and parasympathetic activation (Toledo 2003). Changes of the LF peak cannot be interpreted independently of the HF peak. Therefore, when associating changes in LF and HF peaks to changes in autonomic tone, the effect of the sympathetic and vagal activities on the indexes of HRV must be considered. A change in vagal activity is reflected by a parallel change in both the LF and HF peaks. However, a change in sympathetic activity is reflected mainly by a change in the LF peak (Saul 1991). Although the sympathetic and parasympathetic systems usually act in opposition, the two systems may work in unison. The LF: HF ratio is also commonly used in order to represent the controlled and balanced behaviour of the sympathetic and parasympathetic branches of the autonomic nervous system (Task Force 1996). Stimulation of the ANS resulting in an increase in heart rate can induce either an increase in sympathetic activity or a reduction in vagal activity, or both. Similarly, a decrease in heart rate is mediated by either sympathetic withdrawal or an increase in vagal activity, or both.

Toledo (2003) cites changes in HRV parameters indicating a shift in sympatho-vagal balance toward vagal enhancement follow one of the following patterns: *"1a) LF decreases and HF is unchanged or increased, indicates a reduction in sympathetic activity; vagal activity is unchanged or increased. 1b) LF is unchanged and HF increases, indicating a reduction in sympathetic activity and an increase in vagal activity. 1c) Both LF and HF increase, but their ratio is unchanged or reduced, indicating increased vagal activity and unchanged or reduced sympathetic activity,*

respectively. 1d) Both LF and HF decrease and their ratio decreases, indicating decreased vagal and sympathetic activities, with a shift in balance toward relative vagal enhancement."

The changes in HRV parameters indicating a shift in balance toward sympathetic enhancement are the converse of the above changes and conform to one of the following patterns: *"2a) HF decreases and LF increases or is unchanged, indicating a reduction in vagal activity and increase in sympathetic activity. 2b) LF increases and HF is unchanged, indicating increased sympathetic activity and unchanged vagal activity. 2c) Both LF and HF decrease, and their ratio is unchanged, indicating reduction of vagal activity without considerable change of sympathetic activity. 2d) Both LF and HF increase and their ratio increases, indicating increased vagal and sympathetic activities, with a shift in balance toward relative sympathetic enhancement."*

In this research we examine the association of low-grade inflammation with subtle changes in HRV using a battery of autonomic tests, including metronome guided breathing, where the frequency peak during breathing at six breaths per minute is on the threshold of low and high frequency power.

2.2.18 Time (temporal) Domain Measures of Heart Rate Variability

"Time domain measures of heart rate variability are based on either statistical analyses of the heart rate or the intervals between successive normal complexes" (Task Force 1996). The standard deviation of R-R intervals (SDNN) is the simplest variable for calculating the statistical time domain measure. The SDNN reflects all the cyclic components responsible for variability in the period of recordings. The duration of the recordings used to determine the SDNN should be standardised, as it depends on the length of the time period. The square root of the mean square differences of successive R-R intervals and the number of interval differences of successive R-R intervals greater than 50ms (Ewing et al 1985), are the most commonly used measures derived from R-

R interval differences, and are estimates of high frequency variations of heart rate (Task Force 1996).

2.2.19 Photoplethysmography (PPG)

Photoplethysmography is a low cost optical technique that can be used in the assessment of cardiac intervals through the measurement of blood volume changes (Allen 2007). The technique involves shining a light of infrared or near infrared wavelength into peripheral tissue of the body and detecting light that either passes through or is reflected back from that tissue. Typically a probe is placed on the finger, toe or ear lobe. The light is directed towards the blood flow. Left ventricular systolic contraction leads to an arterial pressure pulse and blood flow through arteries into the periphery. The blood volume in the finger increases during the systolic phase, and decreases in the diastolic phase. The proportion of the light signal that is absorbed or reflected back to the sensor represents these changes in blood volume. This produces a slow changing signal represented by a waveform identifying the pulse wave (Figure 4), which can be used if the ECG signal is distorted.

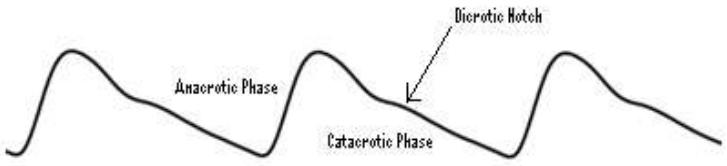


Figure 4 A typical segment of PPG signal. The rising phase is known as the anacrotic phase and the falling phase is known as the catacrotic phase. In healthy vessels a dicrotic notch can be seen.

Figure 5 below displays the same segment of data, with the heart rate calculated using the ECG signal (a) and the PPG signal (b). In this instance, the PPG was much more reliable for calculating beat to beat heart rate. This is due to the noise on the ECG trace at maximum inspiration.

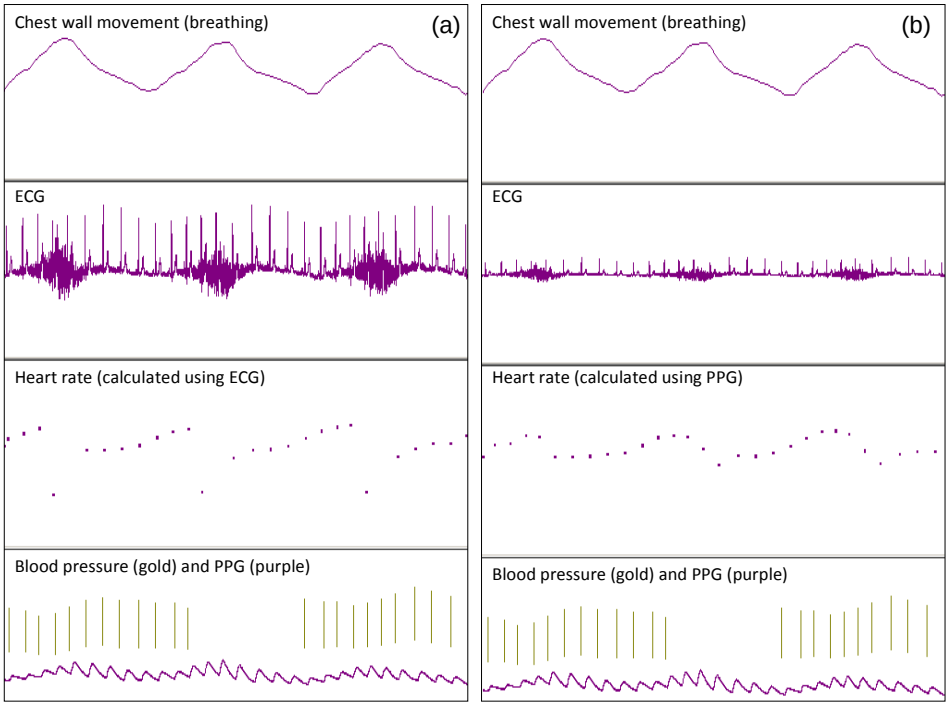


Figure 5 The same segment of heart rate variability data with the heart rate calculated using ECG (a) and PPG (b).

The ECG signal is corrupted with noise at maximum inspiration leading to more consistent identification of heart beats using PPG. The amplitude of the ECG data (a) has been adjusted to display the ECG noise more clearly.

The PPG signal has been used as an alternative to the Electrocardiography (ECG) signal to identify heart beats and for estimating heart rate (Nakajima et al 1996). This has particular clinical relevance in heart rate variability studies for testing the autonomic nervous system, during which subtle beat to beat variations in heart rate are measured. Autonomic function testing is generally performed using the ECG signal to measure heart rate but there are situations where the ECG signal can be corrupted. Sources of noise on the ECG recording can be electromyography (EMG) signals of

nearby muscle groups, baseline drift related to respiration, mains power line interference and poor electrode contact (Lu et al. 2009). In cases where the ECG signal is poor, using PPG to measure heart rate variability can be a useful alternative. Using PPG to measure heart rate variability has been previously investigated during an extended resting period, with good correlation found between the techniques (Lu et al. 2009).

2.3 Distinguishing Cardiac Autonomic Neuropathy from Autonomic Imbalance

Typically in cardiac autonomic neuropathy it is documented that there is a reduction or loss of parasympathetic autonomic control culminating in abnormal autonomic control over heart rate variability (HRV) in response to deep breathing. This loss or reduction in parasympathetic tone may not be as significant as first thought and the attenuation in autonomic tone may be as a result of early augmentation of sympathetic tone (Vinik 2011). Measurement of the two branches of the autonomic nervous system is possible through spectral analysis (high frequency) of HRV during respiration. HRV derived from ECG will give an overall impression of autonomic tone but will not identify which branch is activated. "Respiratory analysis can be used in addition to identify parasympathetic activity that generates respiratory sinus arrhythmia" (Vinik et al 2011). Figure 6 shows how the respiratory frequency on the RA spectrum is aligned with the respiratory activity on the heart rate spectrum representing vagal activity.

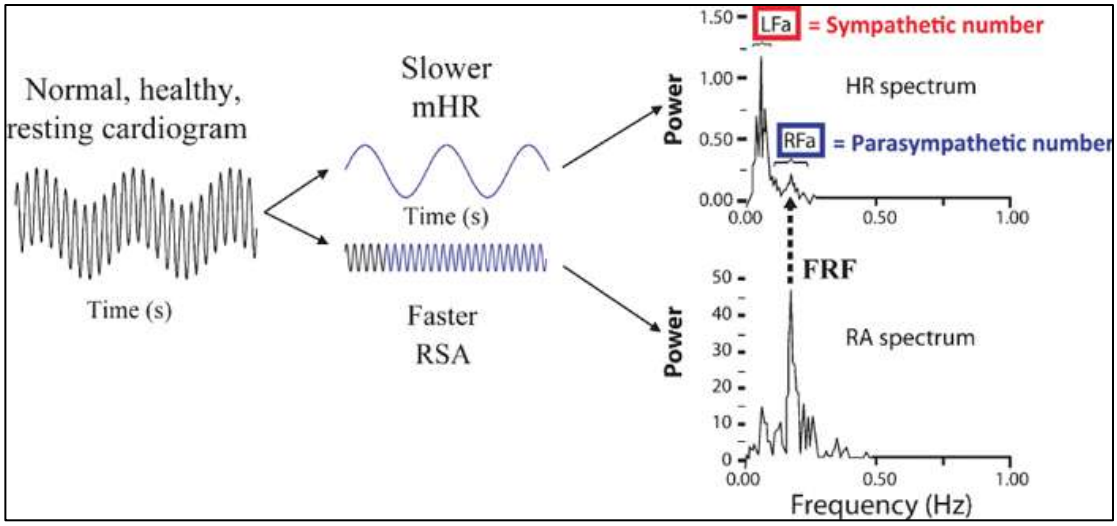


Figure 6 Parasympathetic and sympathetic measurement model.

FRF, fundamental respiratory frequency; HR, heart rate; Lfa, low frequency area; Lfa/Rfa, sympathovagal balance; mHR, mean heart rate; RA, respiratory activity; Rfa, respiration frequency area; RSA, respiratory sinus arrhythmia. (Taken from: Vinik et al 2011).

2.3.1 Neuropathy: Damage to Autonomic Nerve Fibres

Depending on the nerve fibres involved, neuropathy can present in many different ways. Small fibre (2-6µm) dysfunction usually precedes large fibre (12 µm) neuropathy (Mathias and Bannister 2006) and is usually noted first in the lower limb. It also occurs in the autonomic nervous system. Large fibre neuropathy is typically associated with muscle wasting and weakness, and loss of proprioception, deep tendon reflexes and vibratory sense. Damage to A-Delta fibres can result in numbness; C fibre dysfunction can result in burning pain and increased sensation to moderate pain. Vinik (2002) lists three stages of neuropathy:

- Functional neuropathy is reversible and without pathology.
- Structural neuropathy involves loss of structural change and may be reversible.
- Nerve death occurs in critical disease and is irreversible.

2.3.1.1 Diabetes and Nerve Damage.

Diabetic neuropathy is common in the early stages of disease with small nerve fibre involvement. The mechanism underlying the development of neuropathy is not fully understood but it is known that in insulin dependent diabetes vagal denervation becomes increasingly common with lengthening duration of disease. Metabolic changes resulting in hypoxia could be important in the development of neuropathy Stevens (1995). Guy et al (1984) suggested an immunological mechanism underlying neuropathy. Neil et al (1988) suggested symptomatic neuropathy is present in 12% of insulin dependent diabetes. Mathias and Bannister (2006) suggest it is more likely to be far lower (~1%).

2.4 Autonomic Function and Inflammation

2.4.1 The Inflammatory Response and Disease

The physical response to an invasion from pathogens, damaged cells, bacterium or irritants starts by the immune system recognising and responding to antigens on the cell surface which then triggers an immune cascade and depending on the site of the invasion will determine which part of the immune system is activated (Firestein 2011). An invasion will trigger a complicated process causing, macrophages, mastocytes, dendrites, histiocytes and Kuppfer cells to release inflammatory mediators responsible for inflammation and for macrophages to migrate to the site and destroy the pathogen or bacteria (Tortora 2011). The phagocytes will also produce cytokines, which act as chemical messengers activating other areas of the immune system (Sherwood 2010). Inflammation is a protective measure to remove the invading stimuli. The classic signs of acute inflammation are redness, swelling, heat, pain, and loss of function (Sherwood 2010). The primary effect of the inflammatory response is for blood flow to increase to the affected area, promoting a heightened immune response. Once the inflammatory

response has started it continues until the source of the invasion has been removed (Firestein 2011).

The inflammatory response is primarily governed by the efferent arm of the autonomic nervous system, which is initiated by IL-1 binding to glomus cells. Detection of injury and infection activates an anti-inflammatory pathway (Tracey 2007).

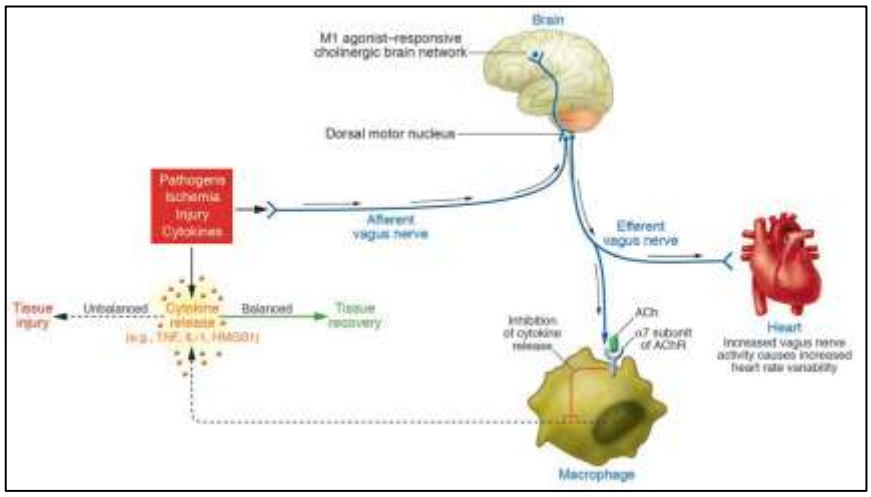


Figure 7 The Cholinergic Anti-inflammatory Pathway and the Vagus Nerve (Tracey 2007)

Wiring of the cholinergic anti-inflammatory pathway, which balances cytokine production. If the cytokine response is unbalanced the same mediators can cause disease. Efferent signals inhibit cytokine production and increase HRV. Afferent signals carried to the brain via the vagus can activate the efferent response (the inhibitory reflex)

Ligands from the immune response activate toll-like receptors and lead to increased expression of TNF-α and interleukin-6. "*Thus the nervous system is capable of initiating a response to tissue injury and inflammation and can per se initiate a pro inflammatory response*" (Vinik 2012). In Vinik's paper (2012) he describes in detail, autonomic control over inflammation. "*The efferent arm of this inflammatory response is termed the cholinergic anti-inflammatory pathway*". Acetylcholine interacts with immune cells that express nicotinic acetylcholine receptor subunit α7, which has an inhibitory role. Acetylcholine activates the Janus kinase and signal transducer and activator of transcription pathway (JAK-STAT), affecting the inflammatory responses

mediated by Nuclear Factor-KappaB (NF-kB) and initiating the release of inflammatory cytokines as a defensive reflex (see Figure 8).

New research by Martelli et al (2014) supports the theory that inflammation is under the control of an inhibitory neural reflex. However, they found in anaesthetised rats, sympathetic nerves mediate this reflex rather than the theory put forward by Tracey that the parasympathetic and sympathetic systems work together to maintain immunological homeostasis. Martelli et al (2014) found that vagotomy caused no increase in response from inflammatory mediators and concluded that the "*cholinergic-anti-inflammatory pathway and the vagus nerve do not constitute the efferent arm of the inflammatory reflex, when stimulated with moderate or high doses of lipopolysaccharide*," although they do not deny that vagal pathways can exert anti-inflammatory actions in acute inflammation

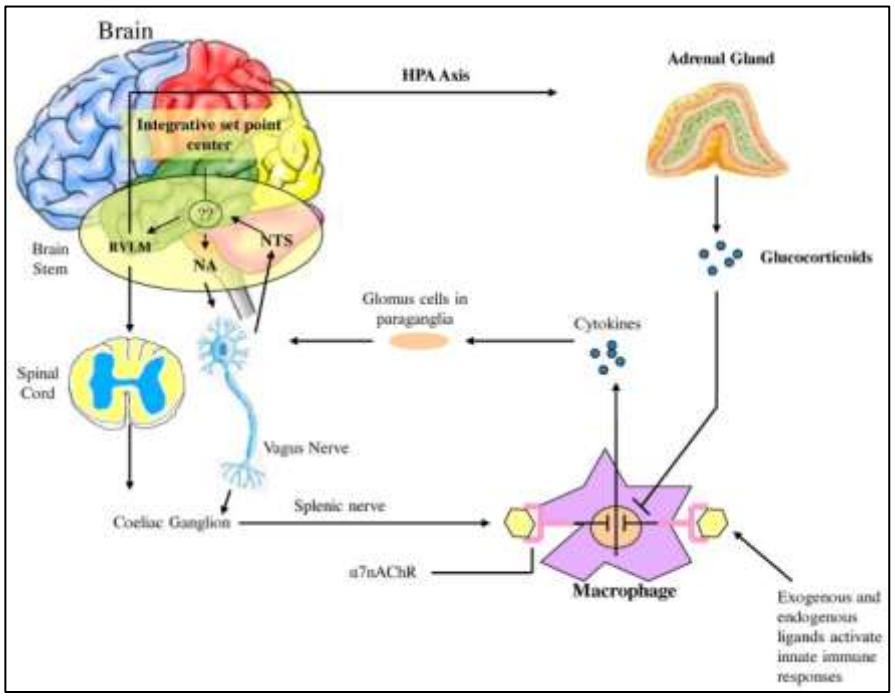


Figure 8 Watkins et al. (1995) discovered that sensory neurons detect the presence of inflammation in tissues cited in Vinik (2012).

An inverse relationship between increased serum markers for inflammation such as tumour necrosis factor-alpha (TNFα), (Vinik 2012) C reactive protein (CRP) and

interleukin-6 (IL6) has been demonstrated in patients with coronary heart disease and a reduction in heart rate variability (Aronson et al 2001). The inflammatory process is complex, and research by Lampert et al (2008) suggests that inflammatory and autonomic processes are almost certainly linked and that the risk factors for coronary heart disease may be pro-inflammatory associated with autonomic dysregulation. Vinik (2012) states, "*autonomic dysfunction and neuropathy have become the most powerful predictors of risk of mortality*." In disease states such as Diabetes Mellitus there has been a recent increase in the focus on inflammation related to the pathogenesis and long term effects on diabetes. Newly diagnosed diabetics (type 2) and pre-diabetics are associated with attenuation in parasympathetic tone and an increase in sympathetic tone (Lieb et al 2011). Haensel et al (2008) found that heart rate variability is inversely correlated with inflammatory markers in healthy individuals as well as in those with cardiovascular disease. Lombardi (2004) also found increased measures of inflammation such as CRP or IL6 have been proven as risk factors for cardiovascular mortality in both healthy subjects and patients with different cardiovascular diseases. "*Growing experimental and clinical evidence indicates that inflammation plays a determinant role in the pathogenesis and progression of atherosclerosis*: a finding that may well explain the strong epidemiological association between inflammation and cardiovascular morbidity and mortality" (Libby et al 2002). Lieb et al (2011) posit that inflammation and autonomic function may be intrinsically linked due to dysautonomia being associated with an increase in inflammatory cytokines.

A host of research has been published reporting a link between inflammatory disease states such as rheumatoid arthritis (Pontet et al 2003), diabetes mellitus, and other autoimmune disorders and autonomic impairment (Toussirot et al 1993). In most clinical conditions characterized by an increase of inflammatory markers (for example, diabetes or acute coronary syndromes), a reduction in heart-rate variability parameters is consistently observed in high risk patients, strengthening the connection between inflammation and autonomic dysfunction (Lombardi 2004).

2.4.2 Mechanism of Inflammation and Autonomic Dysfunction

The mechanisms involved in inflammation and autonomic dysfunction are still not fully understood. Infection and respiratory disease can all have a negative effect on autonomic tone. Vassallo and Allen (1997) discuss a link between infection and respiratory disorders and a reduction in autonomic function. They referenced Heath et al (1982) citing "*possible damage to fibres in the terminal bronchioles and alveoli from the inflammatory process accompanying pneumonia*." Corrales-Medina et al (2013) also discuss the detrimental effect of acute pneumonia on the cardiovascular system.

It is still unclear the exact mechanism between acute inflammation and temporary dysautonomia, however it is likely to be multifactorial. Sajadieh et al (2004) give two possible explanations. The first is that autonomic imbalance and inflammation may potentiate one another. A direct relationship may exist between heart rate variability and inflammation. It is possible that an imbalance of the autonomic nervous system in favour of sympathetic activity could influence and increase the inflammatory response. Inflammation may in turn influence autonomic tone. "Interleukin-6 has been found in the brain and may influence autonomic balance by affecting the hypothalamic-pituitary adrenal axis" (Juttler et al 2002). The second explanation could be that reduced heart-rate variability and inflammation are an additional complication of atherosclerosis. Even though the majority of evidence supports the effect of reduced heart-rate variability on cardiovascular mortality and morbidity, a direct aetiological relationship has not been proven. The mechanisms of activation of inflammation in diabetes mellitus, hypertension, obesity, coronary artery disease, mental depression, and other states are not clear. Sajadieh et al (2004 page 369) suggests, "*Sympathetic over-activity or dominance could be a mechanism. Hypertriglyceridaemia; a metabolic consequence of sympathetic activity, is seen in many of these situations and is inversely related to heart-rate variability. Autonomic imbalance could be secondary to other conditions like congestive heart failure, acute myocardial infarction, and stress, as well as a primary condition*." Recent studies support the notion that autonomic reflexes may control the inflammatory response (Tracey 2009).

Tracey (2009) and Vinik (2012) correlate vagal nerve activity and inflammatory disease states, suggesting a reflex arc may be involved in inflammation. Vinik (2012) implicates the "hypothalamus as the conductor of the endocrine orchestra" and estimates that "the earliest detectable changes in diabetes are those of a change in HRV." It may therefore be appropriate to follow-up or screen patients with long-term chronic inflammatory conditions (such as cardiovascular disease or diabetes) to assess their autonomic status and identify those at increased risk of sudden death associated with dysautonomia. Vinik and Erbas (2001) and Vinik (2012) suggest patients with type 1 diabetes should be tested for autonomic function after diagnosis and 5 yearly thereafter; patients with type 2 diabetes should be tested at diagnosis and yearly thereafter.

2.5 Anti-Inflammatory Response

There is a corresponding anti-inflammatory response to every inflammatory process. Anti-inflammatory mechanisms are also stimulated in the same way as an inflammatory response to host invasion; the anti-inflammatory process exists to attenuate inflammation. One of the primary anti-inflammatory interleukins is interleukin 10 (IL-10) (De Vries 1995), which is produced by T lymphocytes, some B lymphocytes, monocytes and mastocytes resulting in inhibition of t lymphocyte proliferation, prevention of macrophage activation and protection against endotoxins. *IL-10 also reduces the production of inflammatory cytokines by Th1 lymphocytes and promotes Th2-type immune response, which is essential in the fight against inflammation* (Garcia-Moll 2005 page 616). De Vries (1995 page 537) suggests *IL-10 has great potential therapeutic utility in the treatment of diseases, such as chronic inflammation, autoimmune diseases; transplant rejection, graft-versus-host disease and sepsis.*

2.6 Influenza Vaccination

In this project we are looking at the effects on autonomic tone resulting from three types of low-level inflammatory provocations. The first inflammatory provocation investigated for the effects on autonomic tone was the routine influenza vaccination.

2.6.1 The Influenza Virus

Influenza is a highly contagious viral infection resulting in acute respiratory disease caused by a ribose nucleic acid (RNA) virus of the orthomyxoviridae family (Cox and Subbarao 2000). Influenza typically occurs in winter months in temperate regions and is one of the most severe illnesses of the winter season (Centre for Disease Control and Prevention 2006). It affects all age groups and is spread in respiratory droplets from coughing and sneezing.

According to the World Health Organisation (2014) annual influenza epidemics affect between 5-10 per cent of the adult population. Each year there are between 3-5 million cases of severe illness and between 250,000 and 500,000 deaths around the world due to influenza or associated complications. In healthy people the disease is usually self-limiting and usually resolves in 7-10 days. Complications can arise in people with underlying cardiopulmonary and other chronic disease, in children less than 2 years, pregnant women and all elderly people (Whitley and Monto 2006).

There are three strains of influenza viruses, type A, B and C. Both strain A and B are known to cause illnesses of varying severity in humans (Cox and Subbarao 2000). Strain A is likely to be more virulent and is known to have caused all of the influenza pandemics to date. The influenza virus is subject to rapid mutation. A mutation in the influenza viral strain (antigenic drift) occurs approximately every 10 - 50 years (Potter 2001). The virus mutates significantly enough to become resistant to known vaccine combinations. When a marked change in the influenza virus occurs a pandemic inevitably follows.

2.6.2 Influenza Vaccination and Chronic Disease

Vaccination programmes and rapid response to potential threats have significantly limited the spread of influenza.. According to the Health Protection Agency the number of people in the UK vaccinated in 2011/12 increased to 45% compared to the previous year. Vaccination can reduce hospitalisation and deaths among adult diabetics by 70% (Looijmans-Van Den Akker 2006). According to The Centre for Disease Control and Prevention 2006 (CDC), diabetics are three times more likely to die of flu complications than people without diabetes and six times more likely to be hospitalised. It could be argued that reduced autonomic tone in these already compromised patient groups is likely to be further reduced by the inflammatory response to the influenza virus. This will consequently place them at far greater risk from morbidity and mortality (diabetes.org.uk 2012) and is presumably the reason why so many patients are hospitalised as a result. It is therefore vital that annual vaccination is encouraged to all those who fall into the "at-risk" groups.

2.6.3 The Influenza Vaccine

The Occupational Therapy Department at Poole Hospital NHS Foundation Trust administers approximately 1500 influenza vaccines per year within the trust. The aim is to vaccinate all front line staff and any staff with pre-existing medical conditions such as diabetes or heart disease, which would put them at risk if they contracted influenza. The vaccination period in the UK runs from mid-October through to December.

The World Health Organisation (WHO) makes a decision in February of each year on the influenza strains to be included in the following winter's vaccination for the Northern Hemisphere. The prototype viruses (currently 3) originally isolated from humans suffering with influenza are propagated in hens eggs to produce larger amounts of the virus. The liquid containing the virus is extracted and the viral RNA is inactivated and is unable to replicate. The virus is clarified and separated from the membrane and the viral core by ultra-centrifugation. The 3 components are combined

in the correct proportion and the mixture is filled into syringes of 0.5ml. A syringe contains one dose of trivalent vaccine.

Both the vaccine documentation from Solvay Biologicals, who supply the vaccine to the hospital and the information from the World Health Organisation list the composition of the vaccine for the 2008/09 and 2009/10 flu season.

Influvac ® Solvay Biologicals 2008/2009 vaccine
The recommendation from the world health organisation for the composition of influenza virus vaccines for use in the 2008/09 season in the northern hemisphere to include:

- Viral strain A / Brisbane/59/2007 (H1N1) like virus
- Viral strain A / Brisbane/10/2007 (H3N2) like virus
- Viral strain B / Florida/4/2006 like virus

Influvac Solvay Biologicals 2009/2010 vaccine
The recommendation from the world health organisation for the composition of influenza virus vaccines for use in the 2008/09 season in the northern hemisphere to include:

- Viral strain A / Brisbane/59/2007 (H1N1) like virus
- Viral strain A / Brisbane/10/2007 (H3N2) like virus
- Viral strain B / Brisbane/60/2008 like virus

2.6.4 Side Effects from the Influenza Vaccine

According to the medicines information product leaflet and the medicines information department at Solvay Biologicals who produce the Influvac vaccine which was used in this research project, 95% of people vaccinated experience none or very mild side effects. The most frequent side effect of vaccination is soreness at the injection site, which is intramuscular in the upper arm lasting less than two days.

Systemic symptoms can arise after vaccination as part of the body's immune reaction when the body produces interferon. The most frequent symptom would be headache. Occasionally fever, malaise, myalgia and other systemic symptoms can occur after the vaccination. These reactions are usually mild and begin 6-12 hours post vaccination and can persist for 1-2 days.

2.6.5 The Immune Response to the Influenza Vaccine

Vaccination with an influenza vaccine such as Influvac stimulates B lymphocytes to produce antibodies without causing Influenza. When the body is exposed to the antigens in the vaccine the immune response is the same as occurs in an influenza infection. These antibodies are specific to the antigens in the vaccine and protective immunity occurs within two to three weeks post vaccination and lasts up to twelve months. The vaccine is effective in protecting against disease in approximately 70-90% of people vaccinated (Stohr 2003). It is well known that inflammatory markers such as the liver derived plasma proteins increase in concentration in response to an event such as infection (Steele 1994 and Gabay and Kushner 1999). Acute phase reactants C-reactive protein (CRP) and serum amyloid A protein (SAA) are elevated in the common cold and influenza (Whicher 1985 and Miwata et al 1993). Carty et al (2006) found that the influenza vaccination resulted in a small but measureable acute phase response in men with and without carotid artery disease. However, little information is available on the response of these inflammatory markers to influenza vaccination. Research by Tsai et al (2005) and Lieb et al (2001) showed transient changes in inflammatory blood markers specifically C-reactive protein and plasma lipid concentrations at day one and day three after vaccination. These finding suggest that an inflammatory response is occurring at a chemical level, which is most significant for IL-6 and CRP on days one to three. Posthouwer et al (2004) also found a similar response after vaccination with values peaking at two days post vaccination. Tsai et al (2005, page 236) quote in their conclusion that "*Our findings indicate that influenza vaccination, like yellow fever vaccination; can be used as a model to study the*

response of mild stimulation of the inflammatory system." We decided to use the influenza vaccination as a mild but measureable inflammatory provocation in an attempt to establish whether heart rate variability changes as a measure of autonomic tone pre and post vaccination.

Chapter 3: Methods (Influenza Study)

3.1 Rationale for Methods Used in this Study

Within the Medical Physics Department, assessment of patient autonomic function is routinely performed. A battery of tests including forced breathing and the Valsalva manoeuvre is carried out. From clinical experience of autonomic function testing, the provocation that consistently produces the most pronounced and reliable measure of heart rate response is the forced breathing exercise. The Valsalva manoeuvre produces a more dramatic heart rate response when present, but patient capability (particularly in the elderly) often determines how successful it is as a measure of autonomic function (Mathias and Bannister 2006). Many studies have used deep breathing (forced breathing) and / or the Valsalva Manoeuvre as the "gold standard tests" for autonomic function (Ryder and Hardisty 1990) (Low 1996). We decided, based on results from previous studies and clinical experience that 2 minutes of metronomic guided breathing would be the principal provocation used in this study.

Monitoring of chest wall motion during forced breathing as a means of determining the degree of co-operation with the procedure in less compliant patients has been adopted in the routine assessment technique. We have developed a technique for short-term measurement of heart rate (HR) and chest plethysmography with metronome-guided breathing for assessment of cardio-ventilatory coupling (Perring and Jones, 2003). In previous research, we established that a breathing rate of 6 breaths per minute (0.1 Hz) gave optimum amplitude of heart rate variation and optimum correlation between heart rate and chest wall movement (Perring and Jones 2003). We have incorporated the six breaths per minute breathing rate over two minutes into the routine clinical assessment.

3.1.1 Developments to the Current Measurement Technique

Blood pressure (BP) also displays rhythmical and non-rhythmical variations from beat to beat. It appears that HR and BP variations at approximately 0.1Hz may reflect sympathetic vascular and cardiac autonomic modulation and may be used to obtain a measure of Baroreflex Sensitivity (Parati et al, 1995). We now have the capability of non-invasive beat-to-beat measurement of blood pressure in the finger using a Portapres system.

Photoplethysmography (PPG) has been suggested as a potential measure of autonomic function, both as an alternative measure of HR and by assessment of signal amplitude (Nitzan et al, 1998). Pulse transit time (PTT) is the time delay between a proximal pulse trigger (ECG R-Wave) and a peripheral trigger (characteristic shape in the PPG signal). PTT has been suggested as an alternative to measurement of beat-to-beat systolic blood pressure in short-term studies (Chiu et al 1991 and Payne et al 2006). Davies et al (1999) specifically use metronome guided breathing rate of 6 breaths per minute. Other groups have suggested forced breathing at higher rates to avoid a breathing component to the HR and BP variability at 0.1 Hz (Fredericks et al, 2000). We have implemented both 6 breaths and 10 breaths per minute for a two-minute period.

Detailing the relationship between PPG signal, PTT and BP in healthy participants and patients with autonomic neuropathy, which has been documented by established techniques, is difficult, particularly the derivation of timing information in a relatively slow changing signal like the PPG signal. We have applied the technique of rolling correlation coefficient (Buttfield and Bolton, 2005) to accurate timing of a variety of physiological signals including ECG and oesophageal manometry for timing oesophageal peristalsis (Perring and Jones 2009). We have used this technique to establish the effectiveness of PPG measurement for HR variability studies. This is continuing work that will be concluded by another researcher within the department.

3.1.2 Evaluating the Measurement Techniques by Examining an Inflammatory Response to the Influenza Vaccine

Researchers have shown a temporary fall in cardiovascular autonomic responses following pneumonia using simple autonomic reflex testing and posit an inflammatory link to autonomic failure (Vassallo and Allen 1997). Corrales-Medina et al (2012) reported a high incidence of cardiac complications during the course of community acquired pneumonia and have shown these events are independently associated with increased mortality. It is also known that HRV is reduced in patients during ventilator weaning (Shen et al 2003). Pontet et al (2003) established reduced HRV in patients with rheumatoid arthritis, and also in separate research into sepsis. Von Kanel et al (2008) identified a link between decreased HRV and atheroma. Lieb et al (2011) found reduced HRV before the advent of inflammation in diabetes. Lee et al (2006) found changes in autonomic tone in gastro-oesophageal reflux.

This study looks at the short-term effects on autonomic function of an inflammatory provocation. The cohort we examined were normal healthy participants having a routine influenza vaccination. Work has been published on the variation of inflammatory markers following the influenza vaccination, but initial research suggests that no one has assessed autonomic responsiveness variation in a similar fashion (Posthouwera et al 2004). Using the measurement techniques discussed above (PPG, PTT and BP) we assessed the mild and short term effects of the inflammation from the influenza vaccine on autonomic tone.

3.2 Study Design

A descriptive, cross sectional, pre-test / post-test design was used to assess the effect of the Influenza Vaccination on heart rate variability (autonomic function) in 71 healthy volunteer's pre and post vaccination.

This study assessed the degree of loss of autonomic control over heart rate variability in healthy volunteers and whether the novel measurement regime described, proved to

be a more sensitive measure of the degree of attenuation of autonomic control than the methods presently used.

3.3 Recruitment

Seventy one healthy participants, age 18 to 80 years, who elected to have the annual vaccination for influenza, which is routinely offered and administered by the Occupational Health department within Poole Hospital, were recruited. Participants were selected on the basis that they were having the vaccination because of their occupation, rather than for health reasons. Therefore all of the participants were hospital employees or voluntary workers within the hospital. We assessed their autonomic function between one and five days prior to the vaccination and between two and five days after the vaccination.

Initial application to Dorset REC was for 35 volunteers in the study. Recruitment was extremely successful and it was decided to further increase the number of participants. The protocol was subject to a successful application for a substantial amendment to increase numbers to 50 volunteers. Analysis of data suggested in some cases that there were differences in autonomic tone pre and post vaccination. Based on results from a post vaccination questionnaire we subdivided the total cohort into two symptomatic groups and have found that we needed to increase the data to give more power to the statistics. We reapplied to the Ethics committee to further increase the participant numbers to 100. We continued recruitment in October – December 2009 during the Influenza vaccination period to further improve the statistics.

We recruited participants using either a direct approach or by placement of posters around the hospital. We talked with the Poole Occupational Health Department and established that they were happy to help with the recruitment process by displaying one of our posters in the department. When the Occupational Health Department advertised the availability of the Flu vaccine we asked them to display the recruitment poster. Each year the occupational health department place posters around the hospital offering

the flu vaccine and advertises the list of session dates in the hospital magazine. We submitted our recruitment poster to the hospital magazine along with an invitation to participate to coincide with the announcement of the availability of the vaccine. We established that the occupational health department expected to vaccinate approximately one thousand people in a two month period leading up to the "flu season."

There were a large number of people potentially available to take part in our study. Members of staff were encouraged to sign up for the vaccination sessions in advance. We asked the Occupational Health Team to send out an "Invitation to take part in the research" and the participant information sheet. This meant that confidentiality of the people signing up for the vaccination was maintained. They were left to decide whether they wanted to make contact with our department.

We also approached members of staff known to us in other departments around the hospital including the many elderly volunteer staff. They were left with the information sheet about the research project and it was up to them to contact us if they wanted to participate.

3.3.1 Inclusion Criterion:

1) Healthy participants between the ages of 18 – 80 years.
2 All participants filled out a health questionnaire and did not suffer from any of the listed inflammatory conditions.
3) Members of staff / voluntary staff offered the flu vaccine at Poole hospital.
4) Other participants eligible to take part such as the healthy partners of people visiting the hospital.
5) People able to fully consent to taking part.

3.3.2 Exclusion Criteria

A health questionnaire was given to each participant. Anyone who had any of the following pre-existing conditions or who were unable to fully consent were excluded:

- Diabetes Mellitus
- Degenerative disease, such as: Alzheimer's disease
- Heart disease
- Arthritis
- Gastrointestinal disease (see section 7.2.2).
- Respiratory disease
- Allergies, hives or anaphylaxis
- Lupus
- Multiple Sclerosis
- Tuberculosis
- Chronic Cholecystitis
- Chronic Prostatitis
- Glomerulonephritis
- Pelvic Inflammatory Disease
- Vasculitis (phlebitis, arteritis)
- Vascular disease
- Any patients taking the following medications:

1. Alpha Blockers
2. Beta Blockers
3. ACE Inhibitors
4. Calcium Channel Blockers
5. Tri-Cyclic Antidepressants
6. Opiates
7. Anticholinergics

3.4 Instrumentation

3.4.1 Procomp Infiniti Biosignal Recorder.

A ProComp Infiniti (Thought Technology Ltd) was used to collect ECG, PPG and chest plethysmography signals. This is a microprocessor controlled encoder unit with 8 recording channels, which receives signals from the sensors. These signals are then digitised, encoded and transmitted to a TT-USB interface unit. A fibre optic cable is used for transmission of the signals from the ProComp Infiniti unit to the TT-USB interface, which converts the data from optical form to USB format, thereby electrically isolating the subject from the electrical mains supply.

3.4.1.1 ECG

Patients were connected to a 3 lead ECG. The ECG lead was connected to a ProComp Infiniti a multiple biosignal detector. The ECG pulse was sampled at 2048Hz. Heart rate was captured in real time using a thresholding technique using the peak of the QRS complex as the trigger, a schematic of instrumentation is shown in Figure 10. The data is displayed in real time (see Figure 9). Recording software was written in Microsoft Visual Basic V6.0 (Microsoft Corp, Redmond, Washington).

3.4.1.2 PPG

A ProComp Infiniti PPG sensor was positioned and secured against the palmar surface of the left index finger. PPG was sampled at intervals of 2048Hz. Blood volume pulse or PPG is a relative measure, it did not have a standard unit.

3.4.1.3 Chest Plethysmography

Chest wall movement was detected using a ProComp Infiniti respiration-flex/pro sensor. The sensor consists mainly of a long strap that was stretched around the patient's chest. The sensor was placed during a full expiration and quickly fastened with just a small amount of tension. The sensor should not be loose when breathing out completely. The sensor was placed on top of clothing, if not too bulky. The sensor is sensitive to stretch during expansion and contraction of the ribcage during breathing; it is however, not a strain gauge.

The respiration signal is a relative measure of chest expansion. The ProComp Infiniti does not generate standard units of measure for respiration. From the raw signal waveform, the Infiniti software is able to calculate the respiration rate and relative breath amplitude. Chest movement voltages were sampled at intervals of 256Hz and the data recorded to disk.

The breathing channel was displayed on screen in real time as a graphical display with an overwrite period of 20 seconds (see Figure 9). The breathing cycles were monitored to ensure that the amplitude of the breaths were approximately the same for each cycle. The rate and depth of breathing were carefully monitored since the maximum change in heart rate has been demonstrated at frequencies between 5.5 and 7.0 cycles (breaths) per minute and at a maximum voluntary depth (Freedman 1993).

Expiration to inspiration ratio (E: I ratio) was determined by dividing the mean value for longest R-R interval during each expiration, by the mean value for shortest R-R interval during each inspiration. Normal limits proposed are equal to or greater than a ratio of 1.1 (Bannister 1988). This method is dependent on heart rate variability in beats per minute and can be used regardless of resting heart rate.

3.4.2 Portapres Beat to Beat Blood Pressure Measurement.

The Portapres® is the ambulatory Finapres technology solution from FMS, (Finapres Medical Systems). The Portapres model-2® (TNO-TPD Biomedical Instrumentation 2001) offers the ability to perform continuous non-invasive ambulatory finger blood pressure monitoring via equipment incorporating an air pressure control valve, a pressure transducer, an infrared cuff, and a photo-diode amplifier and is carried at the wrist. The data can be directly transferred from the flash card via a serial port to a PC, onto which the beat-to-beat analyses can be run (Figure 11).

Non-invasive blood pressure in the finger is measured using the clamp volume method, which was originally described by Penaz (1973). A finger cuff is placed around a finger, which incorporates an inflatable bladder, an infrared light emitting diode (LED) and an infrared photodiode. The desired arterial diameter is the unloaded diameter, the point where the pressure inside the artery equals the pressure in the finger cuff (zero transmural pressure). This can be calculated by applying periods of constant cuff pressure and observing the PPG signals during this time. The arterial diameter is measured using PPG, the pressure required to keep the diameter constant is then recorded. This pressure mirrors the intravascular pressure.

During measurement periods, the unloaded diameter is unlikely to remain constant. The Portapres uses an algorithm called Physiocal (Wesseling et al 1995) to assess the amplitude and shape of the PPG signal during periods of constant cuff pressure to ensure that the correct unloaded diameter is being used. A calibration is performed at frequent intervals (after every 10^{th} heartbeat) at the start of a measurement period and as the measurements stabilise, this interval increases.

To correct for any hydrostatic differential from finger placement and therefore attempt to approximate the systemic blood pressure, a height correction unit is used. This consists of a liquid filled tube with one end, placed at heart level, containing a very

compliant plastic bag and the other end connected to a pressure transducer, placed on the finger (TNO TPD Biomedical Instrumentation).

The systolic and diastolic blood pressure data recorded using the Portapres was transmitted to the laptop via a RS232 serial comport.

An automated blood pressure monitor (Microlife BP 3AC1) was used at the end of each procedure to measure systemic blood pressure.

Figure 9 shows a typical recording in real time displaying the chest plethysmography signal, raw ECG signal, beat to beat heart rate calculated using a thresholding technique identifying the peak of the QRS complex, beat to beat finger blood pressure and Photoplethysmography signal. After recalculation of the heart rate using the technique described in section 3.9 the data are processed and compared with age matched normal values.

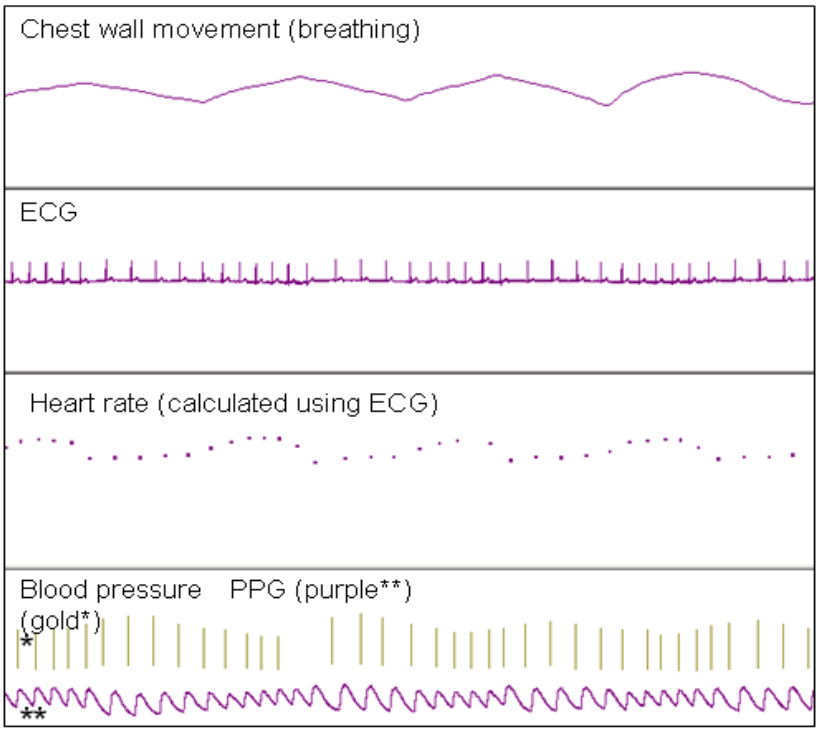


Figure 9 Typical autonomic recording showing chest plethysmography signal, ECG, beat to beat heart rate, finger blood pressure, and PPG blood volume.

3.4.3 Schematic for Instrumentation

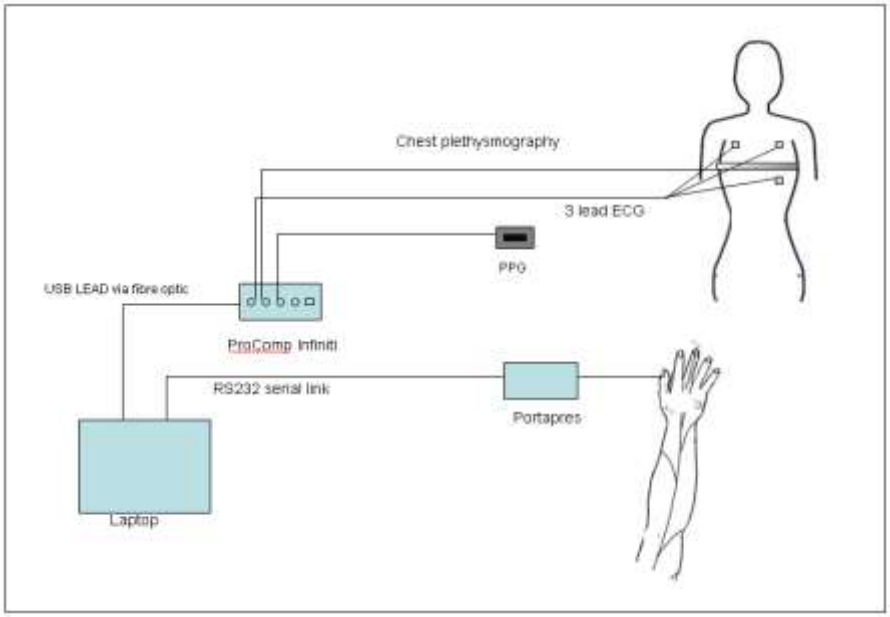


Figure 10 Schematic for Instrumentation showing connections between the patient and the equipment

3.4.4 Parameters Measured

The parameters measured included ECG, PPG, beat-to-beat BP, chest plethysmography and pupil reaction to light and dark. ECG, PPG, BP and chest plethysmography were measured by simple non-invasive sensors placed on the chest and on the fingers. The recording devices were commercial units, which are CE marked and connected to the recording PC using opto-isolated connections. Pupil reaction to light and dark was assessed using a pair of goggles incorporating two infrared cameras.

Time taken for each visit was 20 minutes. All measurements were completely non-invasive.

Autonomic function testing was performed on healthy participants within one to five days prior to and between two and five days following the influenza vaccination. The optimum timing was two days post vaccination. This is mainly due to the evidence emerging from a number of studies which suggest the optimal time for assessment of mild inflammation when looking at C-reactive protein responses is most significant at

two days post vaccination (Posthouwera 2004). Other research suggests testing for CRP inflammatory markers at one and three days post vaccination (Tsai 2005). The volunteer recorded the degree of adverse reaction to vaccination on a "Vaccine Evaluation Centre Questionnaire" (Scheifele 1990), see appendix 16.2.

3.4.5 Preparations required for the investigation:

- No food for three hours prior to testing.
- No caffeine or nicotine for at least three hours prior to testing.
- The test was performed (where possible) at the same time of day, thus avoiding diurnal variation.
- A health questionnaire was issued to exclude anyone with any of the listed diseases.

Informed consent was obtained. Volunteers were given the opportunity to ask questions.

The volunteer's date of birth and the date of the initial visit and the date of the vaccination were written on the consent form. Each volunteer was issued with a sequential "V" number, which made him or her anonymous on the computer. The only piece of personal data linking them to the "V" number was their date of birth.

A record of the gender of each volunteer was assigned to the "V" number for the purposes of sorting the data for statistical analysis.

The clinical room used during the study was maintained using climate control at an ambient temperature of between 22 and 23 degrees Celsius as suggested by Low and Pfeiffer (1997). Lighting was set at a normal level; variable control over this was not possible. Adequate time was given to those volunteers with cold hands and a poor PPG and BP signal due to vasoconstriction of the peripheral vasculature.

The room was quiet and subjects were familiarised with the equipment and the environment (Bloomfield 2001). Subjects were asked to read through the health questionnaire and declare that they were not affected by any of the diseases listed. They were given the opportunity to ask any questions about the test and then asked to sign a consent form. If participants answered yes to any of the questions on the questionnaire they did not have to disclose any health information and could declare themselves exempt from the research project while maintaining their privacy.

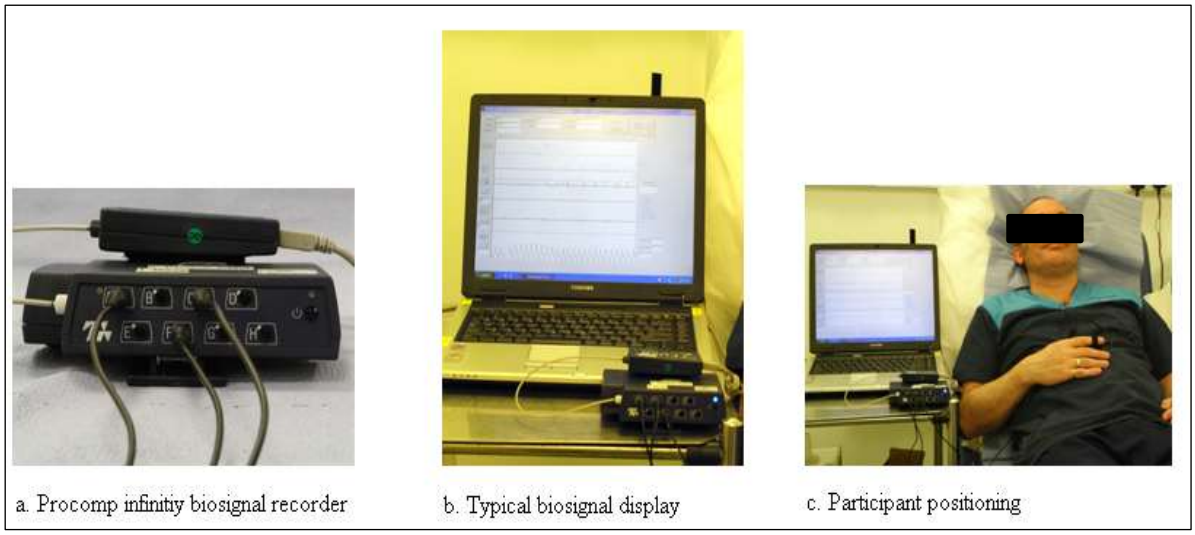


Figure 11. Equipment used in this research

3.5 Autonomic Assessment

Volunteers were positioned on the examination couch in a supine position and connected to the equipment (see Figure 11c).

- A rest period of at least 2 minutes was given to eliminate the effects of anxiety or anticipation (white coat syndrome) (Owens et al 1999).

- 2 cycles of slow deep breathing were performed to establish the correct breathing pattern and technique at 6 breaths per minute.

- The Valsalva Manoeuvre was performed making sure an airway pressure of approximately 30mmHg was obtained. This was held for 8 -10 seconds.

- A maximal hand grip was performed using a grip strength meter. The hand grip was sustained for 10 seconds. Grip strength (kg) was recorded

- An on-screen digital timer was used to pace the breathing cycle. The subject was required to inhale for a count of 5 seconds and exhale for a count of 5 seconds. The breathing rate thus obtained corresponded to 6 breaths per minute. This was performed for two minutes.

- A breathing pattern corresponding to 10 breaths per minute for two minutes was also paced using a digital metronome. The subject was required to inhale for a count of 3 seconds and exhale for a count of 3 seconds. This breathing rate is more similar to normal spontaneous breathing and generally easier to maintain.

The breathing exercises were always performed in the same order for consistency.

An automated brachial blood pressure was taken using a digital blood pressure device. The left arm was always used for this unless there was a clinical reason for not performing it for example in the case of mastectomy. In this instance the opposite arm was used.

At the end of this period the data collection was complete. Subjects were asked to remain still for a short period of time to allow them to overcome any ill effects of the breathing exercises.

The final measurement was dynamic pupillometry. The volunteer was asked to wear Medical Physics designed and built infrared goggles. Images were taken of the right eye while the volunteer was sat in a completely darkened room. After 30 seconds in the dark a light was flashed into the left eye and the response of the Iris in the right eye was

recorded, after another 30 seconds in the dark another flash occurred in the same eye. The response was again recorded.

3.6 Statistical Analysis

Data were analysed using Kwikstat Winks SAS 4.62 statistical analysis software (Texasoft 1991-2001).Parametric and non-parametric statistical analysis was performed as appropriate. The parametric statistical analysis are known as parametric because they require estimation of the parameters that define the underlying distribution of the data which for a paired t-test is the standard deviation and the mean (Whitley and Ball 2002). A paired t-test is an example of a repeated measures design used for testing differences by comparing the means for two variables for a single group, where each individual in a group is their own control giving a smaller error term and larger t-value. Repeated measure t tests often benefit from needing fewer subjects to give statistical power however they give n-1 degrees of freedom, meaning a higher t-value is needed for significance to be reached.

The non-parametric tests make no assumption about the data. Non parametric statistical comparison used in the study was the Wilcoxon signed rank test, which was used for comparison of two independent groups. Non parametric tests are often used to analyse ordered categorical data. Analysis may lack statistical power compared with parametric analysis and are generally geared towards testing a hypothesis rather than estimation of effect.

For correlation analysis of data a Pearson's product moment correlation coefficient was used for parametric data and a Spearman's rank correlation coefficient was used for non-parametric data. Correlation is a measure of the statistical strength of linear association between two variables. However, one disadvantage with correlation analysis is that it gives no indication of causality.

Analysis of variance (ANOVA) was used to analyse the difference in mean values over successive visits. ANOVA can test hypotheses that a t-test cannot by examining what the variation is within groups then examines how the variation translates into variation between groups taking into account the number of degrees of freedom (Moore and

McCabe 2003). ANOVA assumes the data is normally distributed and that the standard deviation in each data set is similar. A limitation of using ANOVA is that it will only determine that there is a difference between at least one group from at least one other. It will not identify which group or how many groups differ statistically. In this instance multivariate analysis of variance (MANOVA) would be appropriate to measure multiple dependant variables simultaneously which will reduce the incidence of statistical errors from repeated testing where the ANOVA allows for only one variable.

Fishers Exact was used to determine whether there was a significant difference between the two vaccination periods with respect to the gender of participants by measuring the association between the variables. Fishers exact can be used for small sample sizes in place of a chi-squared test.

Study results are displayed in tables with mean, standard deviation, T values and P values. Correlational data is represented as scatter plots with line of best fit, tabular results of correlation data give p and r values, for the significant results the slope is also given. Statistically significant results are displayed as either histograms including standard error bars or box and whiskers plots which show a summary of a large amount of data by displaying minimum, first quartile, median, third quartile and maximum values. The exact values and distribution of results are not retained in a boxplot; however it is a visually effective way of presenting data allowing a graphical display of distribution of results and provides an indication of the degree of asymmetry in the distribution of data.

3.7 Ethical Considerations

Approval for the study protocol was granted by the East Dorset Local Research and Ethics Committee and Poole Hospital Trust (see appendix 16.4.1)

3.8 Data Security

Unique study identification was established at the time of taking consent. All pro-forma data and study information stored on computer were identified only by this number. No

further patient-specific information was held in connection with this project. Consent forms were kept securely in a locked filing cabinet. Pro-forma data and automated analysis data were held on the secure hospital shared network. All recorded data were held in accordance with the Data protection act of 1998 and in conjunction with the hospital policy on data security.

3.9 Heart Rate Analysis

Raw ECG, PPG and chest wall motion data was captured using the ProComp Infiniti hardware and capture and analysis software written in Visual basic version 6.
Raw ECG data was processed and instantaneous heart rate recalculated using the visual basic Autonomic analysis software after the rolling correlation technique of Buttfield and Bolton (2005) was applied (See Figure 12).

3.9.1 Rolling Correlation Coefficient Technique

A suitable archetypal QRS complex within the ECG signal was identified as typical and used as a template to define the shape of the QRS complex for each volunteer. The most obvious segment of data to select as the archetype signal is the R-wave. A single point is selected within the signal, and then the data 0.2 seconds prior to and following the mouse click is used as the archetype. QRS pulses in the data were identified in the raw ECG data by a strong positive correlation with the archetypal ECG template. Precise timing of the ECG complex was defined by a maximum of the correlation coefficient. Nearly all heart rate artefacts (erroneous data) were removed from the recording trace in this way. The beat to beat (b-b) instantaneous heart rate was calculated for each adjacent heartbeat from the time of onset of each QRS complex identified using Buttfield and Bolton technique, which is shown in Figure 12. This method was not used in real-time during acquisition, but implemented in software and used to process the data prior to analysis. During data acquisition, a threshold

measurement is used to identify R-waves, with a heartbeat being identified when the ECG signal exceeds a threshold value.

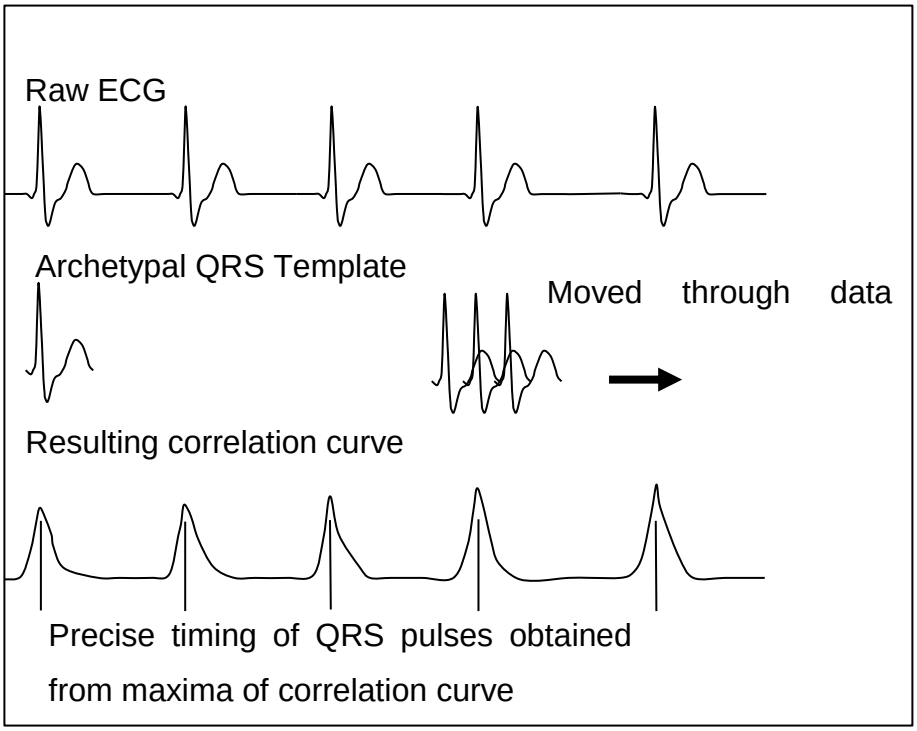


Figure 12. Principle of Buttfield and Bolton technique

For automated analysis of segments of the heart rate data train e.g. frequency analysis and fractal dimension analysis, a cubic spline interpolation was used to re-sample the instantaneous heart rate to a regular sample interval of 8 samples per second. The chest wall movement breathing data was also re-sampled to match the regularised heart rate data. Any additional artefactual instantaneous heart rate data due to ECG noise or ectopic beats were manually removed and substituted with interpolated heart rate data using a cubic spline interpolation technique.

Fast Fourier Transform (FFT) is a computational calculation using an efficient algorithm for time frequency analysis to determine the frequency of a discreet signal. Analysis was performed both in the temporal domain, and in the frequency domain following Fast Fourier Transform.

3.9.2 Fractal Dimension Analysis

The analysis of heart rate variability is a well-established technique for the investigation into cardiac autonomic dysfunction. In recent years non-linear parameters obtained from heart rate variability data analyses have given an alternative insight into the interpretation of heart rate variability. Fractal behaviour of heart rate variability can be assessed by extracting fractal dimension using Higuchi's algorithm. This method has been quoted by Addio et al (2007) as allowing better fractal estimation, eliminating the errors due to the indirect estimation of fractal dimension from spectral power.

Fractal analysis assesses the relationship between heart rate variability and the scale at which this variation is reviewed (see Figure 13). When the data is sampled at a lower resolution, smaller scale variations are overlooked and the total variation in heart rate falls. By plotting Log of the total variation measured against Log of the resolution, a graph representing the fractal characteristics of the HRV is obtained. The gradient of the graph is the fractal dimension, which ranges from 1-2 (see Figure 14).

$$\text{Fractal dimension} = \frac{\text{Log (measured length at resolution)}}{\text{Log (total variation)}}$$

= 1 for straight line

= 2 for plane

= between 1 and 2 for variable plane

If there is random noise the fractal dimension ≈ 2

If there is a sine wave the fractal dimension ≈1.6

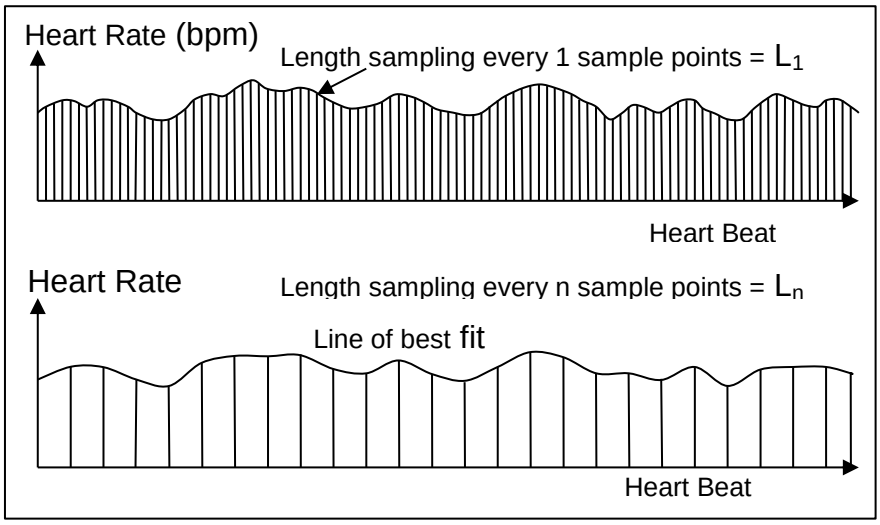


Figure 13. Illustration of sampling during fractal analysis

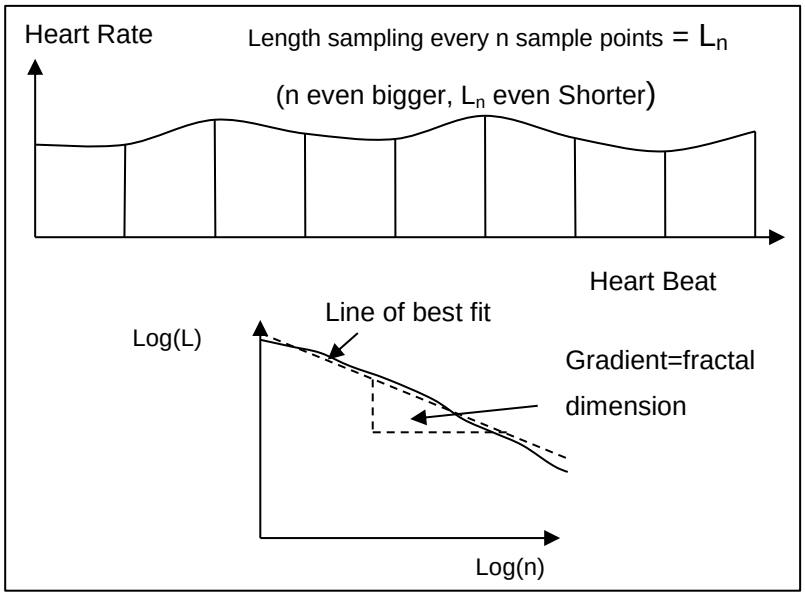


Figure 14. Calculating fractal dimension

3.10 Measurement Parameters Used

3.10.1 Ewing Analysis

The Ewing measures of heart rate variability are generally time domain measures, which assess variations in instantaneous heart rate secondary to provocations such as

the Valsalva manoeuvre. These measurements allow assessment of changes in heart rate variability governed by different branches of the ANS, for example heart rate response to deep breathing is under parasympathetic control, hand grip under sympathetic control and Valsalva Manoeuvre mediated by both branches of the ANS (Kimattila et al 1997).

The following calculations were made for provocations undertaken as recommended by Ewing (1985)

- Slow breathing. Maximum to minimum heart rate during 1 cycle of a slow breathing regimen (6 breaths/min).
- Maximum to minimum heart rate ratio during performance of the Valsalva manoeuvre
- Maximum to minimum heart rate ratio during maximal hand grip.

3.10.2 Frequency Domain

Frequency domain measurement of heart rate variability is an alternative method of assessment. Various spectral methods have been used in the analysis of the HRV. Power spectral density (PSD) analysis provides the basic information about how variance distributes as a function of frequency, however only an estimate can be obtained by mathematical algorithms (Task Force 1996).

Frequency analysis of heart rate data during resting can be used to determine the effect of the branches of the ANS.

- High Frequency (HF) represents vagally (parasympathetic) mediated autonomic tone associated with respiration,
- Low frequency (LF) related to sympathetic autonomic control.
- HF/LF Ratio can be used to represent the balance between the two branches of the ANS.
- Fractal dimension analysis (see section 3.9.2)

- Short-term maximum to minimum heart rate difference during resting
- Spectral Power (see Figure 15).

The total spectral power of the Heart Rate Variation was measured in the following frequency ranges:

Very Low Frequency – 0.01-0.04 Hz
Low Frequency – 0.04-0.15 Hz
High Frequency – 0.15 – 0.4 Hz
LF/HF ratio

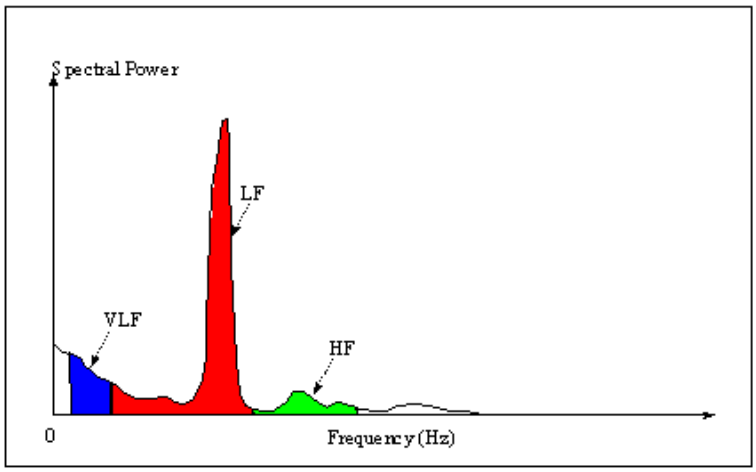


Figure 15. A representation of a typical spectral power curve for heart rate variability.
The resulting spectral power values that can be obtained are labelled very low frequency power (VLFP), low frequency power (LFP), and high frequency power (HFP).

3.10.3 Metronome Guided Breathing Period

A set of heart rate variability measurements were made over a period of 2 minutes during which, breathing was controlled by a metronome. This enabled us to tightly control breathing and assess the short term effects of breathing on autonomic tone. The parameters measured are detailed overleaf.

3.10.4 Temporal Domain

3.10.4.1 Mean Amplitude of Heart Rate Variation (A)

The amplitude of heart rate variation was measured for each cycle of heart rate change from maximum to minimum instantaneous heart rate as indicated in Figure 16. The mean value was calculated, the resulting parameter we have labelled **(A).** Standard deviation of heart rate (±SD) was also calculated.

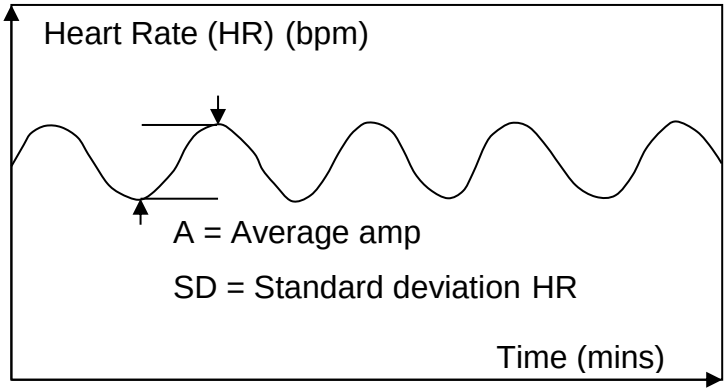


Figure 16. Diagram showing the parameter Average Amplitude

3.10.4.2 Maximum Correlation of Heart Rate to Chest Wall Motion (CORR)

In order to establish the maximum correlation in synchronicity between breathing and heart rate, the heart rate curve was shifted in time by 0.125 s intervals within the range +/- 5 seconds to find the optimum shift required to maximise correlation of heart rate and chest wall movement during breathing (see Figure 17). This was automated through the autonomic analysis software. The maximum correlation coefficient obtained was labelled CORR, (this is shown in Figure 17).

In previous research by Perring and Jones (2003) the technique of shifting the heart rate data was used to determine the maximum correlation between heart rate and breathing

cycle for both normal and diabetic groups. There did not appear to be any difference in time shift values obtained for normal or diabetic group and the time shift necessary to maximise correlation between heart rate and chest wall movement was not related to age.

We would expect a time delay when assessing the phase relationship between heart rate and measurement of chest wall movement using plethysmography as a first approximation of breathing as we are not measuring changes in internal thoracic pressure or pulmonary stretch. It is valid to shift the heart rate data to correlate maximally with the breathing curve.

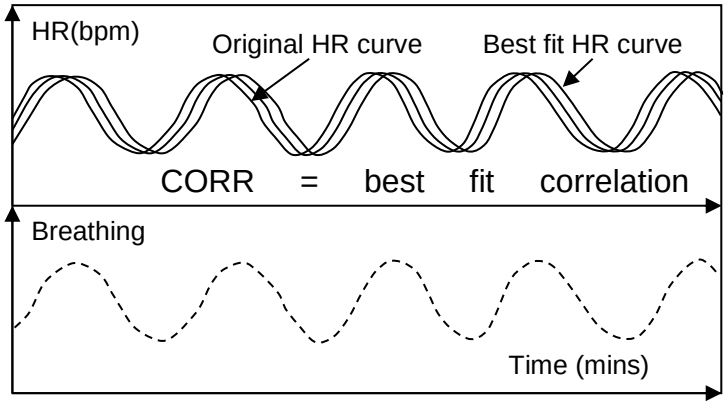


Figure 17. Optimum shift to achieve maximum alignment of HR and breathing

3.10.4.3 Comparison of Spirometry and Chest Plethysmography

In a previous study by Perring and Jones (2003), eight healthy volunteers were asked to perform 2 minutes of metronome guided breathing using the chest plethysmography system while breathing into a spirometer system (Morgan Benchmark). The tidal breathing curve obtained from the spirometer was compared with the chest wall movement curve produced by the chest plethysmography system. Correlation between the breathing cycle measurements using spirometry and plethysmography was

consistently good (mean correlation coefficient 0.94 ± 0.03). This comparison of the two techniques gives confidence in the chest wall measurement.

3.10.5 Frequency Domain Measures

The result of power spectral density (PSD) analysis provides the basic information about how variance distributes as a function of frequency. The nonparametric calculation for PSD is fast Fourier transform (FFT). The result of FFT was a graph of the variation of spectral power with frequency. In periods of metronome breathing, the frequency spectrum was dominated by the spectral peak at the frequency corresponding to the rate of respiration (see Figure 18).

The spectral power within the peak corresponding to the breathing frequency was also measured as the log of the integral of the breathing peak in the frequency spectrum. This was labelled LPP (Log peak power) (see Figure 18).

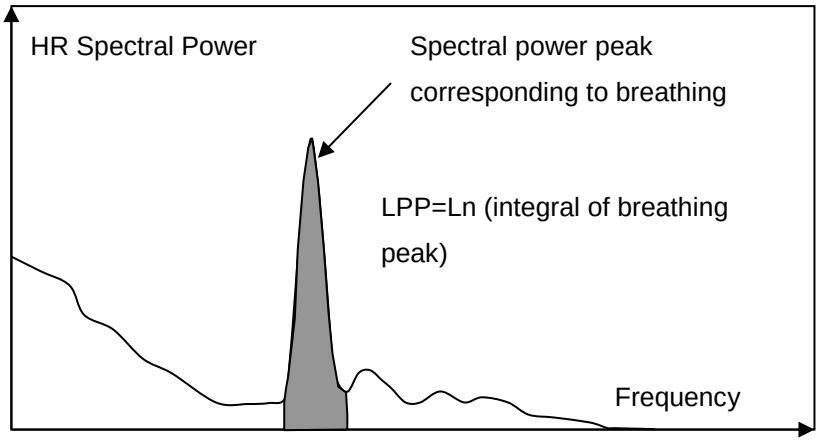


Figure 18. Peak power within the frequency peak corresponding to breathing

The spectral power curve for heart rate variation was also compared with the spectral power curve for chest wall movement and the correlation coefficient calculated. This was labelled FCORR (see Figure 19). As for breathing heart rate correlation in the time domain, there was no evidence of any decay of correlation with age.

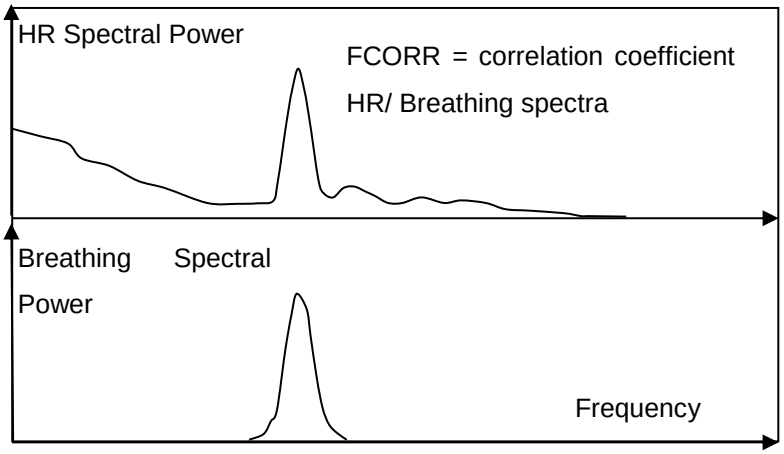


Figure 19.The FFT Correlation parameter represents the correlation coefficient between the values of the two spectral power curves shown above for heart rate and breathing variation,

We have published the results of previous research on a sample of 44 normal healthy volunteers (age range 20-77 years SD+/- 13.6) using chest plethysmography and metronome guided breathing at 6 breaths per minute (Perring and Jones 2003). Using this original normal data we have established an age-matched normal range for the temporal and frequency parameters described below.

We established the lines of best fit with age and the standard deviation from this line of best fit for each parameter as below.

- CORR = 0.765. Standard deviation from line of best fit = 0.104
- A = 22.9 - 0.2297 *Age (years). SD from line of best fit = 5.467
- SD = 9.04 – 0.094*Age (years). SD from line of best fit = 1.851
- LPP = 7.715 – 0.0233*Age (years). SD from line of best fit = 0.4553
- FCORR = 0.922. SD from line of best fit = 0.149

We then obtained an overall deviation from the age-matched normal range by adding the number of SD's from the norm of each parameter. We obtained a standard deviation for this parameter in order to quote a total deviation from the age matched norm for all parameters combined (labelled DEV, the unit being the number of standard

deviations from the normal age matched range). This gives a good indication of overall parasympathetic or vagal tone compared to an age matched normal range.

3.10.6 Parameter Labels (Overview)

The amplitude of heart rate variation was measured for each cycle of heart rate change from maximum to minimum instantaneous heart rate as indicated in Figure 16. The mean value was calculated, the resulting parameter we have labelled (**A)**.

Standard deviation of heart rate (**SD)** was also calculated.

The spectral power within the peak corresponding to the breathing frequency was also measured as the log of the integral of the breathing peak in the frequency spectrum. This was labelled **LPP** (see Figure 18).

The heart rate curve was shifted in time by 0.125 s intervals within the range +/- 5 seconds to find the optimum shift required to maximise correlation of heart rate and chest wall movement during breathing. The maximum correlation coefficient obtained was labelled **CORR** (see Figure 17).

The spectral power curve for heart rate variation was also compared with the spectral power curve for chest wall movement and the correlation coefficient calculated. This was labelled **FCORR** (see Figure 19).

For each of these parameters, the value was expressed as a number of standard deviations away from the (age match if required) normal value. An overall deviation from the age-matched norm was established by adding the number of standard deviations from the norm of each parameter. A standard deviation for this parameter was obtained in order to quote a total deviation from the age matched norm for all parameter combined (**DEV).**

3.10.7 Blood Pressure Analysis (Baroreflex Sensitivity)

Continuous blood pressure monitoring was performed during these investigations using the Portapres system. The systolic blood pressure (BP) was obtained for each beat where it was recorded during the metronome guided breathing period. A cubic spline interpolation was then performed in order to produce a continuous dataset of systolic BP at 8 samples per second. Frequency spectra were then produced for both heart rate and systolic BP using FFT and the peak corresponding to the 6 breaths/minute metronome breathing rate. The integrated peak power was then calculated for the breathing peak in both heart rate and BP spectra and the ratio of the integrated peak power for HR/ BP was calculated (see Figure 20. This represented a measure of the baroreflex sensitivity.

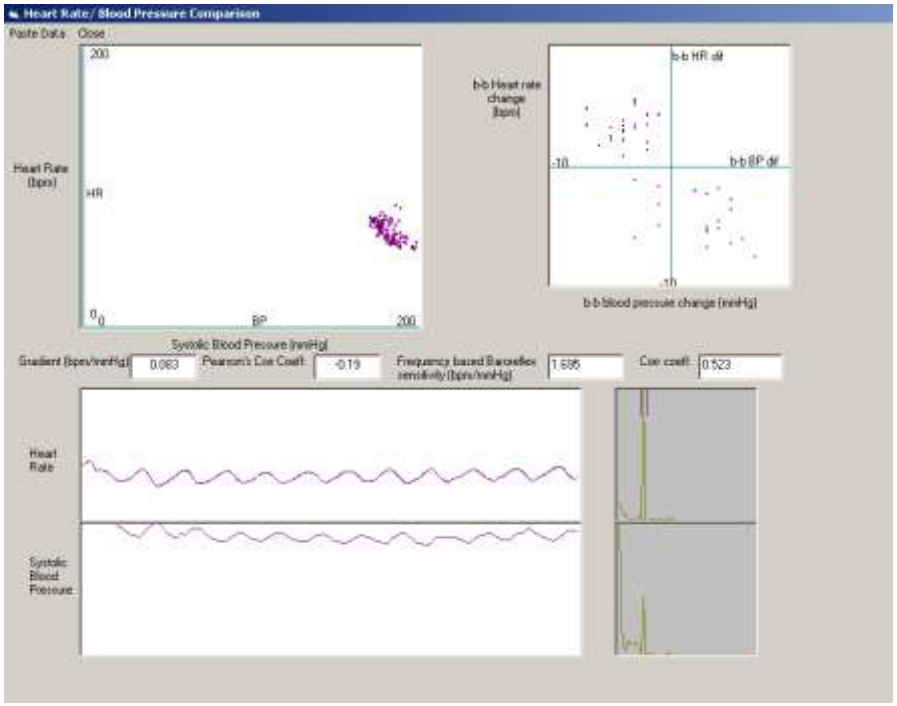


Figure 20 A typical screen shot of blood pressure and heart rate date (baroreflex)

Heart rate and blood pressure are shown in the lower panel of this figure detailing the relationship between the coupling of the physiological signals. The spectral power of the signals is shown to the right and the correlation coefficient is displayed. In this trace it is clear that heart rate and blood pressure remain coupled.

3.10.8 Pupillometry

3.10.8.1 Instrumentation

Pupillometry was performed using medical physics designed and built goggles. These comprised of a standard adult diving mask. The Perspex lenses were removed and replaced with metal plates to fill the spaces. Housed within each eyepiece was a Phillips SPC200NC web camera. The infra-red filters were removed and approximately 280 pictures per minute / four frames per second (4Hz) were captured while the volunteer sat in the dark. While this was not a particularly fast capture rate, it was sufficient to show changes in sympathetic tone in the autonomic nervous system.

Software was written in Visual basic 6 using XVideo OCX image capture software. The goggles are connected to a laptop via USB connection.

3.10.8.2 Pupillometry Method

We assess pupillometry by placing infrared goggles comfortably against the face by the volunteer. The driver for the right eye is selected on the pupilometry programme and the eye is displayed in real time on the screen. The position of the eye is checked to make sure it is in the centre of the field of view. It is important to be able to clearly define the margin of the iris and the pupil in the vertical plane. The lights are switched off and the patient is allowed to acclimatise to the darkness. It is vital that extraneous light is eliminated from the room.

The recording period is started via the software. One eye is filmed. After filming in the dark for 30 seconds a one second flash of light is shone into the contra-lateral eye, a period of 30 seconds in the dark follows and then another one second flash, followed by 30 seconds of darkness. The response to the flash is seen in the eye that is being filmed.

3.10.8.3 Pupillometry Measurement

The pupilometry measurement software is written in Visual basic 6. The volunteer file is selected. The image with the largest pupil size is identified, corresponding to a point at which the eyes are fully accommodated to darkness (see Figure 21). This can be manually adjusted if a better image is required. The patient's date of birth is entered to allow for result to be compared with the age matched normal range database.

Pupil response to dark adaptation was measured in a manner described by Smith and Dewhirst (1986). The software prompts to identify the left and right margins of the pupil and then the left and right margins of the iris see (Figure 21). The diameter of the pupil and iris were measured and the pupil/iris ratio calculated. The result is automatically compared against an age-matched normal range which was established by a previous unpublished normal sample performed in Poole, and is embedded in the analysis software.

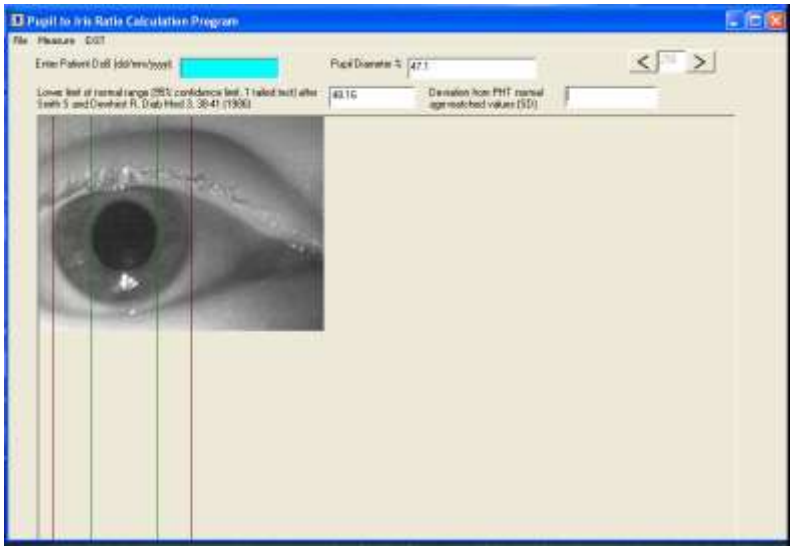


Figure 21 Pupillometry software showing the margins of the iris and pupil.

3.10.9 Summary table of autonomic function testing

Table 1 Summary table of autonomic function testing. *Provocations used for testing HRV and the branch of the ANS that is activated.*

Test Name	Summary	ANS Branch
Resting	Spontaneous heart rate changes during extended resting period are recorded. The maximum and minimum heart rate rise is identified in a typical 20 second period. The percentage change is quoted. >6% is normal.	Gives an indication of the balance between the two branches of the ANS
Valsalva Manoeuvre	The Valsalva manoeuvre consists of respiratory strain that increases intra-thoracic and intra-abdominal pressures and alters hemodynamic and cardiac functions. Heart rate during the Valsalva Manoeuvre is recorded and a ratio of the fastest heart rate to the slowest is calculated as a way of noninvasively quantifying the procedure. The result is >1.2	Multimodal (parasympathetic and sympathetic branches)
Hand grip	Isometric exercise increases heart rate. The Maximum HR in BPM and the minimum are identified. The result is age matched and displayed as a ratio (>1.1)	Sympathetic
Lying / standing **Blood pressure response to lying standing**	Heart rate recorded the ratio of the 15:30 beat after standing is recorded. Blood pressure recorded at rest and after 2 minutes of standing	Parasympathetic Sympathetic
BP response to 5 minute Sustained hand grip	Persistent muscle contraction causes blood pressure and heart rate to increase. The mechanism involves the exercise reflex, which withdraws parasympathetic activity and increases sympathetic activity. This test requires the patient to apply and maintain grip at 30% maximal activity for up to 5 minutes; the diastolic blood pressure should rise more than 16 mm Hg	Sympathetic
2 minute metronome guided	The variation of heart rate with respiration is known as sinus arrhythmia and is generated by autonomic reflexes. Inspiration increases heart rate, and expiration decreases it. The variation is primarily mediated by the vagus innervation of the heart.	Parasympathetic

breathing	Pulmonary stretch receptors as well as cardiac mechanoreceptors and possibly baroreceptors contribute to regulating the heart rate variation. It increases with slower respiratory rates and reaches a maximum around 5 or 6 respirations per minute	
Pupillometry	Measurement of iris: pupil ratio in the dark compared with age matched normal values	Sympathetic and parasympathetic

Chapter 4: Results (Influenza Study)

This chapter will examine the results of the influenza study, including addressing demographics of the study population, the decision to separate the cohort into subgroups, examining the mild inflammatory effect of vaccination on resting heart rate, the effect of handgrip strength on HRV, breathing depth analysis, the gold standard Ewing measurements of autonomic function and HRV during two minutes of metronome guided breathing. All or some of the assessments may be used depending on the physical ability and compliance of the individual. Each component of the test will stress either of the two branches of the ANS; these are listed in section 3.10.9.

4.1 Relevant Study Information

4.1.1 Statistical Assessment

Prior to analysis, all data was checked to see whether or not it fitted a normal distribution. Software called OpenStat was used to assess this and the test used within this software was a Lilliefors test for normality. Following this the data were analysed using Kwikstat Winks SAS 4.62 statistical analysis software (Texasoft 1991-2001). Parametric and non-parametric statistical analysis were performed as appropriate.

4.2 Demographic and Lifestyle Factors

The age of participants in this study ranged from 23 – 73 years which closely reflects the working age of the hospital population and volunteers. Data were analysed for the healthy volunteers collected over the two vaccination periods (2008/09 and 2009/10). The demographics for the groups were assessed to establish whether they are evenly matched between the two years. Data for age was analysed from the two vaccination periods using an unpaired t-test. There was no significant difference between the two cohorts (P=0.47).

The data was disproportionately weighted in favour of females over males (3:1). This was unintentional but is likely to reflect the ratio of female to male employees within the hospital. We have performed statistical analysis using Fishers Exact on the number of men and women in each cohort from each vaccination year and there is no statistically significant difference between the two cohorts (p=0.961).

Resting heart rate (beats per minute) was established prior to vaccination for males (63 ±19.5) and females (72.9 ±11.1) to determine whether there is a difference in heart rate related to gender. Our results show that female participants in general have a higher resting heart rate than males. However, analysis using an unpaired t-test found there was no statistical difference between the data (p=0.061).

Table 2. Table showing the age and gender distribution for the two vaccination periods.

	Recruitment numbers		**Mean Age (±SD)**		**Age Range**	
Vaccination period	**2008/09**	**2009/10**	**2008/09**	**2009/10**	**2008/09**	**2009/10**
Male	12	6	45.8 (14.4)	47.8 (6.6)	20 – 64	37- 55
Female	35	18	51.1 (11.8)	47 (12.1)	23 - 73	23- 63
Total Group	47	24	49.8 (12.6)	47.2 (10.8)	20 - 73	23 - 63

Mean (±SD) values for males, females and the total group. The data indicates a distribution skewed towards females in both vaccination periods with a similar distribution of age for each cohort for both vaccination periods with a similar distribution of age for each cohort for both vaccination periods. P value was calculated using an unpaired t-test with no significance between visits.

4.2.1 Drop-out rate

Data for two subjects were not used in the study (2.8%). One volunteer's ECG trace contained erroneous HR data, which could not be substituted using the PPG data. The second failed to attend the post vaccination assessment.

4.2.2 Sub-grouping of the Volunteers According to the Post Vaccination Symptom Questionnaire

The total group (N=71) was divided into two major categories; volunteers who reported no symptoms after vaccination, these were termed category A (N=31) and volunteers

who reported various degrees of symptoms after receiving the vaccine, called category B (N=40) This symptomatic category was further subdivided into two sub-groups according to the extent and severity of their symptoms in response to the vaccination; Category B, subgroup 1 (N=25) consisted of volunteers with only mild symptoms and Category B, subgroup 2 (N=15) consisted of volunteers who experienced more significant symptoms than subgroup 1 as demonstrated in table 3.

Table 3. Groupings of the volunteers based on post vaccination symptom questionnaire.

Group	Number	Symptoms
Full cohort	**N=71**	**Total group post vaccination**
Category A	**N=31**	Asymptomatic
Category B	**N=40**	**Symptomatic**
Sub group 1	**N=25**	Volunteers reporting mild symptoms after vaccination. Typically sore arm lasting less than 24 hours.
Sub group 2	**N=15**	**Significant symptoms. Including sore arm, headache, malaise and raised temperature, lasting > 24 hours**

Table showing the number of participants in the full cohort and subgroups according to post vaccination symptom questionnaire. Symptoms have been listed for each subgroup.

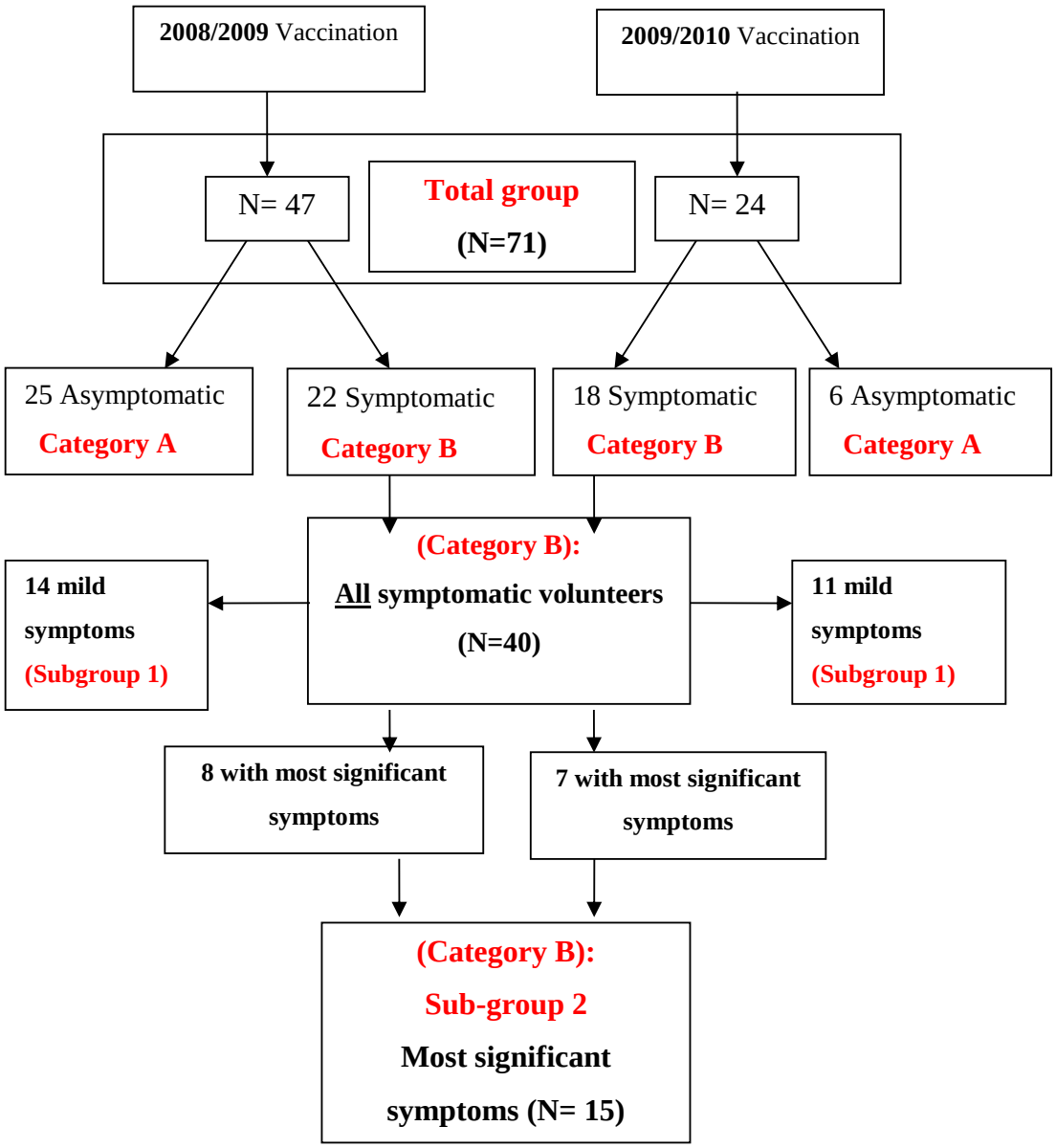


Figure 22. Flow chart showing grouping of participants

This schematic shows the recruitment of participants to the study. The number of participants in each vaccination period and in each subgroup according to symptom questionnaire.

4.3 The Effects of Vaccination on Physiological Measurement Parameters

This study examined the effect of the influenza vaccination on physiological measures such as resting heart rate, blood pressure, measures of breathing depth analysis and spontaneous breathing rate. Results of analyses are shown in the following sections.

4.3.1 Finger Blood Pressure Measures

Beat to beat finger blood pressure was measured continuously throughout the autonomic assessment. Systolic and diastolic blood pressure (mmHg) pre and post vaccination was recorded. For both systolic and diastolic blood pressure, pre and post vaccination the percentage difference (v1 – v2) was reduced for all groups, the largest percentage change was for category B subgroup 2 (systolic -6.8%). There were statistically significant differences noted in the full cohort for both systolic and diastolic blood pressure, which decreased (see Table 4). The reason for this is unclear however it may be related to the inflammatory process following the vaccination. There were no statistical differences seen in either of the subgroups pre and post vaccination despite the percentage changes noted (see Table 4).

Table 4. Systolic and diastolic blood pressure pre and post vaccination.

	Total Group N=71	**Category B N=40**	**Category B Sub-group 2 N=15**
Mean Systolic (mmHg) Pre vaccination (±SD)	124.1 (30.1)	131.2 (19.5)	124.1 (26.8)
Mean Systolic (mmHg) Post Vaccination (±SD)	117.8 (32.2)	128.5 (18.1)	115.6 (24.6)
% Difference Systolic BP (V1-V3)	-5.07	-2.05	-6.8
P Value	**0.103**	0.390	0.225
Mean Diastolic (mmHg) Pre vaccination (±SD)	82.01 (8.6)	82.32 (7.67)	82.8 (9.79)
Mean Diastolic (mmHg) Post Vaccination (±SD)	79.1 (12.3)	80.47 (9.23)	82.06 (11.06)
% Difference Diastolic BP (V1-V3)	-3.55%	-2.22%	-0.89%
P Value	**0.02**	0.07	0.69

The systolic and diastolic blood pressure (mmHg) measures pre and post vaccination for the total group and 2 symptomatic subgroups are shown in the table below. Mean (±SD) values and percentage difference (V1-V3) are given for all participants' pre and post vaccination. P values were calculated using a paired t-test. Significant values (p=<0.05) are shown in bold.

4.3.2 Breathing Depth Analysis Pre and Post Vaccination

Two minutes of breathing data was recorded for each participant before and after vaccination, each set of data was tightly controlled using a metronome to guide the timing of each breath cycle. Measurements were made taking the amplitude of the waveform from peak to trough for each breath cycle during the two minutes. The measurement represents average chest wall movement in arbitrary units. Analysis of breathing depth was performed before and after vaccination to assess whether participants had performed the breathing provocation differently between visits. The mean percentage difference between the two visits was -1.08% showing the breathing depth was slightly reduced after vaccination. However, there was no significant statistical difference in performance of the metronome-breathing task between the 2 visits (P=0.853), (see Table 5).

Table 5 Breathing depth analysis for the full cohort.

	Pre vaccination (SD)	**Post vaccination (SD)**	**% Difference (V1- V2)**	**P Value**
Mean (+/-SD)	111.3 (64.4)	110.1 (69.5)	-1.08	0.853

Breathing depth measured in arbitrary units for the full cohort. Mean breathing depth and SD are shown for pre and post vaccination visits. Analysis was performed using a t-test. No significant difference was seen between the visits.

4.3.3 Measures of Spontaneous (resting) Breathing Rate Pre and Post Vaccination

Measures of breathing rate during an extended period of rest were made for each participant pre and post vaccination. The number of breaths (defined by the peak of the chest wall waveform) was counted for the resting period and an average compared pre and post vaccination (see Table 6). There was a 2% reduction observed between visit 1 and visit 2, however there was no significant difference in spontaneous breathing rate between the resting investigation period prior to and following vaccination (p=0.85)

(resting breathing rate (mean +/-SD) cycles/min prior to and cycle/min following vaccination.

Table 6 Measures of Spontaneous (resting) Breathing Rate Pre and Post Vaccination

	Pre vaccination (SD)	**Post vaccination (SD)**	**% Difference (V1- V2)**	**P Value**
Mean Breaths per minute (+/-SD)	16.4 +/- 3.9	16.08 +/-3.3	-1.95	P = 0.85

Table showing mean, +/-SD, % difference (V1-V2) for resting breathing rate cycles/min prior to and cycle/min following vaccination.

4.4 Results of the "Gold Standard" Ewing Assessment of Autonomic Function

During assessment of autonomic function pre and post influenza vaccination the standard beat to beat Ewing measurements were made along with other provocations. The data were analysed to see if differences in heart rate variability exist pre and post vaccination. The percentage difference between data for visit 1 and visit 2 shows a drop in nearly all parameters post vaccination, particularly for subgroup 2 where all HRV measures were reduced (see Table 7). Despite this, the results of paired t-test show that there are no significant statistical changes to the parameters as a result of the influenza vaccination apart from the resting parameter for subgroup 2 (p=0.05). It could be argued that, I) the sample size was too small to discern a difference in heart rate changes using the Ewing measures for all parameters other than resting or II) that no differences actually exist between pre and post vaccination measures due to the weak inflammatory effect of the vaccination, or III) the Ewing measures are insufficiently sensitive to the small changes that we might expect as a result of this study.

Table 7. Autonomic function testing using Ewing assessments pre and post vaccination.

	Total Group (N=71)				Category B (N=40)				Sub-group 2 (N=15)			
	Mean V1 (±SD)	**Mean V2 (±SD)**	**% Diff v1-v2**	**P Val**	**Mean V1 (±SD)**	**Mean V2 (±SD)**	**% Diff v1-v2**	**P Val**	**Mean V1 (±SD)**	**Mean V2 (±SD)**	**% Diff v1-v2**	**P Val**
Resting (%)	9.2 (5.7)	8.5 (4.4)	-7.6	0.34	9.6 (5.9)	8.09 (3.2)	-15.7	0.09	9.67 (5.55)	7.4 (3.09)	-23.5	**0.05**
Forced Breathing (bpm)	19.5 (0.6)	19.7 (10.3)	1.03	0.82	19.9 (10.5)	19.7 (10.4)	-1.01	0.82	19.9 (9.3)	18.9 (10.2)	-5.03	0.47
Insp/Exp Diff (bpm)	14.17 (7.7)	14.05 (7.8)	-0.85	0.84	14.68 (8.0)	14.2 (7.8)	-3.27	0.46	14.76 (7.58)	13.35 (7.47)	-9.55	0.15
Valsalva (ratio)	1.32 (0.2)	1.33 (0.2)	0.76	0.76	1.32 (0.2)	1.34 (0.2)	1.52	0.55	1.33 (0.24)	1.31 (0.19)	-1.5	0.68
Hand grip (ratio)	1.17 (0.1)	1.16 (0.1)	-0.85	0.79	1.17 (0.1)	1.17 (0.1)	0	0.55	1.15 (0.07)	1.14 (0.05)	-0.87	0.54

Mean (±SD) values and % difference (v1-v2) for HRV parameters for the total group and symptomatic subgroups pre and post vaccination. P values calculated using a paired t-test. Significant values (p=≤0.05) are shown in bold.

4.5 Two Minutes Metronome Guided Breathing at 6 Breaths Per Minute Breathing Heart Rate Variability Pre and Post Vaccination.

Results from the total group were first analysed using Pearson's correlation coefficient to determine whether there was an inverse correlation between Age and Frequency Domain Parameters. We saw a moderate but statistically significant negative correlation between age and three heart rate variability parameters (A, SD and LPP) for all volunteers breathing at six breaths per minute over two minutes (see Table 8).

Table 8. Correlation between Age and HRV Parameters for all volunteers during metronome guided breathing at six breaths per minute.

Parameters measured	Pearson's Correlation Coefficient	t-value	P value
A (bpm)	-0.5239	5.07	**P= <0.001**
SD (bpm)	-0.4948	4.69	**P =<0.001**
CORR (unitless)	-0.1318	1.1	P= 0.277
LPP (unitless)	-0.5241	5.07	**P =<0.001**
FCORR (unitless)	-0.1059	0.88	P= 0.383

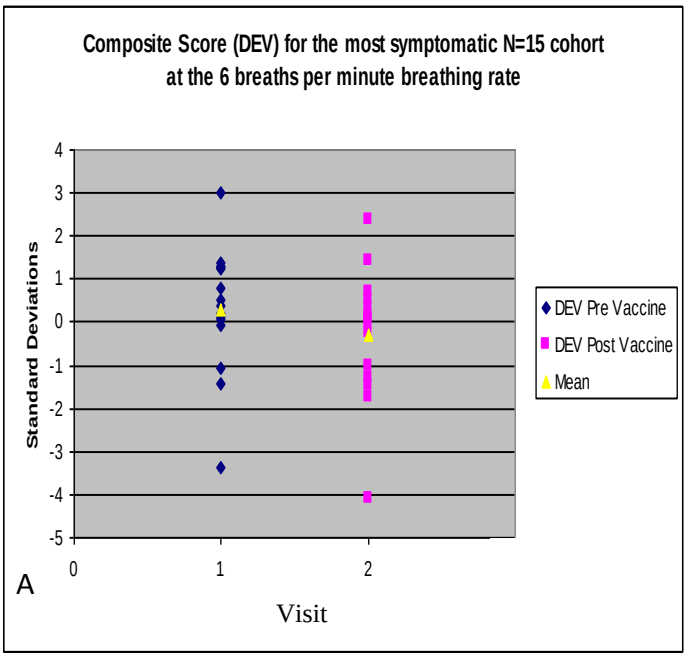
Correlation was performed using Pearson's correlation coefficient. P values were calculated at P=<0.05 significance level. The significant results are shown in bold. HR Parameters shown are listed in 3.10.6.

Secondly data collected during two minutes of metronome breathing at 6 breaths per minute were analysed to determine whether there was a difference in the total cohort and the 2 subgroups (pre and post vaccination). There was a percentage change between the two visits for most of the HRV parameters for the full group, however no statistically significant difference resulting from the influenza vaccination in any of the parameters during the 2 minute metronome breathing period in the full sample group were observed. This also included the overall deviation composite score (DEV) (see Table 9). The percentage difference in heart rate data between the two visits reduced for the two subgroups for all of the HRV parameters. The results of statistical analysis using a paired t-test for the two symptomatic groups are of particular interest and results are displayed in Table 9. In Category B (n=40) where volunteers experienced a symptomatic response to vaccination there is some evidence of deterioration in heart rate variation as a response to metronome breathing. In two parameters, SD (p=0.029) and LPP (p=0.028) the fall in heart rate response was statistically significant although this deterioration was not sufficient to display a significant drop in the composite overall deviation score (DEV), (see Table 9). By contrast in subgroup 2 (N=15) a significant deterioration in all heart rate variability frequency parameters apart from the FCORR was observed. This was reflected clearly in the composite overall deviation score (DEV) (see Table 9 and Figure 23).

Table 9. Comparison of HRV parameters pre and post influenza vaccination for two minutes of metronome breathing at 6 breaths per minute.

	Total Group (N=71)				Category B (N=40)				Sub-group 2 (N=15)			
	Mean V1 (±SD)	**Mean V2 (±SD)**	**% Diff V1-V2**	**P Val**	**Mean V1 (±SD)**	**Mean V2 (±SD)**	**% Diff V1-V2**	**P Val**	**Mean V1 (±SD)**	**Mean V2 (±SD)**	**% Diff V1-V2**	**P Val**
CORR (unitless)	-0.28 (1.8)	-0.37 (2.1)	-32	0.48	-0.22 (2.1)	-0.34 (1.9)	-54.5	0.71	0.17 (1.7)	0.09 (2.23)	-47.1	**0.01**
A (bpm)	0.33 (1.0)	0.34 (1.1)	3.03	0.41	0.37 (1.1)	0.31 (1.2)	-16.2	0.07	0.44 (1.3)	0.39 (1.43)	-9.3	**0.02**
SD (bpm)	0.63 (1.1)	0.53 (1.1)	-15.8	0.16	0.67 (1.2)	0.38 (1.2)	-43	**0.029**	0.43 (1.4)	0.18 (1.47)	-58.1	**0.02**
LPP (unitless)	0.65 (0.8)	0.55 (1.0)	-15.4	0.28	0.66 (0.9)	0.53 (1.1)	-11.7	**0.028**	0.69 (1.0)	0.15 (1.27)	-78	**0.01**
FCORR (unitless)	-0.10 (1.2)	-0.07 (1.3)	30	0.36	-0.09 (1.3)	-0.04 (1.2)	-55.6	0.80	-0.12 (1.1)	-0.13 (1.75)	-8.3	0.193
DEV	0.29 (1.2)	0.25 (1.2)	-13.8	0.35	0.33 (1.3)	0.14 (1.2)	-57.6	0.19	0.26 (1.5)	0.18 (1.52)	3-0.7	**<0.001**

Mean (±SD) results for the full cohort and symptomatic subgroups are shown. Analysis was performed using Wilcoxon's signed rank. Significant P values calculated at P=≤0.05 significance level. Results that differed significantly from the starting value are shown in bold. Parameters shown in section 3.10.6.



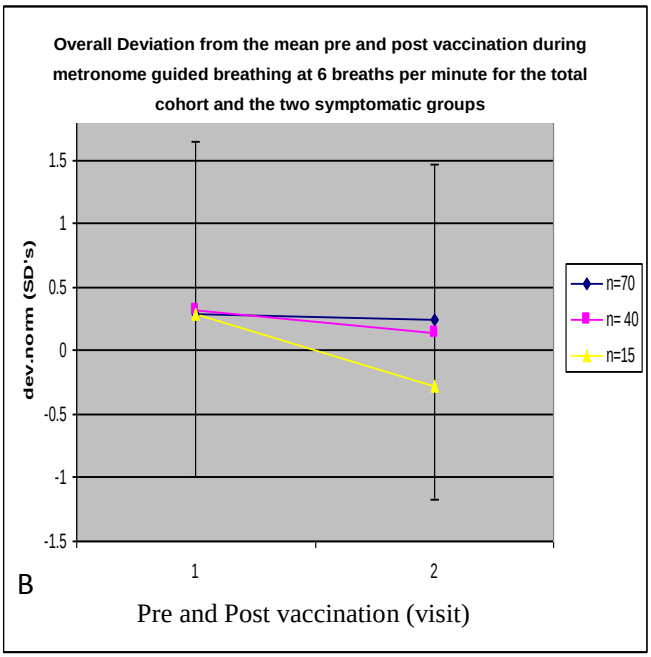


Figure 23 Graphs showing HRV parameter (DEV) after 2 minutes of metronome breathing pre and post vaccination.

Pre-vaccination is shown as visit 1 and post vaccination is shown as visit 2. The (DEV) HRV parameter for Category B, subgroup 2 for each visit is shown in graph (A) A statistically significant difference was seen for this parameter (p=≤0.001) between visits. The mean value for each group is shown

Graph (B) shows the (DEV) parameter for the total group, Category B, and Subgroup 2 pre and post vaccination at the 6 breaths per minute breathing rate. The mean DEV value is shown for each group. There is a statistically significant difference (p=≤0.001) between visits in the DEV parameter for subgroup 2

4.6 Analysis of Raw HRV data

In order to confirm the validity of our HRV measures we assessed changes in the raw HR data for each parameter from which the alternative aged matched deviation data is based. When analysing the heart rate data, the raw data was used in place of the standard deviation from the line of best fit for each parameter during two minutes of forced breathing at 6 breaths per minute for the full cohort and for subgroup 2, (see Table 10). No statistical difference was found when comparing the raw heart rate data for the full group (N=71). However, some of the raw heart rate data for spectral

parameters in subgroup 2 (N=15) were significantly different before and after the influenza vaccination.

Table 10. Raw HRV data from metronome guided breathing at 6 breaths per minute rate.

Parameter	Full Group (N=71 volunteers)		Subgroup 2 (N=15 volunteers)	
	t value	P value	t value	P value
SD (bpm)	0.75	0.4535	1.829	0.088
A (bpm)	0.09	0.921	2.471	**0.026**
LPP (unitless)	0.99	0.3771	3.626	**0.002**
CORR (unitless)	0.43	0.6668	2.739	**0.016**
FCORR (unitless)	0.39	0.6965	0.964	0.351

The raw heart rate data was analysed for the full group and subgroup 2 pre and post vaccination. Parameters shown are described in section 3.10.6. HR data was analysed using a paired t-test. Significant results are shown in bold.

4.7 Summary of Resting HRV prior to and post vaccination

The data was analysed to determine the percentage change in values between visit one and two for the full group and subgroup 2. For the full group, the percentage change in HR values is variable with a reduction in systolic blood pressure, fractal dimension and LF/HF ratio (see table 11). For subgroup 2, the results were again variable with the largest percentage change for PNN50 (28.06%). The same number of parameters for this group showed a percentage reduction post vaccination.

Analysis of resting HRV was performed using a paired t-test. A table of results (Table 11) summarises the differences between measures of resting heart rate variability and blood pressure prior to and following influenza vaccination for the full volunteer group of 71 volunteers and the 15 volunteers who subsequently reported significant post-vaccination symptoms. Other measures include Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. There

were no statistically significant differences between any measures pre and post vaccination for the full cohort or for subgroup 2.

Table 11 Summary of the differences between measures of resting heart rate variability and blood pressure prior to and following influenza vaccination for the full group, and Category B subgroup 2.

Parameter	Pre-vaccination Mean (±SD)	Post-vaccination Mean (±SD)	% diff (V1-V2)	P value
Full group (71 volunteers)				
**Resting Heart Rate (bpm)	73.45 (10.91)	74.34 (10.45)	1.21	0.285
*Systolic Blood Pressure (mmHg)	124.1 (30.1)	117.8 (32.2)	-5.08	0.103
Fractal Dimension	1.39 (0.12)	1.36 (0.19)	-0.03	0.166
SDNN (ms)	46.66 (25.84)	57.45 (59.46)	23.12	0.137
PNN50 (%)	8.80 (11.61)	8.87 (11.63)	0.8	0.960
LF/HF Ratio	1.07 (0.08)	1.05 (0.14)	-1.87	0.341
Category B, Subgroup 2 (15 volunteers)				
Resting Heart Rate (bpm)	76.90 (13.65)	76.86 (14.56)	-0.05	0.977
Systolic Blood Pressure (mmHg)	124.1 (26.8)	115.6 (24.6)	-6.85	0.225
Fractal Dimension	1.390 (0.09)	1.43 (0.12)	2.88	0.176
SDNN (ms)	42.60 (24.69)	46.61 (19.68)	9.39	0.551
PNN50 (%)	8.09 (9.09)	10.36 (13.88)	28.06	0.459
LF/HF Ratio	1.37 (0.66)	1.36 (0.61)	-0.73	0.703

Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. BP taken with automated device. ** Over extended resting period*

4.8 Frequency Domain Parameters during 2 Minutes Metronome Guided Breathing at 10 breaths per Minute Breathing Rate Pre and Post Vaccination.

A two minute breathing exercise at a fixed rate of 10 breaths per minute was also performed. There are fewer participants in the group for the 10 breaths per minute

protocol, as this particular test has not been performed for all participants at the start of recruitment and data collection.

Firstly the HRV results during breathing at 10 breaths per minute were correlated against age. The results show an inverse correlation for all of the parameters with age (see Table 12), which is in keeping with the 6 breaths per minute results and with previously, published research (Perring and Jones 2003). All parameters show a statistically significant deterioration with age; including CORR and FCORR at 10 breaths per minute (see Table 12). By contrast CORR and FCORR showed no significant decay with age at 6 breaths per minute, (see Table 8).

Table 12 Correlation between Age and HRV Parameters for all volunteers during metronome guided breathing at 10 breaths per minute.

Parameter	Pearson's Correlation Coefficient	t-value	P value
SD (bpm)	-0.5149	4.8	**P = 0.001**
A (bpm)	-0.5569	5.36	**P = 0.001**
LPP (unitless)	-0.5267	4.96	**P = 0.001**
CORR (unitless)	-0.3101	2.61	**P= 0.011**
FCORR (unitless)	-0.3083	2.59	**P= 0.012**

Correlation was performed using Pearson's correlation coefficient. Parameters shown are explained in section 3.10.6. Significant values are shown in bold.

Secondly we did not see significant differences between many of the frequency parameters before and after the vaccination however, analysis shows a statistically significant result in the full group for LPP (P=0.03) and category B, subgroup 1 (P=0.01) for LPP, which showed a decay greater than would be expected as a result of random fluctuations at the 5% level. The reasons for this are unclear; however it is likely that the 10 breaths per minute breathing rate is not enough of a provocation to promote large changes in HRV. No parameters showed any significant deterioration as a result of the influenza vaccination in Category B, subgroup 2.

Table 13. Table showing comparison of HRV parameters pre and post influenza vaccination for two minutes of metronome breathing at 10 breaths per minute.

	Total Group N=66	**Category B N=38**	**Category B, Sub-group 2 N=14**
SD (bpm)	P=0.217	P=0.058	P = 0.226
A (bpm)	P = 0.325	P=0.127	P = 0.351
LPP (unitless)	**P=0.029**	P =0.009	P = 0.091
CORR (unitless)	P=0.958	P=0.894	P = 0.665
FCORR (unitless)	P=0.665	P= 0.838	P = 0.613

A paired t-test was performed for HRV data for the full group and two subgroups. P values were calculated at P=<0.05 significance level. The significant results are shown in bold. There is no statistically significant difference between results. Parameters shown are described in section 3.10.6

4.9 Iris/pupil response to dark pre and post vaccination

The right eye was photographed while in the dark using infrared goggles. The pupillary adjustment to dark and to a flash of light in the contra-lateral eye was recorded. The iris: pupil ratio was calculated using the medical physics pupillometry computer programme. The result is compared to an age matched normal range and the number of standard deviations from the normal range is quoted. The iris: pupil ratio was calculated for all of the volunteers. The percentage change in iris: pupil ratio was calculated between visit one and two. The largest percentage change was seen in Category B (N=40), which was reduced by 46.15% (see table 14).

The pre and post vaccination values were statistically analysed using a paired t-test. The results show there was no evidence of a statistically significant decrease in the pupil response to darkness in the full sample (N=71) and in Subgroup 2 (N=15), despite a 23.9% and 34.7% reduction. Interestingly enough there was statistically significant difference (P=0.027) in pupil/iris responses was seen in Category B (N=40) (see Table 14 and Figure 24). Subgroup 2 was too small to show a significant difference pre and post vaccination. This is discussed further in chapter 5.

Table 14. Iris/pupil response to dark pre and post influenza vaccination for all volunteers

	Mean (standard deviation from normal mean) Pre vaccination (±SD)	**Mean (standard deviation from normal mean) Post vaccination (±SD)**	**% Diff (V1-V2)**	**P Val**
Total Group N=71	-0.46 (1.32)	-0.57 (1.44)	-23.9	P = 0.171
Category B N=40	-0.39 (1.47)	-0.57 (1.35)	-46.15	**P = 0.027**
Category B Sub-group 2 N=15	-0.46 (1.3)	-0.62 (1.4)	-34.7	P= 0.237

Mean and SD data for the full cohort and symptomatic subgroups. Analysis was performed. P values were calculated using a paired t-test. Significant results (P=≤0.05) are shown in bold.

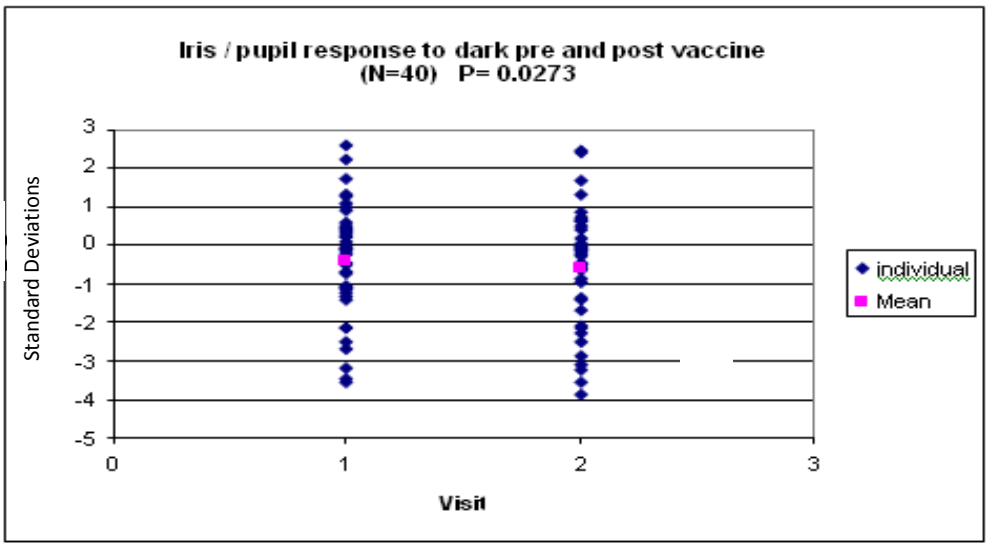


Figure 24. Mean difference in Iris/pupil response to dark pre (visit 1) and post (visit 2) vaccination for the symptomatic group B (N=40).

*Mean values are shown for visit one and two, there is a significant statistical difference between the pupillometry data (p=≤0.027**3**)This graph shows the mean difference in the number of standard deviations above or below the age matched normal range for iris:pupil ratio measures for subgroup category B (N=40) pre and post vaccination.*

4.9.1 Inter-operative Variability for Assessment of Iris/Pupil Ratio Measurements

The pupillometry data from the original cohort of volunteers from the first vaccination period (n=45) were assessed to establish age matched mean prior to and post vaccination with a view to investigating inter-operator variability. The same assessment was performed by two independent assessors for the same data to determine the repeatability of the technique. The standard deviation results were then compared to determine whether there was variability between assessors. There is no significant statistical difference between the assessors for the data for either of the visits (p=0.62 and 0.61 respectively). (The results are shown in Figure 25).

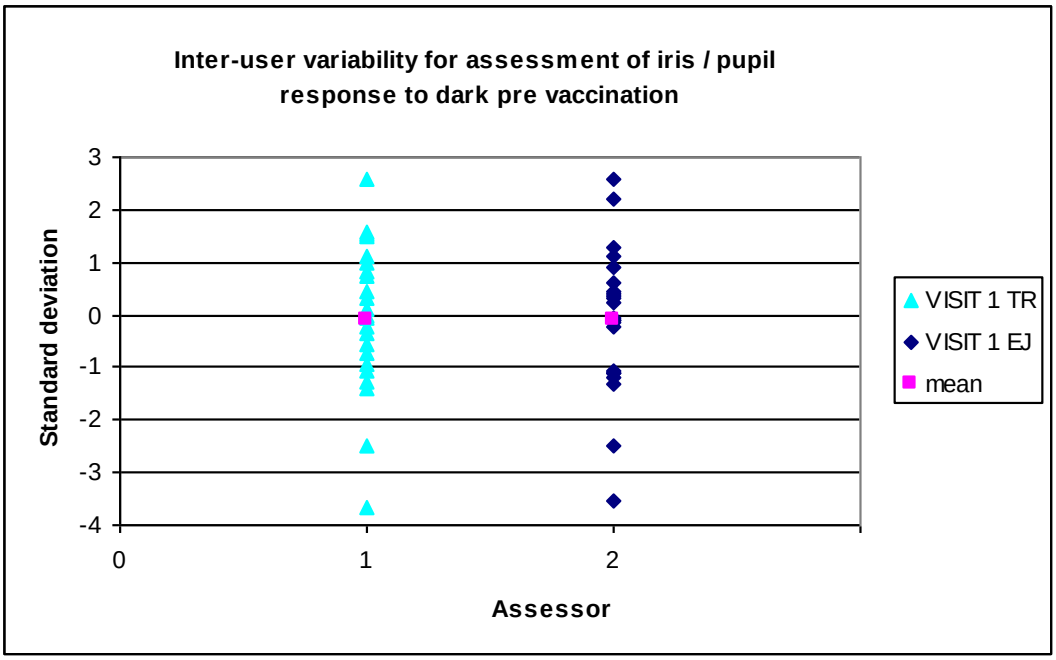


Figure 25. Graph showing inter-operative variability for assessment of Iris/pupil ratio measurements.

Graph showing pupillometry results for 2 independent reviewers assessing the same 45 participants for visit one. There was no statistical difference between reviewers the mean of the data for both assessors is shown.

4.9.2 Factors Affecting the Outcomes of the Influenza Study:

There were a number of factors affecting the outcome of the study. These are examined in turn in the following segments.

A: Reliability (reproducibility) of the data

In order to assess the reliability (reproducibility) of our collected data, we conducted repeat autonomic function testing on thirteen healthy volunteers separated by 2-5 days, where no vaccination was given. The percentage change for all measures of AF was variable with the majority of parameters reducing between visits. The results of statistical analysis show that the resting parameter and LPP were statistically different pre and post vaccination.

Table 15 Results for autonomic function testing on 13 healthy volunteers tested and retested 2-5 days apart.

Parameter	V1 Mean (±SD)	V2 Mean (±SD)	% Diff (V1-V2)	P Val
Ewing Measures				
Resting (%)	8.8 (5.0)	10.7 (5.37)	21.59	**0.05**
Forced breathing (%)	23.3 (10.8)	22.7 (11.7)	-2.58	0.72
I/E Diff (bpm)	16.66 (7.14)	15.63 (7.2)	-6.16	0.47
Valsalva (ratio)	1.39 (0.17)	1.38 (0.24)	-0.72	0.55
Handgrip (ratio)	1.16 (0.09)	1.17 (0.1)	0.86	0.51
Metronome breathing				
SD (bpm)	1.31 (1.66)	1.2 (1.49)	-8.4	0.36
A (bpm)	1.08 (1.62)	0.99 (1.51)	-8.3	0.11
CORR (unitless)	-0.038 (1.06)	-0.028 (1.55)	26.3	0.83
LPP (unitless)	0.87 (0.96)	0.71 (1.08)	-18.4	**0.01**
FCORR (unitless)	0.09 (0.42)	0.1 (1.26)	11.1	0.24
DEV	0.92 (1.24)	0.71 (1.43)	-22.8	0.16

Mean, SD, and % difference for the 13 volunteers for repeat visits. Analysis performed using a paired t-test (P=<0.05) Significant values are shown in bold. Parameters are described in section 3.10.6.

B: Validity

I Comparison of normal data from influenza study and previous normal range data

In order to fulfil the second aim of the project, the autonomic function data collected from 71 healthy individual's pre vaccination was compared with previous data collected for 44 healthy volunteers to validate our existing normal range. After statistical comparison, there was no statistically significant difference between parameters for the full group n=71 compared to the previous normal range data (n=44) apart from the SD parameter (p=0.007) (see Table 16).

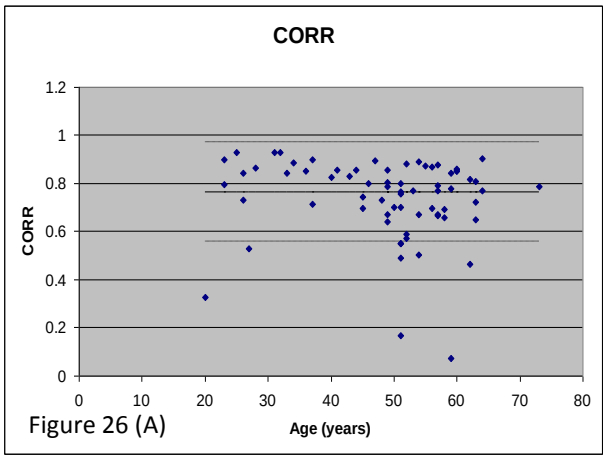
We conclude that:

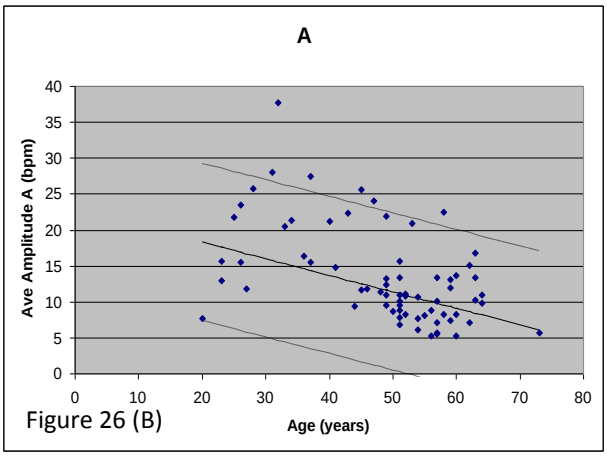
- The calculations used to generate the overall deviation (DEV) parameter appear to be valid.
- The correlation coefficient for all parameters measured for the 6 breaths per minute provocation appears to be similar in the new dataset compared to the original normal dataset.
- The automated calculation is valid based on the original normal dataset for future normal and patient studies.

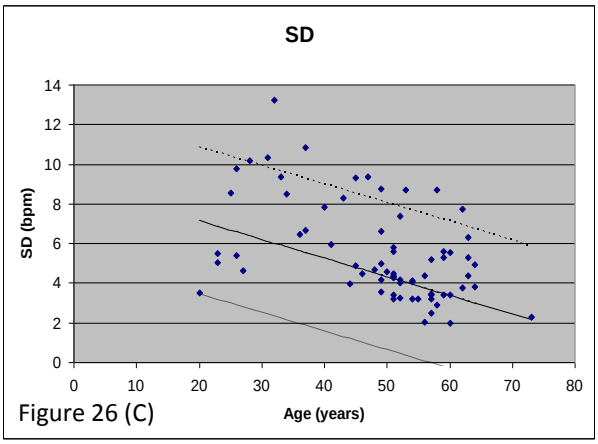
Table 16. Comparison of present normal (pre-flu) sample (N=71) with past normal data (n=44)

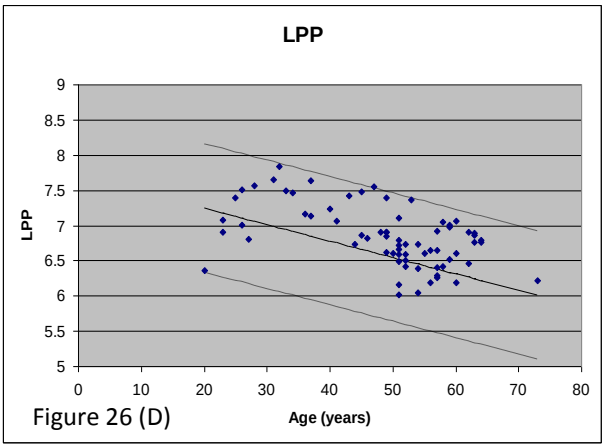
Parameter	Original normal group N=44 (SD's from age-matched norm)	New normal group N=71, 1st visit (SD's from age-matched norm)	t value	P value
A (bpm)	0+/- 1.0	0.373 +/-1.032	1.69	0.093
SD (bpm)	0+/- 1.0	0.631 +/-1.122	2.75	**0.007**
CORR (unitless)	0+/- 1.0	-0.289 +/-1.799	1.13	0.259
LPP (unitless)	0 +/- 1.0	0.652 +/-0.837	0.89	0.375
FCORR (unitless)	0 +/- 1.0	-0.104 +/-1.192	0.43	0.668
DEV	0 +/- 1.0	0.292 +/-1.188	0.45	0.654

SD from age matched normal value is shown for the original group (N=44) and the pre vaccination influenza group (N=71). Statistical analysis was performed using a student's t test for unpaired data. Significant results in bold.









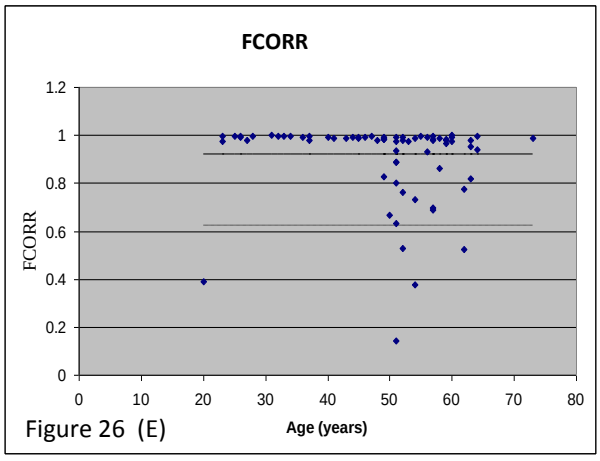


Figure 26 Graphs representing HRV parameters showing the line of best fit for each parameter and +/- two standard deviations from the age matched normal range obtained from the original study in 2003.

Graph (A) showing Maximum Correlation (CORR) between HR and chest wall plethysmography breathing. Graph (B) showing Average peak to trough HR amplitude (BPM). Graph (C) showing standard deviation heart rate variation (±SD). Graph (D) showing Log amplitude of peak of the frequency power curve (LPP) following FFT. Graph (E) showing Correlation of breathing and HR following FFT (FCORR).

C: Type of Influenza Vaccine Used

The response to the vaccination was assessed in terms of the level of symptoms experienced. We noted 46% of volunteers in the first vaccination period (2008/09) experienced a degree of symptoms from the vaccination, 17% of these were significant symptoms. Compared with the 2009/10 vaccination, where 75% experienced symptoms, 29% of which were more significant symptoms (see Table 17). Statistical analysis of the data using Fishers Exact did not show any difference in the number of symptomatic volunteers between the 2 vaccinations (P> 0.05).

Table 17. Number of volunteers experiencing symptoms after influenza vaccination.

	2008/09 Vaccination	**2009/10 Vaccination**
Number of volunteers in each cohort	47	24
Number of volunteers experiencing any symptoms post vaccination and percentage of total in each group	22 (46%)	18 (75%)
Number of volunteers experiencing the most significant symptoms and percentage of total in each group	8 (17%)	7 (29%)

Participant numbers in each vaccination period and the percentage of the total in each period. Sub groups of the total shown with definition of the level of symptoms experienced after vaccination

D: Time Delay between Administration of Influenza Vaccination and Assessment of Autonomic Function Testing

The mean time delay between the vaccination and follow up visit was 2.6 days (+/- 1.07). 51% of the volunteers were seen on day two and 84% were seen between day one and three (see Table 18).

Table 18. Time delay (days) between vaccination and autonomic function testing

Number of days between vaccination and assessment	**1**	**2**	**3**	**4**	**5**
% of total group seen within specific time	5.6%	51%	27%	14%	2.8%

Table showing the number of days between vaccination and Autonomic assessment and the percentage of participants who had the autonomic function assessment on each day post vaccination.

E. Age and Gender

Analysis of the ages in both the 2008/09 and 2009/10 cohorts and have shown that there is no significant difference between the cohorts in the two groups ($p=0.471$) (see Table 2).

There was an inverse relationship between HRV parameters at 6 and 10 breaths per minute and increasing age, the results are displayed in Table 8 and Table 12.

There was no significant statistical difference between the male and female subjects for resting heart rate (BPM), however we did not look at all HRV parameters with respect to gender because of the small male sample and bias towards females**.**

Chapter 5: Discussion (Influenza Study)

5.1 The Choice of the Influenza Vaccination and the Outcome of the Study

Data was collected over a two year period incorporating two flu vaccination periods. The trivalent vaccine used during both vaccination periods was produced by the same pharmaceutical company. The vaccine differed slightly in the composition in accordance with the World Health Organisation guidelines. Both contained the same viral A strain. However the Viral B strain in the 2008/09 vaccine was the Florida/4/2006. Whereas the viral B strain in the 2009/10 vaccine was Brisbane/60/2008. This is in accordance with extrapolation of the evidence for the strain most likely to be most virulent in the forthcoming year.

We noted 46% of volunteers in the first vaccination period (2008/09) experienced a degree of symptoms from the vaccination, 17% of these were significant symptoms. Compared with the 2009/10 vaccination where 75% experienced symptoms, 29% of which were more significant symptoms (see Table 17). Despite these findings statistical analysis of the data using Fishers Exact did not show any difference in the number of symptomatic volunteers between the 2 vaccinations ($P > 0.05$).

The protocol for administration of the vaccine was the same for both periods and the personnel in the Occupational Health department were the same. None of the volunteers who were vaccinated in the first year were recruited during the second year.

The flu vaccine was the chosen provocation necessary to induce a mild inflammatory response in the volunteers. We chose it primarily because of the availability within the research setting and logistically it was relatively easy to recruit volunteers, as hospital staff were asked to sign up for the vaccination sessions in advance. This project also had to be approved by the ethics committee and this would have been less likely if we had tried to incorporate a more deleterious provocation. We could have used a travel

vaccination such as yellow fever, in place of the influenza vaccine. Tsai et al (2005) suggest yellow fever is a similar model to study the response of mild stimulation of the inflammatory system. One problem with using the yellow fever vaccination is that it is not routinely administered to large numbers of people at one time in the UK in the same way the influenza vaccination is administered and it tends to be given in the community setting by a practice nurse.

In order to increase our recruitment numbers we had to accept that performing the research over two years would mean that the composition of the vaccine was likely to be different for each year. Using two slightly different vaccines did not affect the results because we were not directly comparing the vaccines for any differences; we were comparing heart rate variability for each participant before and after a vaccination.

5.2 Demographic Factors

Large variation in heart rate variability has been described in healthy volunteers by Molgaard et al (1994). Both age and gender have been identified as primary factors, excluding disease, which may affect autonomic function. Increasing age has been shown to have an attenuating effect on autonomic tone in healthy subjects (Bigger et al (1995) and confirmed in Perring and Jones (2003) and in the present study (see Table 8 and Table 12).

There is research to suggest that gender bias is present for autonomic tone with men displaying higher values of heart rate variability (Cowan et al 1998). We did not see any significant differences between the gender groups for resting heart rate. This might be due to a small sample size of the study and to the large number of female participants in the study.

The group was not ethnically diverse. There was a significant bias as more than 95% of volunteers were Caucasian. A literature search does not suggest any evidence relating ethnicity to heart rate variability.

Where possible autonomic function assessments were performed at the same time of the day to avoid diurnal variation. This was in practice slightly difficult in the work place setting as we were limited with the timing of the vaccination and when the autonomic function testing could be performed but where possible we adhered to the protocol.

Repeatability was addressed by assessing thirteen healthy volunteers on two occasions, separated by 2-5 days who did not have the influenza vaccination (see Table 15). Autonomic function testing was performed on each occasion. The protocol for testing was the same as per section 3.4.5. After statistical analysis there are two parameters that show significant differences between visits, resting heart rate and LPP (see Table 15). It could be argued the difference in the resting parameter between visits (see Table 7) could simply be due to the way in which the analysis was performed. The percentage change in resting heart rate is calculated using a single point to determine a minimum heart rate and a single point to determine a maximum heart rate selected from one heart rate cycle. In a healthy normal trace the heart rate changes are quite marked and it is down to the operator to choose a maximum and minimum point from one cycle in an extended resting period (see Figure 27). Where the markers are positioned may make a difference to the overall percentage change and therefore although both results may have been above the normal threshold, which is 6%, it is possible that markers were not positioned on the cycle with the highest heart rate, as the differences can be subtle when performing the analysis. In the clinical setting it is accepted that this parameter is quite variable and we do not place much emphasis on the resting heart rate result. On the other hand the LPP result is more difficult to explain, however this is likely to be an anomaly, which may be due to a relative small sample size.

There were no significant differences in the other heart rate variability parameters between repeated autonomic function testing (separated by two to five days) for healthy

volunteers who did not have the influenza vaccination. This suggests that the technique is reproducible.

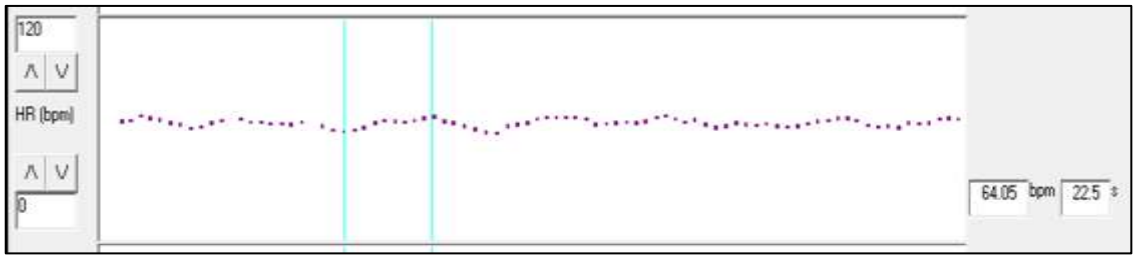


Figure 27 Screenshot of resting heart rate data with markers for minimum and maximum heart rate.

5.3 The Choice of Technique in Assessment of Autonomic Function and the Effect on the Outcome of the Study,

5.3.1 Holter Measurements

Many studies assessing heart rate variability have previously used extended 24 hour Holter measurement (Stein et al 1994, Nada et al 2001). There are several problems surrounding the use of this method. Essentially there is no control over the primary effector parameter on heart rate during the extended measurement period i.e. breathing and no concomitant recording of that parameter measuring any variation during the measurement period. Also there is no control on the other parameters likely to affect heart rate variability i.e. exercise or emotional state.

We opted to use a short sampling time instead of longer Holter measurements. The advantages of short recording periods are that the collection and analysis time is shorter and the data can comply with the assumption made during data analysis that the signal (breathing rate) does not change with time. Short term recordings such as the two minute metronome guided breathing are ideal for following disease progression. One of the problems associated with short recordings is that they only record data on short term reflex responses and they do not include very low frequency (VLF) data. Using

the LF/HF ratio reflects the balance between the two branches of the ANS. This sympatho-vagal balance in a healthy adult is usually between 1 and 2. It was felt that the benefits of controlled metronome guided breathing outweighed the effects on VLF power for the purpose of this project, and that a short sampling time was on balance advantageous.

5.3.2 Limitations of Heart Rate Variability Testing

A limitation of standard heart rate variability testing is that it relies on normal sinus rhythm. Performing autonomic function testing on patients with arrhythmias, frequent ectopic heart beats or significant tremor is often very difficult and may result in an unsatisfactory study. PPG signal can be used in place of ECG where artefact or noise is a limiting factor but may not offer an alternative in all circumstances.

It is indicative that autonomic neuropathy is not universal over all systems uniformly and that assessment of multiple systems in the clinical setting is necessary to ensure best diagnostic power.

5.3.3 Pupillometry

We performed pupillary dark adaptation measures on all volunteers before and after vaccination. During analysis a significant difference was demonstrated in category B (n=40), with respect to the standard deviation of iris/pupil ratio compared to an age matched normal range pre and post vaccination. This difference was not evident in category B, subgroup 2 with the most significant symptoms (n=15) (See Table 14). Possible conclusions that we can draw from this are:

- The sample size for subgroup 2 is too small.
- That the sympathetic branch of the autonomic nervous system controlling the pupil response is less affected by the vaccine and no statistical difference exists between the pre and post vaccination.

- Or that the measurement technique is not sufficiently sensitive to determine low level changes in autonomic tone.

We will continue to collect these data clinically as it is a useful indicator of reduced autonomic tone in patients with more significant autonomic dysfunction and not susceptible to errors as a result of ectopic or erroneous heart rate data.

5.3.4 Heart Rate Variability Findings

We can infer that the mild inflammatory response to the influenza vaccination is associated with a change in HRV between the two visits and had a significant effect on short measures of heart rate variability during metronome guided breathing in the subgroups of symptomatic participants (as shown in Table 9). This is in keeping with work by Lanza et al (2011) who found significant correlation between reduced C-reactive protein (CRP) levels and increased HRV parameters after the administration of atenolol, suggesting a patho-physiological link between inflammation and autonomic regulation.

Analysis of the raw HR data (pre and post vaccination) (shown in Table 10) for subgroup 2, results in significant differences in all metronome guided breathing parameters except for FCORR which does not significantly differ for either the raw heart rate data or the standard deviation data. This may be due to the correlation of the frequency power curves being significantly affected by low level changes in heart rate and the resulting power being spread either side of the signal peak. Care needs to be taken when interpreting the statistical significance of multiple parameters as the likelihood of one reaching the threshold for significance is additive. However, in this case multiple parameters display significant difference pre and post vaccination and we are confident that this is not due to chance.

Analysis of extended period of resting heart rate variability was assessed for the full Influenza group and subgroup 2 prior to and following influenza vaccination. Other

measures include Fractal dimension, SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. There were no statistically significant differences between any measures pre and post vaccination for either group.

We also re-established that the 6 breaths per minute breathing rate is a greater heart rate provocation than spontaneous free breathing during the resting period or breathing at a fixed rate of 10 breaths per minute and resulted in more significant results pre and post vaccination (see Table 9). This is in keeping with previous research performed by Perring and Jones (2003). Several other studies have also documented that the scale of respiratory related fluctuations of HRV significantly changes according to breathing rate, exhibiting the highest response 0.1 Hz and a progressive decrease of the gain as the respiratory frequency increases above this value (Brown et al 1993, Hirsch et al 1981, and Saul et al; 1991).

We have established that there is no statistically significant difference between our previous normal range published in 2003 and the new data collected from healthy volunteers during this study (see Table 16). New normal volunteer data was collected from volunteers who did not have the vaccination. Data analysis suggests the technique is reproducible with no statistical difference between the initial visit and a second visit. However we still need to further refine the normal age matched values during metronome breathing and to establish the reproducibility of these measures.

5.4 Inflammation and Heart Rate Variability

This study showed that acute inflammation in response to the seasonal influenza vaccination lead to statistically significant changes in heart rate variability parameters prior to and post vaccination in the subgroups of symptomatic participants, particularly subgroup 2 (n=15) where participants experienced the most marked symptoms in

response to the vaccination (see Table 9). In keeping with our research, a host of other research has been published reporting a link between other inflammatory disease states and autonomic impairment, Sajadieh et al (2004) found reduced HRV in patients with subclinical inflammation, Libby et al (2002) with atherosclerosis, Vinik (2012) and Lombardi (2004) with Diabetes, Addio et al (2007) with COPD, and Chakraborty et al (1989) with GORD.

Our research found a significant difference in HRV in a subgroup of the most symptomatic participant's i.e. those assumed to have the highest level of inflammation, which is in keeping with other research in the field. We question what physiological mechanisms are involved in this process. It is known that increasing age, medications, infection, and respiratory disease can all have a negative effect on autonomic tone. Corrales-Medina (2012 and 2013) and Vassallo and Allen (1997) discuss a link between infection and respiratory disorders and a reduction in autonomic function. Vassallo and Allen reference Heath et al (1982) citing "*possible damage to fibres in the terminal bronchioles and alveoli from the inflammatory process accompanying pneumonia*." It is still unclear the exact mechanism linking acute inflammation and temporary dysautonomia, however it is likely to be multifactorial. Evidence from Tracey (2007), suggests the presence of the "cholinergic anti-inflammatory pathway" where the vagal nerve is implicated in a "hard wired" connection between nervous and immune systems (Czura and Tracey 2005), with consequent association between heart rate variability and inflammation (Thayer 2009). Stimulation of the vagal nerve attenuates the production of pro-inflammatory cytokines and inhibits the inflammatory process (de Jonge and Ulloa 2007). New research by Martelli et al (2014) supports the theory that inflammation is under the control of an inhibitory neural reflex. However, they refute that parasympathetic and sympathetic systems work together to maintain immunological homeostasis and conclude that the "*cholinergic-anti-inflammatory pathway and the vagus nerve do not constitute the efferent arm of the inflammatory reflex*," although they do not deny that vagal pathways can exert anti-inflammatory actions in acute inflammation.

In clinical conditions characterized by an increase in inflammatory markers, for example diabetes, rheumatoid arthritis, sepsis and acute coronary syndromes, a reduction in heart rate variability is consistently observed in high risk patients, strengthening the connection between inflammation and autonomic dysfunction (Lombardi 2004), this is also in keeping with our findings that showed a decline in HRV in response to the influenza vaccination (Perring and Jones 2012). The connection between inflammation and reduced HRV suggests that potentially many of the inflammatory diseases may be diseases of autonomic dysfunction (Czura and Tracey 2005). We know from previous research (Tsai et al 2005) that after vaccination there is a corresponding increase in inflammatory markers e.g. CRP. Although we did not measure inflammatory markers directly in this project we can infer that the known and published inflammatory response to the influenza vaccine from Tsai et al's work (2005) affected autonomic tone temporarily.

Research by Ziegler et al (1992) suggests regardless of glycaemic control, all patients with diabetes are at risk from developing autonomic complications. Chronic disease such as diabetes results in permanent loss of autonomic tone. The exact cause of this is unknown but it is believed to be a product of demyelinating polyneuropathy (Sharma 2002). It is not clear at what point diabetic dysautonomia is temporary and therefore reversible and at what point it becomes permanent. We do know that short-term changes in autonomic tone were seen in the influenza study (see Table 9 and Table 11). The heart rate changes related to inflammation from the vaccine would be expected to be transient and peak at two days post vaccination and would rapidly diminish, we can therefore measure temporary and reversible changes in HRV related to acute inflammation which may be an important development in the assessment and progression of other inflammatory disease. In the case of diabetes it is chronic disease and poor glycaemic control that is thought to lead to irreversible damage. If assessed and managed early the consequences of the disease to the patient could be potentially reversed or minimised.

Other inflammatory diseases such as asthma, reflux oesophagitis, arthritis, and inflammatory bowel disease may all lead to a temporary reduction in autonomic tone. We have assessed reflux oesophagitis and heart rate variability and the response to proton pump inhibitor therapy, the details are discussed in chapter six.

5.5 The Implications of this Study on Routine Clinical Assessment

Spectral analysis of metronome guided breathing at a rate of six breaths per minute for 2 minutes has shown the most significant difference before and after vaccination. We did not find that other measures were sensitive enough to detect such low level changes. This does not mean that they should be excluded from routine clinical use. Ewing et al (1982) suggested a battery of five tests 3 predominantly parasympathetic (Valsalva Manoeuvre, Deep "forced" breathing and 30:15 ratio after standing) and two sympathetic tests, (blood pressure response to standing and sustained handgrip (30% maximum for 5 minutes). O'Brien et al (1986) suggest a similar regime but with less repetition of provocation making it quicker to perform. Ewing et al (1982) used one normal range regardless of age, which gave false negative results among younger patients and false positives among the elderly patients. O'Brien et al (1986) used an age match normal range to overcome this problem. They suggested maximum-minimum heart rate during single deep breath, maximum – minimum heart rate during Valsalva Manoeuvre, (taking the maximum after the Valsalva has been performed) and heart rate ratio on standing at 15 and 30 beats after standing. In 1996 The Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology defined and published standards for clinical use and interpretation of heart rate variability "*Time domain, geometric measures and frequency domain constitute the standard clinically used parameters."*

5.6 Limitations of the Study

Breathing was measured indirectly through first approximation of chest wall movement measured by changes in a pressure band worn around the chest. Direct measurement of tidal breathing was not measured due to unavailability of equipment and the chest cuff was not calibrated prior to use. The measurement of chest wall movement as an approximation of patient breathing was a limiting factor in this project. To investigate accurately the relationship between Heart Rate and Breathing, a more accurate measure of instantaneous air flow and ideally intra-thoracic pressure would be needed. However as we are looking at heart rate changes relating to controlled cyclical deep breathing, absolute measures of lung function are not necessary and for assessment of autonomic function (heart rate variability) this technique is adequate.

We assessed volunteers prior to the vaccination and as close to two days post vaccination as possible. The occupational health department vaccinated approximately one thousand members of staff on eight set dates over approximately 4 weeks and we tried to fit in around their schedule. Some of the "flu clinics" were drop in clinics, where booking was not required and the other sessions were pre-booked in advance. It was the pre-booked people we were able to contact prior to the vaccination appointment. Vaccination takes approximately two minutes. Our study took approximately 20 minutes on each visit so we were unable to see the volunteers at the same rate as the occupational health department. We struggled to cope with the number of volunteers recruited. On the days following the flu clinics, we were performing autonomic function studies in 20 minute sessions from 8am. This was on top of routine clinical commitments. We also had a problem when the flu clinics fell on a Thursday or Friday as it meant patients wouldn't be seen again for the second assessment until Monday which would put the timing beyond the optimum 2 day follow up. We therefore could not include people vaccinated on Thursday and where possible avoided recruiting people vaccinated on Friday.

We acknowledge a significant drawback for this study was the lack of provision in the protocol to take blood samples for inflammatory markers. The project was performed on a minimal budget and there was no financial provision made for such testing. We did not have staff competent in phlebotomy techniques within the immediate department, or storage and analysis equipment for blood samples, which would have incurred further costs. We also thought that it would be more difficult to recruit if blood samples were part of the protocol. If this study is repeated in the future it would be the first amendment that would be made to the protocol.

5.7 Future Work

Vassallo and Allen (1997) identified that patients often remain non-specifically ill for a significant period following pneumonia and recovery takes much longer than would be expected. Mr Middleton, Consultant Orthopaedic surgeon spoke at a post graduate research meeting about hip replacement and he observed that there appears to be a distinct group of patients who undergo total hip replacement, who do not seem to recover as quickly as might be expected which may be related to a drop in autonomic tone and we posit that we could potentially identify those patients by autonomic screening prior to surgery. A consultant in elderly medicine at Poole hospital has recently contacted our department to discuss a similar phenomenon involving patients under his care. He has noticed anecdotally that elderly patients with pre-existing co-morbidities often seem to take much longer to recover from influenza than would be expected. He has noted that the non-specific ill health cited by Vassallo and Allen following inflammatory response to influenza seems to last for considerably longer than expected but usually resolves before the next follow up appointment several weeks later where the patient reports an improvement. We would like to be involved in the clinical assessment of HRV in this group of patients.

I intend to perform a small study to establish whether changing the shape of the respiratory curve makes any difference to heart rate variability. This is on-going.

There is a need to further refine our age matched normal ranges of autonomic heart rate variation based on metronome guided breathing and simultaneous measurement of chest plethysmography.

This project has been written up and has been published in: Clinical Physiology and Functional Imaging 2012. A copy of the published document is included in the appendices (see appendix 16.1).
A longer term study following up the most symptomatic volunteers at set periods after the vaccination would have been useful to establish when the autonomic tone returns to its pre vaccination state. This was not in the ethics protocol for this study and was not performed.

The influenza vaccination and heart rate variability project looked at the influence of an acute inflammatory response triggered by the influenza vaccination on the autonomic nervous system. It is suggested that this inflammatory state is temporary and blood markers suggest it peaks at two days and diminishes rapidly thereafter. I will look at the data and reanalyse it according to the number of days post vaccination, days 2-5.

The next phase of this research was to look at the effects of a more chronic inflammatory state. The provocation chosen for the next research project was erosive oesophagitis related to gastro-oesophageal reflux disease. It is established in previous research that the presence of erosive oesophagitis significantly reduces autonomic tone. This will be discussed in more detail in chapter 6.

Chapter 6: Evaluation of the Effects of Proton Pump Inhibitors on Autonomic Tone in Patients with Erosive or Non Erosive Oesophagitis.

6.1 Autonomic Nervous System and the Gastrointestinal System

It has been suggested that there is a link between gastroesophageal reflux disease (GORD) and impaired cardiovascular autonomic function, assessed by heart rate variability measurement. Chen (2006) found that the autonomic function appears to differ significantly between patients with and without erosive oesophagitis. Lee et al (2004) suggested that it is the structural state of the oesophagus and not the symptomology, which dictates the autonomic function status.

This study will examine heart rate variability in two patient groups, those with mild erosive reflux (oesophagitis) disease (ERD) and those with non-erosive reflux disease (NERD). It will also assess symptom severity and the change in autonomic tone in the two groups after an eight week healing dose of proton pump inhibitor therapy.

6.2 Gastro-Oesophageal Reflux Disease and Erosive and Non Erosive Oesophagitis

6.2.1 Terminology

Interchangeable terms are frequently used to describe gastro-oesophageal reflux disease and are often abbreviated to either GORD or GERD, the former is English, the latter is the American spelling but both are commonly used to mean the same thing.

6.2.2 GORD: What are the Causes and Symptoms of Acid Reflux?

There are several theories as to the causes of reflux. It is generally thought that the muscle of the lower oesophageal sphincter becomes weak or is prone to Spontaneous Transient Lower Oesophageal Sphincter Relaxations (TLOSR's) (Bredenoord et al 2006)). Several other contributory factors can also promote acid reflux, such as smoking, or drinking too much alcohol. Being overweight or wearing tight clothing can also increase the upward pressure on the gastro-oesophageal junction between the oesophagus and the stomach (Ayazi et al 2007). Some people will notice that certain foods promote reflux, particularly acidic, rich, spicy or fatty foods. In some cases acid reflux is associated with the presence of a hiatus hernia, which prevents the muscle at the base of the oesophagus from functioning (Sontag 1999).

Symptoms of gastroesophageal reflux disease (GORD) are very common, affecting 9% to 17% of the population of Europe, 20% in North America, 12% to 15% of Australia, and 2% to 5% of Asia once a week (Fass 2005). Acid reflux causes a range of symptoms including a burning sensation in the upper abdomen and lower chest, sometimes moving up into the throat. The oesophagus does not have the same protective lining as the stomach and therefore the acid and other digestive enzymes normally produced by the stomach can cause a burning sensation. It is often worse at night or when supine or can be brought on by bending or physical exercise, particularly after a meal. This sensation or discomfort is most often referred to as heartburn. Other typical symptoms associated with reflux are epigastric pain, retrosternal pain, belching, dysphagia, and regurgitation.

6.2.3 Reflux Oesophagitis

If the lower oesophageal sphincter (LOS) is weak or relaxes inappropriately, stomach contents flow into the distal oesophagus causing irritation and inflammation (De Vault et al 1999). Oesophagitis is inflammation of the lining of the oesophagus. In most people this is caused by the digestive enzymes in the stomach, repeatedly moving upwards into the lower oesophagus. The oesophageal mucosa becomes inflamed and has a blistered appearance (Sontag et al 2006).

6.2.3.1 Grading Erosive Oesophagitis.

When the oesophagus is assessed using endoscopy, the practitioner looks for evidence of reflux oesophagitis and the severity? Oesophagitis is graded according to its severity to improve consistency of reporting among clinicians. The Los Angeles Classification for diagnosis and grading of reflux oesophagitis was published in 1999 (Dent 2008) and then further recommended in a paper by Katz et al (2013.).

6.2.3.2 Erosive Oesophagitis: Los Angeles Classification

Grade A
One (or more) mucosal break no longer than 5 mm that does not extend between the tops of two mucosal folds

Grade B
One (or more) mucosal break more than 5 mm that does not extend between the tops of two mucosal folds

Grade C
One (or more) mucosal break that is continuous between the tops of two mucosal folds but which involves less than 75% of the circumference

Grade D
One (or more) mucosal break, which involves at least 75% of the oesophageal circumference.

6.2.3.3 Non Erosive Oesophagitis (endoscopy negative reflux disease).

The number of patients presenting with symptoms of typical gastro-oesophageal reflux disease (GORD) but do not have any endoscopic evidence of erosive oesophagitis is actually higher than those with reflux oesophagitis (Fass 2005). Compared with patients with erosive oesophagitis, the non-erosive reflux disease (NERD) patients tend to be younger, female, and lack a hiatus hernia (Fass 2006). The lack of endoscopic evidence of reflux in the non-erosive group does not mean that these patients are not experiencing GORD. Further assessment via 24 hour pH and impedance measurement often reveals a high level of acid reflux outside of physiological limits.

6.2.3.4 Erosive and Non Erosive Oesophagitis and the Effect on Autonomic Function

Historically the link between Gastroesophageal reflux disease (GORD) and impaired cardiovascular autonomic function has been examined in research by Chakraborty et al (1989). Recent research by Chen (2006) has looked at the differences in autonomic function in patients with and without erosive oesophagitis; assessed by clinical symptoms, endoscopy, and 24hr pH measurement. Research showed that autonomic tone was reduced in the erosive oesophagitis patients. Chen (2006) found that the symptoms severity scoring was the same regardless of the presence or absence of erosive oesophagitis but the autonomic function appears to differ significantly between the two groups, with spectral analysis of heart rate variability significantly reduced in the high frequency power band in patients with erosive oesophagitis (neurogenic inflammation) (Maggi and Meli 1988). Lee et al (2004) also found reduced autonomic tone in patients with erosive oesophagitis (even in patients without symptoms) compared with the non-erosive group. Chakraborty (1989) and Cunningham (1991) both demonstrated reduced autonomic tone in patients with erosive oesophagitis and abnormal ambulatory 24 hour pH measurement.

There is an established link between oesophageal motility and reduced autonomic tone. Autonomic neuropathies constitute disorders of extrinsic innervation. These may affect both the sympathetic and parasympathetic systems or may selectively involve cholinergic or adrenergic function (Khurana 1988). Oesophageal acid exposure is generally associated with a decrease in autonomic tone. A predominant parasympathetic fluctuation during sleeping and a superimposed sympathetic interaction during waking dictate diurnal characteristics of autonomic regulation (Lee et al 2006).

Cunningham et al (1991) studied the prevalence of and relations between autonomic nerve dysfunction (as assessed by cardiovascular reflex tests) and oesophageal transit, oesophageal motility, gastric emptying, and endoscopic grade of oesophagitis. They concluded that in gastro-oesophageal reflux disease there is a high prevalence of parasympathetic nerve dysfunction, which relates to delayed oesophageal transit and abnormal peristalsis and may therefore be of pathogenic importance. They report that there is no correlation between the presence of autonomic dysfunction and the endoscopic grade of oesophagitis.

6.2.4 Proton Pump Inhibitor (PPI) Therapy

Proton Pump Inhibitor (PPI) therapy is a common treatment for GORD related symptoms. PPI therapy has been commercially available since the 1980's and is some of the most widely prescribed medication in the world. Caro et al (2001) performed a Meta-analysis of 53 studies involving acute PPI therapy and maintenance PPI therapy. These studies looked at the PPI healing rates for erosive oesophagitis at eight weeks. All PPI's showed a greater degree of healing oesophagitis at eight weeks than ranitidine (H_2-receptor antagonist) or placebo and all maintained similar rates of symptom control. Chiba et al (1997) show that 4 – 12 week PPI therapy heals oesophagitis twice as fast as Histamine receptor antagonists (H2RA's) peaking at 6 weeks and determined that PPI's irrespective of dose or duration of treatment provided the greatest overall symptom relief. Dean et al (2004) suggest healing at 4 weeks may

not show full therapeutic gain in patients with NERD and that the trend of symptom improvement may well takes at least eight weeks. We opted to follow up patients in this study eight weeks after commencing PPI therapy to allow for complete healing.

6.2.4.1 PPI Therapy and the Autonomic Nervous System.

An extensive literature and Internet search has found no evidence to suggest that PPI therapy has any known effect on the autonomic nervous system. The Medicine Information Department at Astra Zeneca could not find information on any known effects of Proton Pump Inhibitors on the Autonomic Nervous System.

6.3 Rationale

The first part of this PhD research explored the acute inflammatory effects of the influenza vaccine on autonomic function. We were able to perform experimental research on healthy volunteers collecting pre-test and post-test data, looking at subtle and temporary changes in autonomic tone in response to the vaccination provocation. The influenza vaccine allowed us to study a relatively large number of volunteers in a controlled hospital setting, assessing HRV before and after vaccination.

The results of the preceding influenza study suggest a temporary reduction in autonomic tone with respect to temporal and frequency measures of heart rate variability post vaccination. This is particularly significant in a small sub-group of participants (n=15) who reported the most notable symptoms after the vaccination (see Table 9).

Another group of patients we are interested in studying are those with gastro-oesophageal reflux disease. We routinely provide a range of GI physiological assessments and work closely with endoscopy, gastroenterology and surgery. A paper by Farmer et al (2013) recently discussed the role of the autonomic nervous system during oesophageal intubation and the relationship of stress to intubation. We know

that the gut is driven by the autonomic nervous system (Olsson and Holmgren 2011), and hypothesize in a similar fashion to Chen et al (2006) that the presence of erosive oesophagitis and associated inflammation caused by gastroesophageal reflux disease (GORD) may be enough to cause a change in HRV, particularly for those with erosive oesophagitis. This was also investigated by Lee et al (2004), who concluded autonomic tone was lower in the erosive oesophagitis group than for the non-erosive oesophagitis group. Although previous research has shown a relationship between inflammation (oesophagitis) and reduced HRV, none have demonstrated whether or not it is a reversible process. We posit any resulting attenuation in autonomic function may be a temporary process, which is reversible after healing of the inflammation from eight weeks of PPI therapy. We aim to establish whether there is a measureable difference in autonomic tone through measurement of heart rate variability (HRV) prior to, and after eight weeks of PPI therapy which research by Caro et al (2001) suggests should result in complete healing of erosive oesophagitis caused by GORD. After healing of the oesophagitis has occurred we would expect an improvement in autonomic tone (increase in HRV) compared to the baseline visit. This will be supported by the GERD Impact Scale scores, which will be collected before and after eight weeks of PPI treatment for both the NERD and ERD groups, where we expect a significant decrease in symptom scores relating to an improvement in symptoms. Other research in this field has not looked at changes in HRV in this way.

There is also controversy surrounding the non-erosive oesophagitis group with conflicting results relating to the effect of inflammation on HRV in this particular group. We would also like to examine whether there is any change in autonomic tone in patients symptomatic of GORD who are identified endoscopically as having non-erosive reflux disease (endoscopy negative), but have other markers for GORD such as a hiatus hernia or lax lower oesophageal sphincter. In this instance, we would posit that i) autonomic performance in the non-erosive group would be the same prior to and after eight weeks of PPI therapy and ii) In the erosive oesophagitis group we would expect differences in autonomic function before and after eight weeks of PPI therapy.

6.4 Aims

The main aims of this part of this study were to:

1. Discern whether there was a difference in autonomic tone between patients with non-erosive oesophagitis disease (NERD) and erosive oesophagitis reflux disease (ERD)
2. Investigate the effects of an 8 week course of anti-reflux treatment (PPI) on autonomic tone in both patient groups.
3. Establish whether the level of response to PPI treatment according to the GERD Impact Scale was associated with the level of improvement in autonomic tone.

Chapter 7: Methods (GORD Study)

7.1 Study Design

A descriptive, cross sectional, pre-test / post-test design was used to assess the effect of erosive and non-erosive reflux disease and the effect of eight weeks of PPI therapy on autonomic performance.
Routine endoscopic investigation was performed by Consultant Gastroenterologist / Surgeon/ Endoscopist who made a clinical judgement regarding the inclusion / exclusion criteria for recruitment for the research project.

7.2 Recruitment

Patients were referred for endoscopic investigation and diagnosis of symptoms associated with GORD. Endoscopic examination was performed in the normal way. If a patient was identified as a suitable candidate for the research project and fulfilled the inclusion / exclusion criterion (see section 7.2.1) they were approached prior to their discharge. A patient invitation letter was given to the patient introducing them to the study along with the patient information leaflet. If sedation had been used during the endoscopy procedure the patients could not be recruited to the study on the same day and were asked to contact us if they wished to take part on a future date.

Patients were informed that taking part in the research project would not be part of their routine care and that they did not have to take part if they did not wish to do so. They were also assured that any decision they made about taking part would not affect their treatment and continuing care in the hospital.

7.2.1 Inclusion Criteria:

- Heartburn
- Retrosternal pain
- Regurgitation
- Epigastric pain
- Non cardiac chest pain
- Patients to be prescribed PPI
- Grade (A) and (B) oesophagitis
- Hiatus hernia
- Lax lower oesophageal sphincter

7.2.2 Exclusion Criteria:

Exclusions remain as per section 3.3.2. The following are related to this study:

GI related exclusions:

- Oesophageal varices,
- Grade (C) and (D) reflux oesophagitis,
- Barrett's oesophagus,
- Achalasia,
- Upper GI pathology apart from oesophagitis,
- Recent upper GI surgery,
- Dysphagia,
- Long term use of PPI

7.2.3 Description of Population under Study

Patients aged 18 – 80 years were recruited by Gastroenterologists, nurse endoscopist and a GI Surgeon. Patients were assigned to two distinct groups based on the endoscopic findings; the erosive oesophagitis group or the non-erosive oesophagitis

group. Aside from reflux disease we recruited an otherwise healthy group of participants with no pre-existing co morbidities.

Statistical power calculations performed by a statistician in the research and development unit at Bournemouth University, suggested that recruitment of thirty patients would provide enough data for the results to be statistically reliable (see appendix 16.3).

7.2.4 Patient Pathway

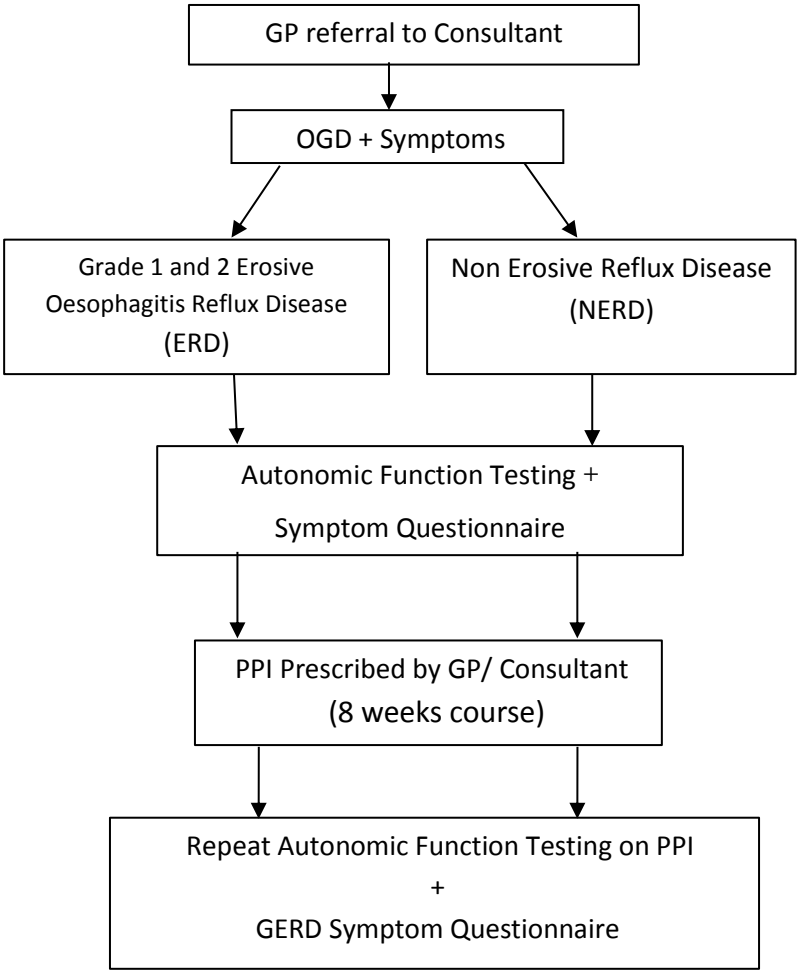


Figure 28. Algorithm to determine route through research pathway.

7.3 Instrumentation

Full details of instrumentation are discussed fully in sections 3.4. The same protocol was followed for this study.

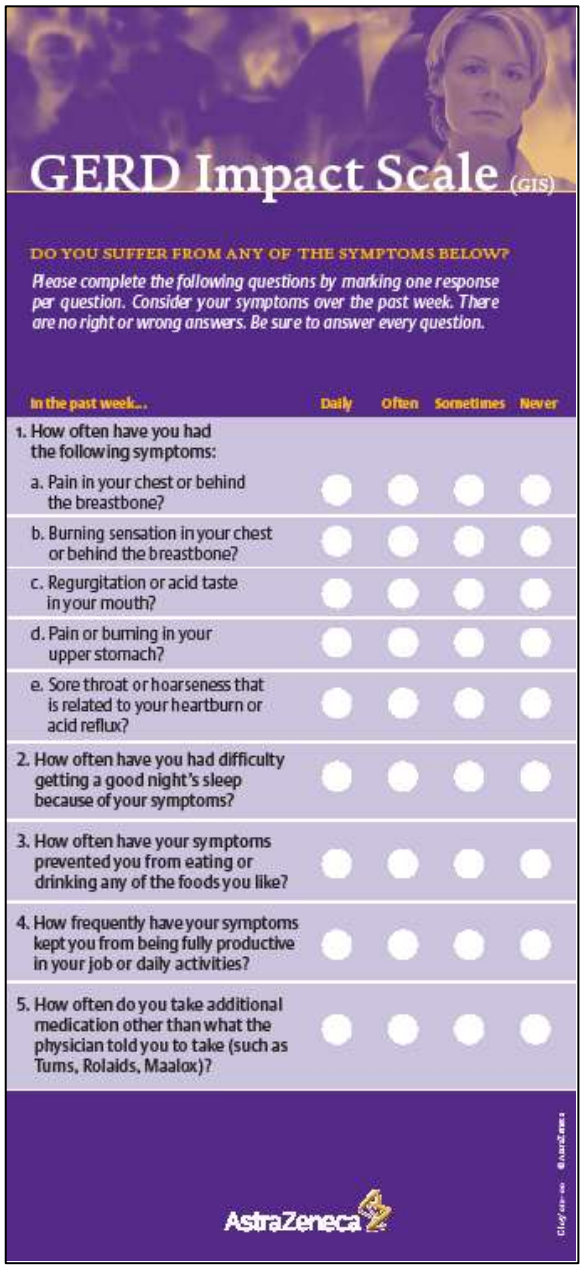
7.4 Preparations Required for the Investigation:

The general preparations for this assessment are as listed in section 3.4.5. The following additional preparations are specific to this study:

- If the patient's GP has previously prescribed a course of PPI treatment this was continued as normal after the initial autonomic function testing has been performed.
- If the patient had not already been prescribed a PPI by the GP the Consultant prescribed an eight week course of PPI treatment.
- Patients were reassessed at approximately eight weeks post endoscopy while still taking PPI medication. Repeat autonomic function testing was performed in the medical physics department.
- A second "GERD Impact Scale" questionnaire was completed after eight weeks of treatment with PPI (see Figure 29).

7.4.1 GERD Impact Scale

The GERD Impact Scale (Figure 29) is a simple method of scoring reflux symptom severity. The symptoms are rated from 1-4 (1) =never (4) =daily. The best possible score would be 9 relating to no reflux symptoms. The worst possible score would be 36 representing daily symptoms.



GERD Impact Scale (GIS)

DO YOU SUFFER FROM ANY OF THE SYMPTOMS BELOW?

Please complete the following questions by marking one response per question. Consider your symptoms over the past week. There are no right or wrong answers. Be sure to answer every question.

In the past week...	Daily	Often	Sometimes	Never
1. How often have you had the following symptoms:				
a. Pain in your chest or behind the breastbone?	○	○	○	○
b. Burning sensation in your chest or behind the breastbone?	○	○	○	○
c. Regurgitation or acid taste in your mouth?	○	○	○	○
d. Pain or burning in your upper stomach?	○	○	○	○
e. Sore throat or hoarseness that is related to your heartburn or acid reflux?	○	○	○	○
2. How often have you had difficulty getting a good night's sleep because of your symptoms?	○	○	○	○
3. How often have your symptoms prevented you from eating or drinking any of the foods you like?	○	○	○	○
4. How frequently have your symptoms kept you from being fully productive in your job or daily activities?	○	○	○	○
5. How often do you take additional medication other than what the physician told you to take (such as Tums, Rolaids, Maalox)?	○	○	○	○

AstraZeneca

Figure 29 GERD Impact Scale

7.5 Ethical Approval

Ethical approval was sought and approved by Hampshire Research Ethics Committee (10/H0504/31) see appendix 16.4.2

Chapter 8: Results (GORD Study)

8.1 Recruitment and Characteristics of the Study Group

This chapter will present the results of the GORD study, including addressing demographics of the study population, examining the mild inflammatory effect of non-erosive and erosive oesophagitis on HRV during autonomic assessment including two minutes of metronome guided breathing and the effect of eight weeks of PPI therapy on autonomic tone.

We recruited 20 participants in total, 12 in the NERD group and 8 in the ERD group; the average age was 50.6 years ±12.2. The average age for the ERD group was slightly higher than the average age for the NERD group however the difference between the two groups was not statistically significant. The recruitment numbers, age and gender of the participants for this study are shown in Table 19.

The age of this cohort was compared with the age of the previous normal range collected from the influenza study (mean 48.6± 11.9) using an unpaired t test. There was no statistically significant difference between the ages of the two groups (P=0.52, t=0.64).

Recruitment was unpredictable and more difficult than expected. We initially recruited 23 patients into the trial. An additional 12 patients were identified but they either declined to take part in the project or were sedated during the gastroscopy and did not contact us. The dropout rate was 13%. Despite best efforts to encourage a repeat visit some of the participants did not attend for follow up.

Table 19. Gender and age for all participants in the GORD project.

	Numbers	Mean Age (yrs.)	SD
Total Group	20	50.6	12.2
NERD Group			
Total	12	50.58	13.67
Female	5	52.2	9.2
Male	7	49.8	16.3
ERD Group			
Total	8	52.5	10.12
Female	4	50.5	9.2
Male	4	53.7	11.8

Mean and standard deviation for age of the males, females and total for the NERD and ERD group.

8.2 Relating the GERD Impact Scale Scores to severity and frequency of symptoms

All participants completed a GERD impact scale questionnaire at both visits. In the ERD and NERD group there was a relationship between a decrease in symptom scale scores and a decrease in the severity or frequency of the symptoms, suggesting after eight weeks of medication the patients experienced less frequent and less severe symptoms related to GERD. Table 20 shows the percentage decrease in symptom scores between the first visit and after eight weeks of PPI treatment.

Analysis of the results for the GERD Impact scale using a paired t-test shows a statistical difference ($p=<0.001$) in the scores for the ERD, and NERD groups between visits one and two, after eight weeks of PPI therapy. The patients reported less frequent and less severe symptoms. The improvement in symptoms are reflected in a lower score on visit 2 representing a reduction of 26.4% and 26.7% in ERD and NERD scores respectively (see Table 20).

Table 20 GERD Impact Scale Scores and percentage reduction in scores for the ERD and NERD groups pre and post 8 week PPI therapy.

	Mean V1 (±SD)	**Mean V2 (±SD)**	**% Difference in GERD Impact scores between visit 1 and 2 (±SD)**	**P Value**
ERD	32.6 (4.3)	24.0 (4.9)	26.4% (7.3)	**P= <0.001**
NERD	30.3 (4.9)	22.2 (4.8)	26.7% (14.2)	**P= <0.001**

Mean (±SD) are given for each group with the analysis performed using a paired t-test. The results are given at (P=0.05) confidence level (in bold). Minimum GERD Impact Scale score = 9 (indicating no reflux symptoms); maximum GERD Impact Scale score = 36 (indicating severe daily symptoms).

8.3 Comparison of GERD Impact Scale scores for ERD and NERD groups for visit one and visit two respectively.

Sub-group analysis showed a statistical difference in scores relating to symptoms pre and post eight week PPI therapy (see Table 20). A lower overall point score on visit two for both groups related to a better outcome i.e. less frequent or less severe symptoms experienced after eight weeks of PPI treatment compared to the initial visit where patients were either not taking their PPI (as per gastroscopy protocol) tablets or had not yet started the course of PPI therapy.

Comparison of the GERD Impact scale scores at baseline for the ERD and NERD group was performed using a Mann Whitney U test. There is no statistical difference between the ERD (32.6 ±4.4) and NERD (30.3 ±4.9) scores collected for visit one (p=0.42). This suggests that neither the ERD nor NERD group were experiencing greater or lesser symptoms than the other.

8.4 Comparison of Baseline Heart Rate Variability for the ERD and NERD Groups with Normal Range Data (N=71)

Heart rate variability at baseline (visit 1) for the ERD and NERD groups was compared with heart rate data from a healthy normal range to establish if HRV is attenuated in either group compared with the healthy normal range (currently used in our medical physics laboratory as normal standard reference values for similar age and gender). The percentage difference between the sets of data is clear, with attenuation in all ERD heart rate variability parameters compared with the normal heart rate data. An unpaired t-test was used to analyse the data. Results show that there are some statistically significant differences between heart rate measures for the ERD group and the normal reference values for the healthy data; in particular, resting ($p=0.05$), Valsalva ($p=0.001$) and CORR ($p=0.03$) as shown in Table 21.

However, the outcome is less clear with the NERD data, as there is either a decrease in half of the HRV parameters or no change in the other half when compared with the normal reference values. Furthermore, there were no statistical differences between NERD data and the normal range data (see Table 21).

Table 21 Comparison of Baseline ERD (N=8) and NERD (N=12) HRV with Normal Range HRV Data (N=71)

	Normal Mean (±SD)	**ERD V1 (Mean (±SD)**	**% Diff**	**P Val**	**Normal Mean (±SD)**	**NERD V1 Mean (±SD)**	**% Diff**	**P Val**
Ewing Measures								
Resting (%)	9.2 (5.7)	5.3 (2.9)	-42.4	**0.05**	9.2 (5.7)	7.0 (4.9)	-23.9	0.20
Forced Breathing (%)	19.5 (0.6)	13.9 (9.1)	-29.1	0.16	19.5 (0.6)	20.6 (14.6)	5.1	0.78
Insp/Exp (bpm)	14.17 (7.7)	9.3 (5.2)	-34.4	0.08	14.17 (7.7)	13.5 (8.9)	-4.7	0.77
Valsalva (ratio)	1.32 (0.2)	1.2 (0.1)	24.2	**0.001**	1.32 (0.2)	1.4 (0.3)	6.0	0.79
Hand grip ratio (ratio)	1.17 (0.1)	1.1 (0.1)	-5.9	0.3	1.17 (0.1)	1.2 (0.1)	2.56	0.13
Metronome Breathing								
SD (bpm)	0.63 (1.8)	-0.34 (1.6)	-153	0.71	0.63 (1.8)	0.89 (1.5)	41	0.5
A (bpm)	0.33 (1.0)	0.16 (1.4)	-51.5	0.6	0.33 (1.0)	0.69 (1.5)	109	0.37
LPP (unitless)	0.63 (1.1)	0.1 (1.0)	-84	0.09	0.63 (1.1)	0.68 (1.0)	7.9	0.90
CORR (unitless)	-0.37 (0.8)	-1.91 (1.9)	-416	**0.03**	-0.37 (0.8)	-0.84 (1.8)	-127	0.42
FCORR (unitless)	-0.10 (1.2)	-0.95 (2.1)	-850	0.07	-0.10 (1.2)	-0.09 (0.6)	-10	0.36
DEV	0.29 (1.2)	-0.46 (1.6)	-258	0.10	0.29 (1.2)	0.27 (1.1)	-6.9	0.74

Mean (±SD) for HRV parameters for the NERD and ERD group and the HRV data from the healthy normal range are shown. Percentage difference is shown between the normal values and the ERD/ NERD values. Analysis using an unpaired t-test was performed (P=<0.05). The significant results are shown in bold. Parameters shown are described in section 3.10.6.

8.5 HRV Parameters for Ewing assessment and during metronome guided breathing for the ERD and NERD Groups

The Ewing tests were performed at baseline and after eight weeks of PPI therapy for the NERD and ERD groups. There was a generalised increase in (i.e., improvement) in HR values for the ERD and NERD group between the two visits indicating an improvement (recovery) in autonomic tone after eight weeks of PPI. Analysis of the results showed no statistically significant differences between the HRV for the ERD group before and after 8 weeks of PPI therapy. There is however an overall trend in the data showing an increase in the mean HRV values, in particular for forced breathing, (see Table 22).

The same observation is also applicable to the NERD group, as we did not see any statistically significant differences in HRV measures in the NERD group. However, there was again evidence of a trend in the data showing an improvement in HRV before and after eight weeks of PPI therapy (see Table 22).

Metronome guided breathing was performed for two minutes at 6 breaths/minute rate. Mean values for the ERD group for certain parameters show a trend toward a recovery in autonomic tone after eight weeks of PPI therapy with a percentage improvement in four of six parameters. However, analysis of the heart rate data showed a statistically significant difference only in the DEV parameter (P=0.05) before and after eight weeks of PPI therapy (see Table 22 and Figure 30).

On the other hand, although certain parameters from the NERD group data also show a trend toward some improvement in autonomic tone after eight weeks of PPI therapy, there were no significant statistical differences in the frequency parameters for the NERD group.

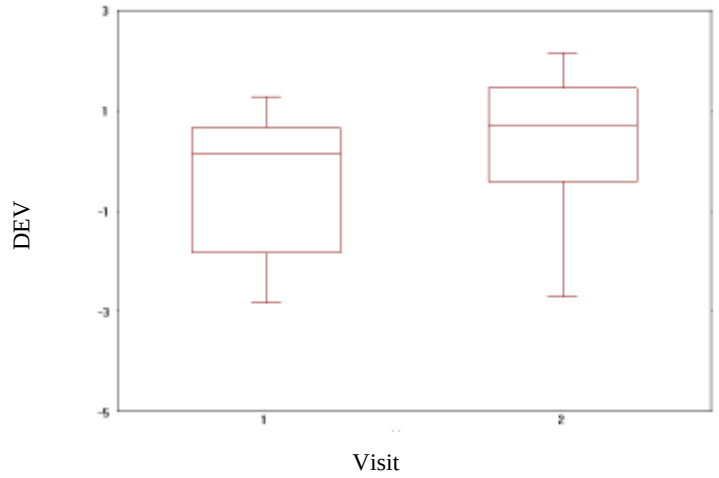


Figure 30 Graph showing the DEV parameter for the ERD group.

Box and whiskers plot showing the DEV parameter before (visit 1) and after eight weeks of PPI (visit 2) for the ERD group. Statistical analysis shows a significant (p=0.05) difference between visits with a higher DEV score (improved HRV) after eight weeks of PPI.

Table 22 Result for all HRV parameters during Ewing assessment and metronome guide breathing for the ERD and NERD group pre and post eight week PPI therapy.

	ERD Group (N=8)				NERD Group (N=12)			
	V1 Mean (±SD)	**V2 (Mean (±SD)**	**% Diff**	**P Val**	**V1 Mean (±SD)**	**V2 Mean (±SD)**	**% Diff**	**P Val**
Ewing Measures								
Resting (%)	5.3 (2.9)	5.4 (3.8)	1.89	0.91	7.0 (4.9)	10.0 (8.4)	42.9	0.12
Forced Breathing (%)	13.9 (9.1)	19.6 (9.7)	41	0.06	20.6 (14.6)	22.7 (13.6)	11.17	0.32
Insp/Exp (bpm)	9.3 (5.2)	12.1 (6.1)	30.1	0.08	13.5 (8.9)	14.6 (9.5)	8.15	0.30
Valsalva (ratio)	1.2 (0.1)	1.3 (0.2)	8.3	0.12	1.4 (0.3)	1.4 (0.23)	0	0.27
Hand grip (ratio)	1.1 (0.1)	1.1 (0.1)	0	0.35	1.2 (0.1)	1.2 (0.08)	0	0.18
Lying / Standing (ratio)	1.0 (0.1)	1.1 (0.1)	10	0.14	1.1 (0.16)	1.0 (0.38)	9.1	0.71
Metronome Breathing								
SD (bpm)	0.34 (1.6)	0.47 (1.7)	38.2	0.55	0.89 (1.5)	0.99 (1.5)	11	0.58
A (bpm)	0.16 (1.4)	0.13 (1.5)	-18.7	0.39	0.69 (1.5)	0.6 (1.5)	-13	0.81
LPP (unitless)	0.1 (1.0)	0.07 (1.1)	-30	0.44	0.68 (1.0)	0.61 (1.0)	10.3	0.62
CORR (unitless)	-1.91 (1.9)	-1.65 (2.2)	13.6	0.80	-0.84 (1.8)	-0.61 (1.8)	27	0.62
FCORR (unitless)	-0.95 (2.1)	-0.64 (2.0)	32	0.49	-0.09 (0.6)	-0.15 (1.3)	-66	0.38
DEV	-0.46 (1.6)	-0.37 (1.6)	19.57	**0.05**	0.27 (1.1)	0.36 (1.4)	33	0.34

Mean (±SD) for HRV parameters for each group are shown. Analysis using a paired t-test was performed (P=<0.05). The significant results are shown in bold. Parameters shown are described in section 3.10.6.

8.6 Summary of Resting Heart Rate Variability Prior to and following eight weeks of PPI therapy

The extended resting heart rate values for both groups were compared before and after PPI therapy. There was an increase in all values between visits apart from SDNN for the ERD group. For the NERD group there was a marginal increase in all values apart from LF/HF ratio. Statistical analysis of the extended period of resting heart rate variability was assessed for the ERD and NERD group. A table of results (Table 23)

summarises the differences between measures of resting heart rate variability prior to and following eight weeks of PPI therapy for the ERD and NERD groups. Other measures include Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. There were no statistically significant differences between any measures pre and post PPI therapy for either group.

Table 23 Summary of the differences between measures of resting heart rate variability prior to and following PPI therapy for the ERD and NERD group.

Parameter	**Mean (±SD) Visit 1**	**Mean (±SD) Visit 2**	**% Diff (V1-V2)**	**P value**
ERD group (N=8)				
*Resting Heart Rate (bpm)	76.9 (12.3)	77.8 (11.65)	1.2	0.89
Fractal Dimension	-1.29 (0.06)	-0.98 (0.87)	24.0	0.36
SDNN (ms)	32.1 (4.07)	30.9 (10.7)	-3.74	0.36
PNN50 (%)	1.66 (1.8)	2.86 (3.6)	72.3	0.34
LF/HF Ratio	2.29 (1.07)	3.55 (1.8)	55	0.12
NERD (N=12)				
*Resting Heart Rate (bpm)	73.8 (13.2)	74.5 (10.91)	0.95	0.28
Fractal Dimension	-1.24 (0.05)	-1.23 (0.06)	0.8	0.92
SDNN (ms)	66.4 (47)	68.6 (72.6)	3.3	0.27
PNN50 (%)	2.7 (2.07)	2.8 (2.7)	3.7	0.15
LF/HF Ratio	3.55 (1.6)	2.93 (1.3)	-16.9	0.28

*Mean and SD values for extended resting HR measures. Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. *Over extended resting period*

8.7 Pupillary dark adaptation response. A comparison of standard deviation from the age matched normal range for the ERD and NERD group.

Pupillometry was performed for each volunteer. The pupillary dark adaptation response increased between visit one and visit two, (after eight weeks of PPI therapy) with a percentage rise in both the ERD and NERD group. Analysis of results using a paired t-test show a statistically significant difference (p=0.037) when comparing the number of standard deviations from age matched normal values for the ERD group (see Table 24). This suggests that with healing of inflammation (erosive oesophagitis) in the ERD group the pupillary dark adaptation response improved with a mean value of 0.43 standard deviations above age matched normal values. For the NERD group there was not such a statistically significant difference, however, pupillometry values at baseline were already higher for this group than the ERD group and rose to a mean value of 0.16 standard deviations below normal age matched values. The subsequent percentage improvement after PPI therapy for the NERD group was half that of the ERD group (70.9% compared to 144.4%).

Table 24 Pupillary dark adaptation response for the ERD and NERD Groups.

Group	**Mean SD Visit 1 (+/- SD)**	**Mean SD Visit 2 (+/- SD)**	**% Diff (V1-V2)**	**P Value**
ERD (n=8)	-0.97 (1.07)	0.43 (1.38)	144.3	**0.037**
NERD (n=12)	-0.55 (1.09)	-0.16 (1.48)	70.9	0.086

A comparison of standard deviation from the age matched normal range for the ERD and NERD group. Mean (±SD) values for pupillometry for each group are shown. Comparison using a paired t-test was performed. Significant results (P=<0.05) are shown in bold.

8.8 Analysis of Blood Pressure Measurements Pre and Post PPI Therapy

A blood pressure measurement was taken at the end of each study using a microlife automated brachial blood pressure monitor to ascertain whether there were any changes between the two visits. Continuous beat to beat measurement was also taken throughout each study using the Portapres. Looking at the percentage difference in

values between visit one and two, the automated measure of systolic blood pressure for both groups reduced. For the other measures all values increased slightly between visits for both groups. Statistical comparison of automated brachial BP and Portapres finger BP at visit one and visit two was performed using a paired t-test for both the ERD and NERD group. Analysis of the blood pressure measures between the first and second visits for both groups showed there was no statistical difference in systolic or diastolic blood pressure between visits using either measurement technique apart from the Portapres measure of diastolic blood pressure for the ERD group (p=0.04) (see Table 25).

Table 25 Comparison of automated blood pressure and Portapres finger blood pressure for visit 1 and visit 2 for both the ERD and NERD groups.

	ERD Group (N=8)				**NERD Group (N=12)**			
	V1 Mean (±SD)	**V2 Mean (±SD)**	**% Diff**	**P Val**	**V1 Mean (±SD)**	**V2 Mean (±SD)**	**% Diff**	**P Val**
Systolic automated mmHg	142.5 (22.2)	141.0 (22.3)	-1.05	0.56	137.6 (17.3)	137 (16.2)	-0.44	0.61
Diastolic automated mmHg	87.5 (12.2)	88.6 (13.1)	1.26	0.64	83.5 (8.8)	84 (7.4)	0.6	0.34
Systolic (Portapres) mmHg	116.6 (23.5)	132.8 (31.3)	13.9	0.14	114.4 (20.5)	120.7 (21.0)	5.5	0.16
Diastolic (Portapres) mmHg	65.1 (8.9)	81.3 (7.5)	24.8	**0.04**	64.3 (12.6)	65.4 (10.1)	1.7	0.36

Mean (±SD) for each visit for the ERD and NERD groups. Analysis performed using a paired t-test (P=<0.05). There were no significant changes in blood pressure between visits for either measurement technique.

Chapter 9: Discussion (GORD Study)

9.1 Demographic Factors and their Effects on Heart Rate Variability

In this study we compared heart rate variability in two distinct groups of patients selected via endoscopic assessment for GORD and associated erosive and non-erosive oesophagitis. Patient HRV was reassessed after an eight week healing dose of PPI therapy to assess for changes in autonomic tone. We recruited 11 males and 9 females with an average age of 50.6yrs (+/-12). For this study this was a lower number of participants recruited than initially intended. The issues surrounding recruitment are discussed in section 9.9.

Research suggests that gender bias is present for autonomic tone with men displaying higher values of heart rate variability (Cowan et al 1998). We did not assess male and female results separately for this study because splitting the data into two more groups would have decreased the sample size further.

Participants in this study were asked to complete a health questionnaire to ascertain whether there were any co-morbidities that would affect the study. The patients included in the study were essentially healthy with no history of inflammatory disease, cardiac disease or diabetes. The only disease present at the time of testing was gastro-oesophageal reflux (with or without the presence of grade one or grade two oesophagitis).

9.1.1 Diversity of the Study Cohort

The study group was not ethnically diverse. There was a significant bias as all study volunteers were Caucasian.

9.1.2 Study Protocol

The autonomic assessment protocol was the same as per the previous influenza study (see section 3.6.3), with the exception of the 10 breaths per minute metronome breathing provocation. Volunteers were asked to re-attend following eight weeks of prescribed PPI therapy. All patients were followed up within the eighth week for autonomic assessment.

9.2 Comparing Baseline Measurements of Autonomic Function in the ERD and NERD Groups with a Healthy Normal Range

Heart rate variability for the ERD and NERD groups at baseline was compared against healthy normal range reference values to examine whether there were significant differences in HRV parameters for either group. There was a marked difference in the percentage change in HRV values for both groups, with all parameters for the ERD group showing attenuation, (see Table 21). Using an unpaired t-test we saw significant differences between some heart rate parameters; suggesting that in an otherwise disease free cohort, heart rate variability in the erosive oesophagitis group was compromised at baseline. This is in contrast with the NERD group, where despite noting attenuation in heart rate variability between baseline data and the normal range, there were no statistically significant differences identified. It could therefore be argued that autonomic tone in the NERD group deviates only minimally to that of the normal healthy range. However, these findings should be interpreted with caution due to the small sample size of the study group.

9.3 Ewing Measures of Heart Rate Variability in the ERD and NERD Groups

Our previous research found that the inflammatory process associated with the Influenza vaccination was not powerful enough to provoke a significant change in autonomic tone assessed using the Ewing measures (except in a small subgroup with more significant post-vaccination symptoms).

Analysis of the heart rate data for the ERD group showed that there was a modest percentage increase in all HRV data (i.e. some recovery in autonomic functions) after 8 weeks of PPI treatment. It is important to note here that although there were no statistically significant differences in all HRV collected data (pre and post PPI therapy), measures of forced breathing and lying/standing parameters in this particular group (i.e. ERD group) were in fact approaching significance (p=0.06 and p=0.08) (see Table 22). The NERD group also showed some improvement in HRV data (pre and post PPI therapy) however; there were no statistically significant differences between all data collected during the two visits. We suggest this may firstly be due to the fact that baseline HRV data for the NERD group –from the outset- were not statistically different from that of the normal range reference values used in our lab, and secondly there was no evidence of endoscopic inflammation in this NERD group. Dobrek et al (2004) "*not only found autonomic dysfunction in patients with inflammatory changes, (ERD group) but also the presence of dysfunction in patients without any morphological changes (the NERD group) during OGD*." Our study does not agree with this research at the current time, as we did not identify statistically significant differences despite a generalised percentage improvement in HRV, (see Table 22). The sample size of both groups in our study is small and may not be large enough to adequately address this particular issue (i.e., the presence or absence of structural endoscopic inflammatory changes in the ERD and NERD groups and their effects on autonomic function), using the Ewing tests.

Analysis of an extended period of resting heart rate was performed for the ERD and NERD group. Assessment of resting heart rate, fractal dimension, SDNN, PNN50 and LF/HF ratio measures were made prior to and after eight weeks of PPI therapy. There was an increase in most measures of resting heart rate between visits, which was only marginal for the NERD group but there was no statistically significant improvement with the intervention of PPI therapy for either group (see Table 23). This suggests that the effect of inflammation (associated with erosive oesophagitis in the ERD group) was not potent enough to elicit measureable changes in resting heart rate in either the sympathetic or parasympathetic branches of the ANS. Rather this suggests an initial reduction in the vegetative balance of the two branches (visit one), leading to a

generalised improvement in responsiveness after PPI therapy (visit 2). For the NERD group the values are only marginally changed between visits, which may be due to the absence of any erosive oesophagitis (inflammation) in this group.

9.4 Heart Rate Variability Measures during Metronome Guided Breathing in the ERD and NERD Groups after 8 weeks of PPI therapy

For heart rate measures during two minutes of metronome guided breathing there was a noticeable improvement in four of the six HRV parameters for the ERD group post PPI therapy. Results of statistical analysis of paired data collected during metronome guided breathing suggest there is a statistically significant difference in the DEV parameter between the baseline and follow up visit for the ERD group (see Table 22 and Figure 30). This finding is in keeping with research, by Lee et al (2004) who found a difference in autonomic tone in the ERD group compared with the NERD group. Chen (2006) also found that autonomic function appears to differ significantly between the two groups, with spectral analysis of heart rate variability significantly reduced in the high frequency power band in patients with erosive oesophagitis. We suggest that the presence of inflammation linked to erosive oesophagitis in this group may be the cause of the attenuated heart rate variability at baseline and may be the cause of I) the attenuation observed in our HRV data when compared to a healthy normal range reference values (see Table 21) and II) may be the reason why in this particular group we see a reversal (improvement) in autonomic tone (DEV parameter) after an eight week healing dose of PPI therapy, (see Table 22). However, the sample size is small (N=8) and more data will need to be collected to establish this relationship further.

For the non-erosive reflux disease (NERD) group there was also an improvement noted in the heart rate data between visits one and two for metronome guided breathing parameters. However, there were no statistically significant changes relating to autonomic tone pre and post PPI, despite a good symptomatic response to the PPI therapy as measured by the GERD impact scale. However, we would not necessarily expect to see a significant improvement in autonomic tone in this particular group, as the baseline heart rate data was not significantly different –from statistical point of

view- to that of a healthy normal range, (see Table 21). Furthermore, it could also be argued that any inflammatory process in the NERD group is so weak to the extent that it is not enough to influence or to cause attenuation in the autonomic function of this particular group.

9.5 The Effect of Erosive Oesophagitis and Associated Inflammation on Heart Rate Variability

The heart rate variability findings discussed in the preceding sections (9.3 and 9.4) are in keeping with our previous research, which suggested that the presence of inflammation related to the influenza vaccination (in a subgroup of the most symptomatic participants) was associated with significantly reduced heart rate variability (Perring and Jones 2012).

All patients recruited to the ERD group were classified as having either grade A or B Mild to moderate oesophagitis. Chakraborty et al (1989) found heart rate variability was related to the grading of oesophagitis. We did not specifically assess this due to the small sample size. Cunningham (1991) states "*The high prevalence of parasympathetic cardiovascular dysfunction, which was unrelated to the grade of oesophagitis, indicates that the vagal nerve impairment in GERD is not a secondary phenomenon*" The results of this study agree that autonomic tone is attenuated during metronome guided breathing in patients with mild to moderate erosive oesophagitis prior to starting eight weeks of PPI therapy with subsequent augmentation in autonomic tone after the inflammation has healed (see Table 22).

In keeping with research by Chen et al (2006), it could be argued that the small but measureable changes in heart rate variability seen in the ERD group may be associated with the inflammatory changes in the oesophagus resulting from erosive oesophagitis. However, research by Dobrek et al (2004) suggests that autonomic tone is disturbed in both NERD and ERD patients. Results from our research do not support these findings. We saw a generalised trend in heart rate data for the NERD group suggesting an increase in heart rate variability post PPI, but this did not reach statistically significant

levels. This may have been due to I) the small sample size or II) the absence of any erosive oesophagitis related inflammation in this particular group.

At present there is a suggestion that the initial reduction and subsequent reversal (improvement) of autonomic tone in the ERD group may be linked to healing of inflammation post PPI. However, there is not enough data to conclusively ascribe the HRV changes solely to the inflammatory process associated with erosive oesophagitis and will continue to recruit more patients to the study to increase statistical power.

9.6 Autonomic Tone in the ERD and NERD groups after eight weeks PPI therapy and GERD Impact Scale Scores.

The GERD impact scale was used to ascertain the frequency and severity of reflux related symptoms prior to and after eight weeks of PPI therapy. There are many validated reflux questionnaires available, some of which may give more in-depth information relating to specific aspects of symptom relief. This scale was used as a simple and quick way to gauge the extent of symptoms and more generalised symptomatic improvement.

For both the ERD and NERD groups there was a significant percentage decrease in "GERD impact scale" questionnaire scores indicating an improvement in symptoms after PPI therapy, (see Table 20). There were statistically significant differences in symptom frequency and severity prior to and after eight weeks of PPI in both groups (P=0.001). Research has shown that eight weeks of PPI therapy is enough time to heal erosive oesophagitis (Caro et al 2001). We saw a statistically significant improvement in autonomic tone in the ERD group (pre and post PPI therapy) during two minutes of metronome guided breathing. This is associated with significant improvement seen in the GERD impact scale scores.

Long and Orlando (2007) suggest PPI's are the most effective agent for the treatment of NERD but are less effective in providing symptom relief than for patients with erosive oesophagitis. We did not see this in our relatively small cohort and the degree of response to PPI was assessed as being the same for both (ERD and NERD) groups

(see Table 20). Chen (2006) found that the symptoms severity scoring was the same regardless of the presence or absence of erosive oesophagitis but the autonomic function appears to differ significantly between the two groups, with spectral analysis of heart rate variability significantly reduced in patients with erosive oesophagitis. This is in agreement with our findings. Lee et al (2004) also found reduced autonomic tone in patients with erosive oesophagitis (even in patients without symptoms) compared with the non-erosive group suggesting, "*It is the structural state of the oesophagus and not the symptomology which dictates the autonomic function status*." This is entirely in keeping with our findings, showing that GERD Impact Scores are not statistically different between the two groups, but HRV is reduced significantly in the erosive oesophagitis group.

9.7 Pupillary Dark Adaptation (Pupillometry)

We performed pupillary dark adaptation measures on all subjects. Statistical analysis was performed for the ERD and NERD group (see Table 24). There was a statistically significant difference in the number of standard deviations below age matched normal values for iris: pupil ratio, before and after eight weeks of PPI therapy in the ERD group (P= 0.037), (see Table 24). This indicates an improvement in autonomic functions after 8 weeks treatment with PPI therapy in the ERD group.

On the other hand, although the NERD group also shows some increase in their pupillary dark adaptation response after 8 weeks of PPI therapy compared with their own baseline measurements (at visit 1), these differences were not statistically significant. Possible conclusions that we can draw from this are:

- That the autonomic nervous system controlling the pupil response is affected by inflammation resulting from erosive oesophagitis in the ERD group.
- It could be suggested that the attenuating effect of inflammation on autonomic function is reversible as evidenced by the improvement in HRV after 8 weeks PPI therapy. However, it could be argued that the possibility of PPI drugs themselves exerting certain unknown (or not yet reported) pharmacological

effects on the improvement (or recovery) in autonomic function cannot be ruled out as a possible confounding factor. This is especially true, as this study did not have a control group to compare the collected data against.

- That the measurement technique on this occasion appears to be sufficiently sensitive to determine changes in autonomic tone.
- However, it is important to note here that the sample size was too small to be confident in the measurement.

9.8 Implications of This Study

This study has demonstrated a link between autonomic dysfunction and GORD. We have established that metronome guided breathing at six breaths per minute implemented during this research was sufficiently sensitive to measure small but statistically significant changes in autonomic tone related to inflammation in the ERD group. The results are in keeping with our earlier research relating to the Influenza vaccination whereby there was deterioration in autonomic tone following vaccination, especially in the severe symptomatic group. We are confident in this technique and have again validated the use of extended forced breathing in conjunction with spectral analysis as a sensitive method of assessing changes on autonomic tone.

Given the reversible nature of the HRV changes seen in this study it could be argued that there may be justification for the treatment of GORD patients, particularly those who are elderly and/or with diabetes, with anti-inflammatory medication alongside conventional PPI therapy to reduce the impact on the autonomic nervous system.

9.9 Limitations of the Study

The project was limited by a number of factors, which were largely outside of our control. The first was the tragic and unexpected death of our consultant and principal investigator (PI). We were able to approach our gastroenterology colleagues for help with recruitment but struggled to recruit patients similar to those having endoscopy for

upper GI reflux who would normally be listed for assessment by the PI. A new consultant was recruited but unfortunately the patients on his endoscopy list were more complicated cases and not suitable for recruitment to the project. A nurse endoscopist performed the majority of the recruitment for the project.

Furthermore it is also important to report here, one limitation related to heart rate variability testing is it relies on a normal sinus rhythm. Performing autonomic function testing on patients with arrhythmias, frequent ectopic heart beats or significant tremor is often very difficult and results in an unsatisfactory study. The study group chosen did not have pre-existing heart disease, diabetes or other co-morbidities and were an essentially healthy group apart from gastro-oesophageal reflux disease.

We encountered a second issue, which was the use of sedation during endoscopy. If a patient had opted to have sedation, ethical approval stipulated we were not allowed to approach them or recruit them on the same day. Patients were identified in the same way during endoscopy and were given patient information leaflets to take home. We did not get any response from the patients who had been sedated.

The third issue we encountered was the dropout rate. We had a number of patients who attended for their first visit following endoscopy and were re-booked at a convenient time and date eight weeks later but did not attend despite a follow up call. We think it was the combination of the time and effort needed to make a repeated visit to the hospital and a delay of eight weeks was long enough for them to forget the appointment or decide they did not wish to continue in the trial, despite a telephone reminder.

A fourth issue was the small sample size; the power of the statistics could be improved by increasing participant numbers. Recruitment is on-going to further delineate whether there is a statistical difference between the two groups. Caution needs to be taken when interpreting the statistical significance of multiple parameters as the likelihood of one reaching the threshold for significance is additive.

The fifth issue is the absence of a control group and the study is not a randomised double-blinded study. The researcher was not blinded to either the ERD or to the

NERD group for ethical and pragmatic reasons (availability of clinical / technical expertise, day to day running of the clinics, limited staffing, and unpredictability of volunteer recruitment).

9.10 On-going Work

We are continuing to recruit patients to the reflux study to give more power to the statistics and hope to publish this work in due course.
We have established there are statistically significant changes in HRV in the ERD group which are likely to result from inflammation which are temporary and reversible after 8 weeks of PPI therapy. The reversible nature of the heart rate changes in this study lead to questions relating to other inflammatory conditions in chronic disease.

The next study is related to the previous work examining the effects of inflammation on autonomic tone and is assessing the effects of obesity related inflammation from increased adiposity in an obese cohort of healthy individuals with a family history of type 1 diabetes. We will assess autonomic tone prior to and after eight months of intensive lifestyle and exercise intervention.

Chapter 10: The Effect of an Intensive 8-month Lifestyle Intervention on Autonomic Function in an Obese Non-diabetic Adult Population with a Familial History of Diabetes

10.1 Introduction to the Project

This project was initiated by the diabetes research team at The Royal Bournemouth Hospital and was a result of an earlier pilot study that showed promising results in a similar field. I was involved with the diabetes team in the development of the study protocol, which was successfully submitted for ethical approval.

There were three distinct areas related to different areas of expertise within the research group. These were provided by the following:

- Physiological measurement and autonomic assessment was performed by myself and another colleague under my supervision.
- Anthropomorphic and nutritional education was performed by research dietician specialising in diabetes.
- Phlebotomy and haematology was performed by research nurses and Royal Bournemouth haematology department.

10.2 Review of Literature

10.2.1 Obesity and Diabetes

The World Health Organisation (2006) cites obesity as becoming a growing and significant public health problem. Evidence from various national surveys show the prevalence of obesity in adults in England has increased threefold since 1980 (NICE 2006).

Obesity promotes abnormal blood glucose metabolism, insulin resistance, abnormal blood lipid metabolism and raised blood pressure and is a major cause of a number of diseases such as Type 2 diabetes and cardiovascular disease, as well as increasing the risk of developing breast, ovarian, cervical, prostate and colorectal cancers (Avenell et al 2004).

In the UK, the rate of diabetes between 1995 and 2005 increased from 2.8% to 4.3% of the adult population (Lujan et al 2009). This increase may primarily be the result of the increased prevalence of obesity, as the number of people newly diagnosed with type 2 diabetes who are obese increased from 46% to 56% over the same time period (Lujan 2009). Family history of diabetes is also a recognised major risk factor for developing the condition, with the risk for people with familial diabetes that is increased by a factor of 2 to 6 as compared with those without familial diabetes (Meigs et al 2000 and Weijnen et al 2002).

There is no clear evidence available to explain the epidemic levels of obesity. A number of neuro-endocrine factors have a role in the regulation of food intake and in the control of insulin secretion. Glucagon-like peptide-1 (GLP-1) is an incretin hormone, the name based on the observation that the insulin response to an oral glucose load was greater than following intravenous administration of an equivalent amount of glucose (McIntyre et al 1964). Incretin hormones are secreted from the small intestine in response to the presence of nutrients in the intestinal lumen. Physiologically, GLP-1 enhances glucose-mediated insulin secretion and suppresses glucagon secretion. GLP-1 also induces satiety and improves gastric emptying.

10.2.2 Inflammation and Obesity

In recent years there has been increasing evidence identifying the role of inflammation in the aetiology of disease states and the mechanism behind the pathogenesis of obesity related disease states (Zulet et al 2007). Hotamisligil (2006) introduced the concept of meta-flammation to describe the low grade inflammatory response to obesity.

Obesity induced inflammation is generally chronic and produces low grade activation of the immune system. It is clear that low grade inflammation is implicated in the link between obesity and disease. "*Few other chronic inflammatory diseases are characterised by the features of pancreatic, liver, adipose, heart, brain and muscle inflammation as seen in obesity*" (Lumeng and Saltiel 2011). Osorio (2013) states that inflammation seen in obesity may be caused by adipocytes. When a high calorie diet is consumed, the excess nutrients are stored as fat and the hormone leptin is produced. Excess leptin stimulates white blood cells known as CD4 T cells to produce another molecule called interferon gamma. This molecule causes adipocytes to produce a group of proteins known as major histocompatibility complex II (MHCII). The presence of MHCII stimulates immune cells in the area and this leads to inflammation.

10.2.3 Weight Loss, Glucagon-like Peptide-1 (GLP-1) and Autonomic Function

In studies of patients with established diabetes, GLP-1 levels and the response to oral glucose have been shown to be attenuated (Nauck et al 1986) and administration of GLP-1 normalized both fasting and post-prandial glucose levels. More recently it has been reported that obese subjects with associated inflammation resulting from adipocytes with normal glycaemic control also seem to have attenuated basal and postprandial GLP-1 concentration, but the evidence is unclear (Verdich et al 2001). Very few studies have looked at the effect of weight loss on GLP-1 concentration and heart rate variability. One study by Adam et al (2006) showed reduced postprandial GLP-1 levels after 6 weeks weight loss, induced by Very Low Calorie Diet (VLCD), however after 3 months weight maintenance period the GLP-1 levels returned to the baseline levels. Other research showed that weight loss of 18.8 kg induced by VLCD resulted in a GLP-1 concentration increase to a level between that of obese and lean subjects at 6-months (Verdich et al 2001). Poirier et al (2003) and (2006) suggest a significant improvement in cardiovascular function related to weight loss in obese individuals. Subjects after jejuno-ileal bypass surgery show that after weight loss there is an increase in postprandial GLP-1 response to a level above that of lean subjects (Naslund et al 1998) effectively resolving diabetes mellitus (Cummings et al 2004|).

A study comparing patients with type 2 diabetes, patients with impaired glucose tolerance and healthy volunteers, suggested that impairment in glucose homeostasis can develop independently of any impairment in GLP-1 levels (Vollmer et al 2008). Therefore it remains uncertain whether lower GLP-1 levels in established diabetes contribute to the pathogenesis of the condition or are a consequence of chronic hyperglycaemia or other hormonal and metabolic changes. The observation that GLP-1 levels are lower in obese subjects suggests the possibility that the former may be the case.

None of the previous research has addressed how gradual healthy lifestyle changes with sensible weight loss goals and increased activity levels affect Heart rate variability and GLP-1 levels in an obese but otherwise healthy population. The aim of this study is to investigate HRV and GLP-1 concentration in obese, non-diabetic subjects with a family history of diabetes during an 8-month lifestyle programme.

10.3 Leptin and Autonomic Function

Leptin is a protein hormone produced by adipose tissue, which has a key role in regulating energy intake and expenditure through a suppressive effect on appetite and an increase in metabolism. Circulating leptin levels reflect body fat mass during energy balance and fall in periods of energy deficit (Weigle et al 1997).

Leptin can enter the central nervous system (CNS) and interact with centres in the regulation of appetite and metabolism (Spiegelman and Flier 2001). Despite these suppressive effects, elevated levels have often been noted in obesity, possibly suggesting a resistance to the hormone, though this may be a result of stimulation by increased insulin levels, which would be expected in obesity. Leptin in turn can inhibit insulin release so a fall in leptin resulting from weight loss may enhance insulin production and may therefore be beneficial in patients with diabetes and patients with impaired glucose tolerance. It could be argued that in newly diagnosed diabetics there is evidence of inflammation with the activation of inflammatory cytokines such as leptin. Vinik (2012) suggests that "*understanding the relationship between autonomic*

dysfunction and adipose tissue inflammation seen early in the development of diabetes will lead to further measures for determining which individuals are at highest risk for cardiovascular disease and mortality, and will also lead to the development of new therapies for reducing that risk." There is strong correlation between vagal nerve activity and inflammatory disease (Tracey 2009) and suggestion that an afferent reflex arc responding to inflammatory markers may be involved in the inflammation seen early in type-two diabetes, "*by eliciting a cholinergic anti-inflammatory effector response, mediated by the parasympathetic nervous system*." (Vinik 2012 and Tracey et al 2009). Research by Vinik shows the earliest detectable changes in the evolution of diabetes is abnormalities in autonomic balance. Leptin has been shown to stimulate sympathetic nervous system (SNS) activity in thermogenic and non-thermogenic organs (Collins et al 1996) and in animal models (Haynes 1997). Paolisso et al (2000) demonstrated that increasing fasting plasma leptin concentrations were associated with a shift of the sympathovagal balance towards an increase in sympathetic activation and an increased response to orthostatic stimulus in non-obese subjects with different body fat content.

10.4 Adiponectin and Autonomic Function

Adiponectin is a collagen like protein that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism, and may have a role in the metabolic derangements of type 2 diabetes (Yamauchi et al 2001). Despite being expressed in adipose tissue, plasma adiponectin levels are decreased in obesity as a consequence of down regulation (Arita et al 1999). Adiponectin plays an important role in insulin sensitivity (Wolfson et al 2012) and has central effects on energy homeostasis, promoting weight reduction. Levels of adiponectin have been found to be reduced in type-2 diabetes compared with non-diabetic controls, hypertension and coronary artery disease. Weight reduction can increase circulating levels of adiponectin. Pischon et al (2004) found doubling the circulating adiponectin levels was associated with a 30% decreased risk for myocardial infarction. Schulze et al (2005) similarly found in type one and two diabetes that there was a corresponding reduction

in coronary risk associated with an increase in adiponectin. These findings suggest, "*adiponectin may have a direct anti-atherogenic role or mediate its effects via obesity-independent mechanism*" (Wolfson et al 2012). Wakabayashi et al (2004) demonstrated that sympathovagal balance favoring relative sympathetic activation is associated with low serum concentrations of adiponectin in type two diabetic patients.

10.5 Role of the Sympathetic Nervous System (SNS) in Inflammation

Both the sympathetic nervous system (SNS) along with the hypothalamic-pituitary-adrenal axis (HPA) represent key peripheral regulators in maintaining internal homeostasis via a mutual positive feedback loop (Koopman et al 2011). When the fine internal balance is disrupted both the SNS and the HPA become activated to restore homeostasis. In response to stimuli, pro-inflammatory cytokines can signal the brain via afferent vagal nerve fibres (Watkins et al 1995) or via the circulation (Steinman 2004), which can trigger central activation of the SNS, stimulating release of adrenalin, dopamine and noradrenaline produced in lymphoid organs, via efferent sympathetic fibres. The role of the sympathetic nervous system in the immune response is complex. "The immune system influences the SNS and the innervation of lymphoid organs allows the SNS to influence the immune cells directly" (Koopman et al 2011). Heightened sympathetic activity is known to be associated with increased mortality.

10.5.1.1 Role of the Parasympathetic Nervous System (PNS) in Inflammation

The parasympathetic nervous system is also jointly responsible for maintaining homeostasis. The role of the PNS in the immune system is still under investigation despite much research. It is widely acknowledged that the PNS plays a key role in the regulation of inflammation. Stimulation of peripheral afferent vagal nerve fibres can activate the HPA axis and the sympathetic nervous system centrally, resulting in release of anti-inflammatory glucocorticoids and noradrenaline (Elenkov et al 2000).

Efferent nerve fibres are also involved in the anti-inflammatory process known as the cholinergic anti-inflammatory pathway (Tracey 2009).

10.5.2 Inflammatory Markers and Autonomic Balance

There is evidence to suggest that there is a reduction or loss of HRV and the loss of sympathetic/parasympathetic balance before any markers of inflammation can be measured (Lieb et al 2011). Thayer and Fischer (2009) found heart rate variability was diminished early in diabetes, this correlated well with increasing levels of CRP and IL-6. Laitinen et al (2011) suggest it is likely that the diabetic process, even before the onset of diabetes, is involved in the progression of parasympathetic dysfunction. Figure 31 shows circulating molecular weight adiponectin and leptin levels in diabetics may be predictive markers for autonomic dysfunction in early asymptomatic diabetics. Vinik (2012) suggests "*there is strong evidence of inflammation with activation of inflammatory cytokines such as IL-6 and leptin in newly diagnosed type 2 diabetes. These changes correlate with abnormalities in sympathetic-vagal balance."*

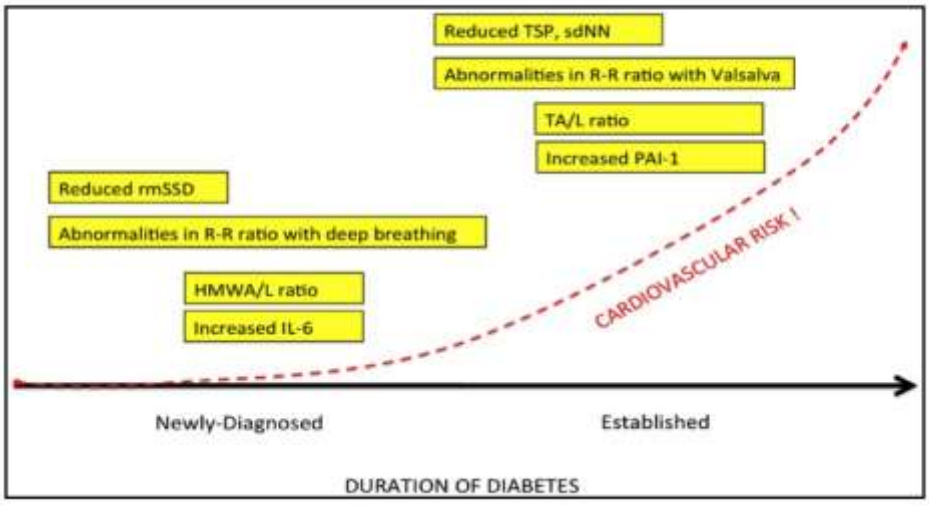


Figure 31 Graph showing development of autonomic dysfunction and early loss of HRV correlating with an increase in inflammatory markers. From Vinik (2012)

KEY:

rmSSD	*Root mean square of the successive differences (time domain tool for measuring HRV)*
R-R Interval	*R wave to R wave timing*
HMWA/L ratio	*High molecular weight adiponectin / Leptin ratio*
IL-6	*Interleukin -6*
TSP	*Total spectral power*
TA/L Ratio	*Total adiponectin to leptin ratio*
PAI-1	*Plasminogen activator inhibitor*

10.5.3 The Link between Diabetes, Obesity and Autonomic Function, and the Association with Exercise

It was previously thought that loss of autonomic tone was irreversible. We now know that this is not necessarily true, Maser and Lenhard (2007) suggest autonomic disturbances appear to be reversible with weight reduction.

It is well established that disease states such as diabetes have a significant impact on the autonomic nervous system. It was cited by Lieb et al (2011) that newly diagnosed type two diabetics and patients with an impaired glucose tolerance test (IGT) would both be associated with a decreased parasympathetic tone and augmented sympathetic tone. Wu et al (2007) also noted a "shift towards augmented sympathetic tone in the

development from normal glucose tolerance to IGT and finally diabetes." Previous research (Perring and Jones 2003) showed a significant difference in autonomic tone between age-matched normal subjects and asymptomatic type 1 diabetics (in the absence of autonomic symptoms e.g. postural hypotension).

There is a host of evidence linking performance of the autonomic function with obesity, weight loss, exercise and diabetes. Despite advances in research, the physiological connection between physical activity and diabetes still remains unclear. Laitinen et al (2011) suggest levels of obesity in men play an important role in the early development of cardiovascular autonomic neuropathy. Carnethon et al (2006) reported an increase in heart rate variability and a decrease in heart rate associated with lifestyle modification compared with therapy and placebo arms of a diabetes prevention programme. Gaede et al (2008) showed multifactorial intervention controlling blood pressure, lipids and hyperglycaemia reduces abnormalities in autonomic function by 68%. Bassuk and Manson (2005) conclude that 30 minutes of daily exercise can reduce the incidence of diabetes and heart disease. They state that the underlying mechanisms of these *"protective effects likely include the regulation of body weight; the reduction of adiposity, insulin resistance, blood pressure, dyslipidemia, and inflammation; and the enhancement of insulin sensitivity, glucose tolerance, and fibrinolytic and endothelial function."* A study by Yates et al (2007) showed group based education promoting increasing activity using a pedometer was effective for improving glucose tolerance compared to the same programme without a pedometer.

In this PhD project, the addition of autonomic function testing allowed us to establish whether it is possible to detect changes in the autonomic tone between the baseline visit and the four and eight month follow-up visits. In subjects where significant weight loss has occurred (average 11%) and a sustained level of increased physical activity has been achieved we may see a positive change in autonomic tone, which should correlate well to these lifestyle changes and with biochemical results of the blood samples. The autonomic nervous system is an important system to evaluate in the association between diabetes and physical activity. One motivation for such a study is the responsiveness of the autonomic nervous system to lifestyle changes. The positive impact of physical activity and weight loss on autonomic function may prove to be a

key benefit in the prevention of diabetes, improving quality of life and reducing morbidity in the long-term.

10.6 Inflammation, Obesity and Autonomic Function

Inflammation is typically described as the principal response of the body to deal with injury. This often short-term adaptive response is a crucial component of tissue repair and involves integration of many complex signals in distinct cells and organs. However, the long-term consequences of prolonged inflammation are often not beneficial. This appears to be the case in metabolic diseases. Although many of the same mediators are involved in obesity and diabetes, few, if any, of the classic features of inflammation are observed (Hotamisligil 2005). *"Unequivocal experimental, epidemiological and clinical evidence produced during the past decade causally links inflammation to the development of metabolic diseases and/or the complications that emerge from these pathologies, particularly in the context of obesity and type 2 diabetes"* (Shoelson 2006). Hotamisligil (2005) described a new term for chronic inflammation as 'meta-flammation" a subclass of inflammation *"which is principally triggered by nutrients and metabolic surplus, and engages a similar set of molecules and signaling pathways to those involved in classical inflammation."* During the past decade, it became clear that inflammation is a key feature of obesity and type 2 diabetes (Wellen and Hotamisligil 2005). The inflammatory response that emerges in the presence of obesity seems to be triggered by and to reside predominantly in adipose tissue, although other metabolically critical sites, particularly the liver, might also be involved during the course of the disease (Shoelson et al 2006). The role of inflammation associated with obesity is an important factor relating to morbidity and mortality. Sajadieh et al (2004) concluded "*increased heart rate and reduced heart-rate variability are associated with subclinical inflammation (meta-flammation) in middle-aged and elderly subjects. An autonomic imbalance in favour of the sympathetic system may interact with inflammatory processes to play a more important role in the process of atherosclerosis than previously thought."* Results from a study by Laitinen et al (2011) suggest that, additional pathological changes related to central obesity (other than impaired glucose tolerance could also be active in cardiovascular autonomic

dysfunction including low-grade inflammation, oxidative stress and a genetic link between autonomic dysfunction and central obesity. Obesity, insulin resistance and type 2 diabetes are closely associated with chronic inflammation characterized by abnormal cytokine production, increased acute-phase reactants and other mediators, and activation of a network of inflammatory signalling pathways (Wellen 2005).

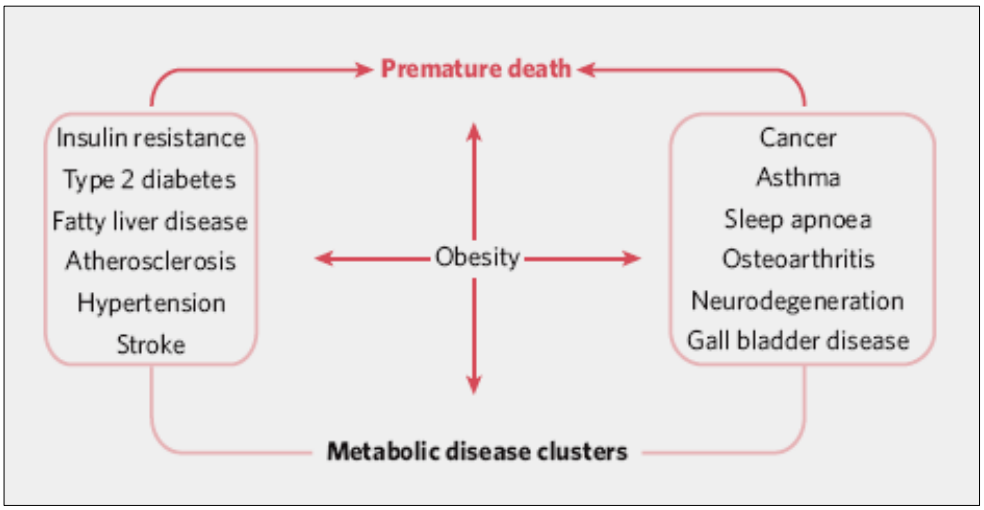


Figure 32 Clustering of metabolic diseases.

Obesity is considered to be a central feature that increases the risk for a vast array of diseases, with significant morbidity and mortality. In general, the mechanistic basis of the link between obesity and disease is poorly understood Cited in (Hotamisligil 2005)

10.7 Aims of the Study

The main aims of this study were to investigate the effects of weight loss achieved after eight months intensive lifestyle programme on: I) autonomic function II) glycaemic indices, III) serum lipid profiles and IV) markers of obesity.

Chapter 11: Methods (Lifestyle Study)

11.1 Study Design:

An experimental, interventional, prospective follow up study design with weight, cardiovascular risk factors, autonomic function testing and hormonal regulators on food intake and glucose homeostasis measured at baseline, and following 4, and 8 months of lifestyle intervention.

11.2 Recruitment:

Subjects with BMI 30.00 – 39.99kg/m^2 (class I and class II obesity) with family history of Diabetes of 1st degree relatives (self-reported) were recruited from patients attending the Hospital Biochemistry department for oral glucose tolerance test (OGTT), direct from local GP practices and through local newspaper and radio advertising.

Subjects who indicated an interest in participating were given a study information sheet with a contact telephone number and invited for an individual appointment with the dietician or nurse. During the appointment the study structure, aims, and procedures were explained. If the subjects indicated they were willing to participate and they fulfilled the study inclusion criteria, they were required to sign a consent form.

11.2.1 Exclusion Criteria

People with a BMI of less than 30 and therefore not categorised as obese were excluded according to the World Health Organisation definition for obesity (World Health Organisation 2013) and people with a BMI over 39.9 kg/m^2 were also excluded firstly, to recruit more homogenous group of subjects and secondly, because the NICE guidelines recommend that for adults with a BMI ≥ 40 bariatric surgery is recommended as a treatment option (NICE 2006).

Individuals who were either: unable to give consent, or who were unable to attend at least 75% of the programme sessions for medical or other reasons, or who were prescribed oral hypoglycaemic, anti-obesity or any other prescription medications that may interfere with the study results, or had no family history of Diabetes in 1st degree relatives, were excluded.

11.3 Study Protocol

11.3.1 Blood Sampling

Following an overnight (8-12 hour) fast, participants were asked to attend for clinical assessment, autonomic function testing and blood sampling. They were required to attend at baseline, 4 and 8 months intervals.

Blood samples were taken for basal measurement of fasting blood glucose, HbA1c, lipids, insulin, glucagon, GLP-1, leptin, ghrelin and adiponectin. Sampling was repeated at 30 minutes after a standard 75 g glucose load (for peak GLP-1 levels).

At the baseline assessment, an additional blood test was taken 2 hours after a standard 75g glucose load as per oral glucose tolerance test (OGTT) protocol to eliminate the possibility of undiagnosed diabetes or Impaired Glucose Tolerance (IGT), (World Health Organisation 1999). If the test confirmed diabetes, the subject was withdrawn from the study and referred to his/her GP to initiate the Diabetes care pathway. In the case where the test confirmed IGT, the subject continued with the study and repeated OGTT at 8-month assessment. If at this stage Diabetes was confirmed, the subject was excluded from the study and referred to their GP for further management. Baseline measurements of weight, height, percentage body fat, waistline circumference, autonomic function, and blood pressure were also taken during the three assessment sessions.

11.3.2 Food / Activity Diary

Subjects were asked to keep a 7-day food and activity diary 1-3 weeks prior to starting the programme and prior to the 4 and 8-month assessments. To help patients record their activity levels they were provided with pedometers. They were also enrolled at a local leisure centre where they took part in group exercise sessions.

11.3.3 Weight Management Programme

A Specialist Research Dietician ran the weight management programme. It consisted of an initial 4 month intensive weight loss phase, followed by a 4 month weight loss / maintenance phase. The initial 4-month programme consisted of 7 group education sessions. The following 4-month programme consisted of 4 group sessions and 1 individual appointment. Each education session lasted 60 minutes. Before the 1st session and, at the 4 month and 8 month sessions, autonomic function testing was performed and blood samples were taken. Prior to the remaining sessions 15 minutes were devoted to weight assessment.

The programme was based on portion control and healthy eating and was supported by behavioural and cognitive change interventions. Interventions included self-monitoring, stimulus control, goal setting, problem solving, and relapse prevention. The specialists from “Bournemouth HealthLink” (a partnership backed by the local NHS, Council and University) took part in helping participants to increase their activity levels.

11.3.4 Schematic for Lifestyle Study Protocol.

The schematic below shows the participant pathway from recruitment, assessment and repeat visits at four and eight months.

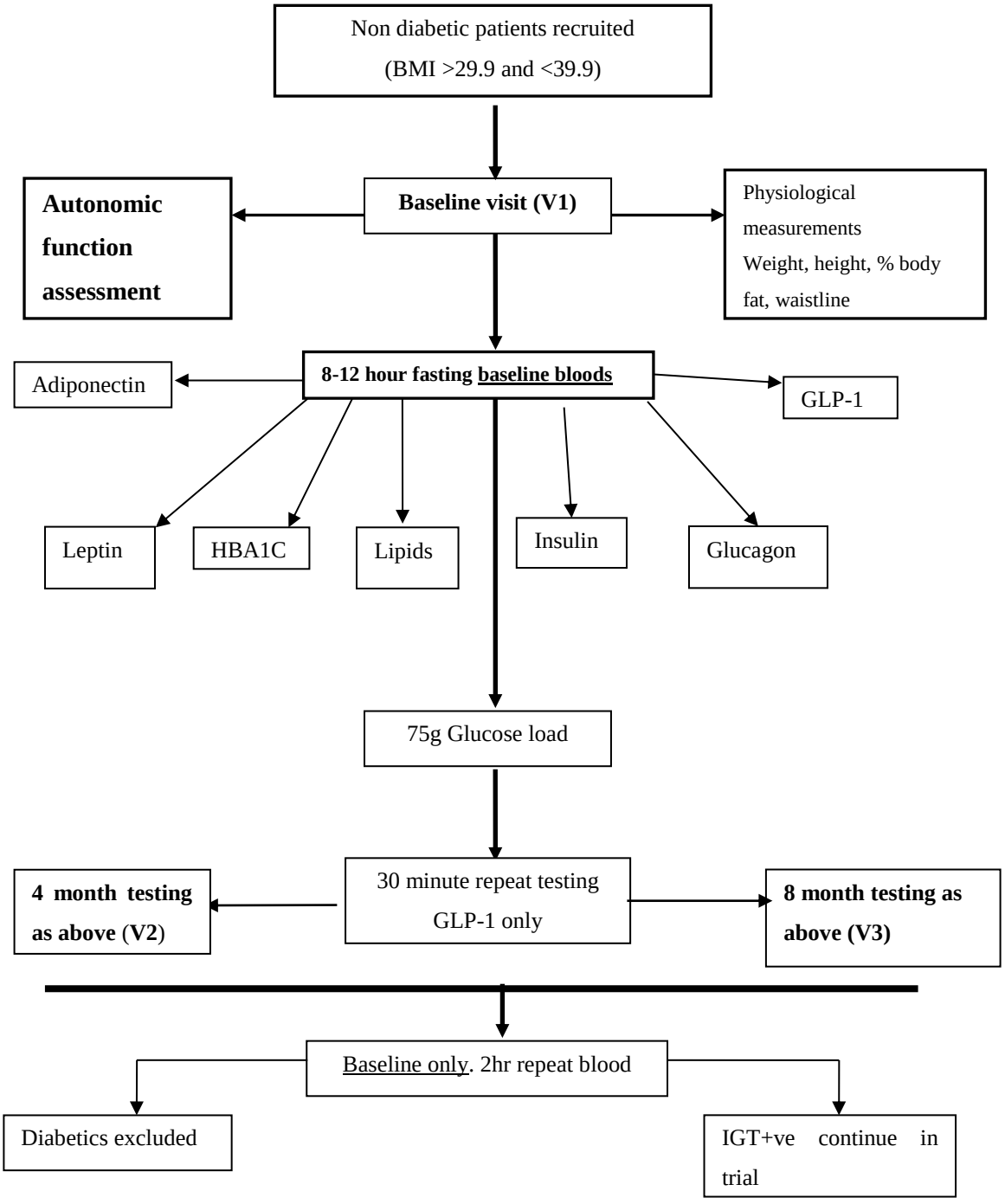


Figure 33 Schematic of the lifestyle study protocol

11.3.5 Autonomic Function Testing

Autonomic function testing took approximately 20 minutes per patient and was performed during the 3 scheduled sessions at the hospital by myself and another Health Scientist from the Medical Physics department. All measurements were completely non-invasive. The measurement protocol was as per the influenza study and the GORD study except for the absence of the 10 breaths per minute provocation.

11.3.6 Ethics

This study received ethics approval by the Dorset Research Ethics Committee (ref 09/H0504/61) and the Royal Bournemouth Hospital Research and Development Committee see Appendix 16.4.3

Chapter 12: Results (Lifestyle Project)

12.1 Recruitment Information

Recruitment for this study was very successful through a range of media including adverts in the local newspapers. We assessed 38 patients over the eight-month period with relatively low drop out and good compliance with the programme.

51 subjects were recruited for the study in total. 38 participants completed the full eight month lifestyle study (74%). 10 subjects did not complete the study, equating to a 19.6% drop out rate. 3 additional participants (5.8%) were found to be diabetic on blood samples taken (see Table 26) and did not continue with the study as per the protocol. 6 participants were initially identified as being "pre diabetic" on blood samples taken at the baseline visit; one of whom dropped out early in the study. The 5 "pre-diabetic" subjects continued with the lifestyle study and were monitored at the scheduled intervals for diabetes.

Table 26 Table showing study recruitment numbers.

	Initial recruitment	**Number completed full study including pre-diabetics**	**Dropout rate from initial 51**	**Diabetics identified and later excluded from study**
Total recruited	51	38	10	3
Percentage of total recruited	100%	74.5%	19.6%	5.8%

Participant recruitment numbers and percentages including drop-out rate and those identified as diabetics

12.2 Variation in Demographic and Lifestyle Factors

The participants were a mixed gender group with an average age of 52.2 years (+/- 9.39). All participants were considered obese with a range of BMI from 30.21 to

39.9kg/m^2 with a mean BMI of 34.85 (+/-3.03). All took part in the eight-month intensive lifestyle programme.

Table 27 Inter-individual variation for males, females and the total group.

	Participant Numbers	**Mean Age (years) (±SD)**	**BMI (kg/m2) Mean (±SD)**	**BMI (kg/m2) Max**	**BMI (kg/m2) Min**
Male	5	45 (11.59)	33.2 (1.56)	34.7	30.73
Female	33	53 (8.78)	35 (3.14)	39.9	30.21
Total	38	52.2 (9.39)	34.85 (3.03)	39.9	30.21

Mean (±SD) age in years, BMI and BMI min /max are shown for the total cohort and for males and females.

12.3 Anthropometric Measurements

12.3.1 Weight Related Parameters at Each Visit

Participants in the study lost a significant amount of weight through increased exercise, measuring activity via a pedometer, group and individual sessions with the dietician, diarising food intake and changing the way they shop, cook and eat. The measurements in Table 28 show the changes in body size at baseline and for the following two visits. The percentage change in all parameters between visit one and three was dramatic. There was an 11.7% mean change in weight in kg equating to a mean loss of 19.01% of body fat (kg). Mean waist circumference changed, reducing by 13.9% equating to a mean loss of 13.86cm. The changes in anthropometric measures between visit 1 (baseline), visit 2 and visit 3 were analysed using analysis of variance (ANOVA). Significant differences were seen over the study period, (the results are shown in Table 29 and Figure 34).

Table 28 Anthropometric measurements for whole group (n=38) at each visit.

N=38	Mean V1 (±SD)	Mean V2 (±SD)	Mean V3 (±SD)	% Diff (v1-v3)	P Val
BMI	34.85 (3.03)	31.85 (2.9)	30.84 (3.5)	-11.5	**<0.001**
Weight (kg)	96.7 (12.8)	88.2 (12.1)	85.4 (23)	-11.7	**<0.001**
% Body fat	44.04 (6.49)	40.6 (6.7)	40.06 (7.3)	-9.04	**<0.001**
Kg body fat	42.6 (9.2)	36.2 (8.5)	34.5 (9.4)	-19.01	**<0.001**
Waist circumference (cm)	109.47 (10.1)	97.86 (9.6)	94.3 (11.7)	-13.9	**<0.001**

Mean (±SD) values for each anthropometric parameter at each visit and the percentage difference in each parameter (V1-V3). ANOVA was performed; the results that reach significance (p=<0.05) are shown in bold.

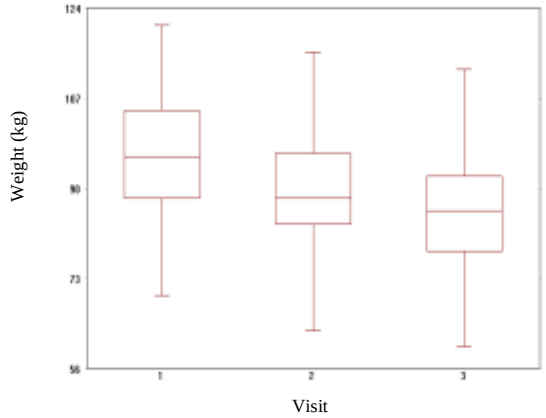


Figure 34 (A) weight (kg) for the total cohort over the 3 visits.

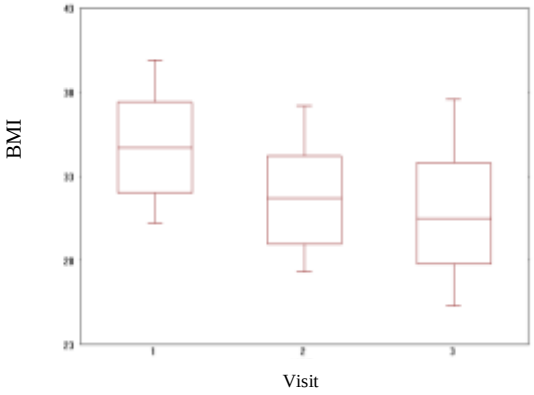


Figure 34 (B) BMI for the total cohort over the 3 visits

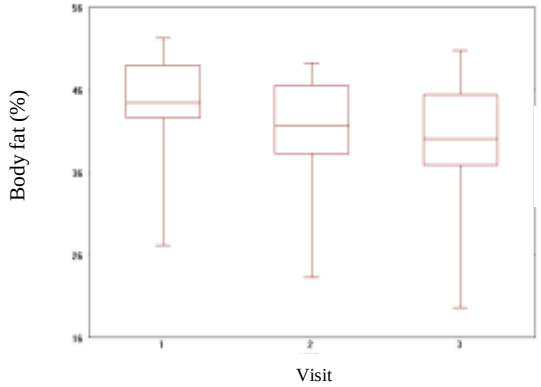


Figure 34 (C) % Body fat for the total cohort over the 3 visits

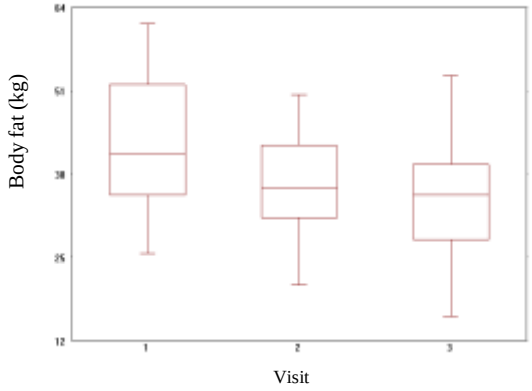


Figure 34 (D) KG Body fat for the total cohort over the 3 visits

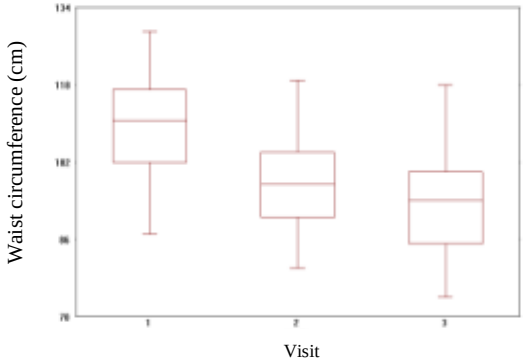


Figure 34 (E) Waist Circumference (cm) for the total cohort over the 3 visits

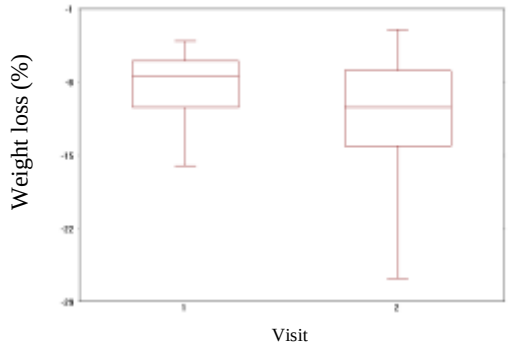


Figure 34 (F) Graph showing percentage weight loss (kg) between baseline and 4 months labelled (1) and baseline and eight months labelled (2)

Figure 34 Anthropometric measures for the total cohort.

(A-E) Box and whiskers plots showing: minimum, first quartile, median, third quartile and maximum values. Mean values for the full cohort for each visit. (F) Box plot showing percentage change in weight (kg) between (v1-v2) labelled (1) and percentage change in weight (kg) between (v1-v3) labelled (2).

All participants lost a considerable amount of weight over the eight month period, with the majority losing between 5% to 26% between visit one and three equating to an average of 11.3kg. Six patients did not lose more than 5% in weight over the eight month period, this loss equated to an average 5kg.

Table 29 Participant weight loss and percentage change in weight loss.

	Weight loss (kg) V1 -V2	**% Weight change V1-V2**	**Weight loss (kg) V2 – V3**	**% Weight change V2 - V3**	**Weight loss (kg) V1 – V3**	**% Weight change V1 – V3**
Mean (±SD)	8.3 (3.55)	8.6% (3.3)	2.8 (3.5)	3.3% (3.9)	11.1 (6.3)	11.7% (6.0)

Mean and Standard deviation for weight loss (kg) and percentage weight loss between visit one and three for the full group.

12.3.2 Blood Pressure Measures for the full cohort

Although we saw a statistically significant change in weight over the eight-month period, when comparing the blood pressure data from baseline and at visit 3 the percentage change was very small. Statistical analysis of blood pressure for the three visits using analysis of variance (ANOVA) shows there was no significant change in blood pressure for the full group (see Table 30).

Table 30 Mean (±SD) blood pressure (mmHg) for total group during all visits.

	Visit 1	**Visit 2**	**Visit 3**	**% Diff (V1-V3)**	**P Value**
Mean Systolic BP mmHg (±SD)	142 (17.3)	142 (18.8)	140.1 (16.7)	-1.34	0.51
Mean Diastolic BP mmHg (±SD)	83.9 (7.01)	84 (7.3)	83.5 (7.04)	-0.48	0.37

Analysis of data using paired data ANOVA. P value (<0.05). There were no significant differences in blood pressure.

12.4 Biochemical Measurements

Percentage change for all biochemical measures reduced significantly between baseline and eight months apart from HDL, which increased 2.04%. For measures of GLP-1, HbA1c (%), HbA1c (mmol), triglycerides and lipid ratio we saw a significant statistical difference using analysis of variance between measures over the eight-month period (The results are shown in Table 31). At 8 months there was a significant improvement in glycaemic control, with a reduction in HbA1c from 5.78% ±0.41 at baseline to 5.66% ±0.35 at 8 months ($p<0.003$), fasting glucose reduction from 8.54 ±01.5mmol/l at baseline to 7.92 ±1.76mmol/l at 8 months ($p<0.001$) and for lipids, triglycerides reduced from 1.39 ±0.58 mg/dL at baseline to 1.16±0.6 mg/dL, total cholesterol reduced from 5.72 ±1.08 to 5.55 ±0.95 and LDL reduced from 3.66 ±0.91 to 3.52 ±0.85 at eight months (see Table 31).

Table 31 Biochemical measures for the full cohort (n=38).

	Mean V1 (±SD)	**Mean V2 (±SD)**	**Mean V3 (±SD)**	**% diff (V1-V3)**	**P Val**
Blood Glucose (0min) (GLP-1)	5.12 (0.46)	4.84 (0.94)	4.89 (0.47)	-4.49	0.081
Blood Glucose (30min) (GLP-1)	8.54 (1.51)	7.85 (1.33)	7.92 (1.76)	-7.26	**0.001**
Total Cholesterol mg/dL	5.72 (1.08)	5.42 (0.96)	5.55 (0.95)	-2.97	**0.006**
Low Density Lipid (mg/dL)	3.66 (0.91)	3.4 (0.84)	3.52 (0.85)	-3.83	**0.007**
High Density Lipid (mg/dL)	1.47 (0.4)	1.45 (0.4)	1.5 (0.38)	+2.04	0.075
Triglycerides (mg/dL)	1.39 (0.58)	1.25 (0.68)	1.16 (0.6)	-16.5	**0.011**
Lipid Ratio	4.23 (1.19)	3.99 (1.19)	3.88 (1.07)	-8.27	**0.002**
HbA1c (%)	5.78 (0.41)	5.72 (0.38)	5.66 (0.35)	-2.08	**0.003**
HbA1c (mmol)	39.76 (4.5)	39.13 (4.22)	38.37 (3.93)	-3.5	**0.001**

Mean standard deviation and percentage difference between visit 1 and visit 3 for biochemical measures at each visit. Analysis of data using paired data ANOVA. P value (<0.05). Significant results are shown in bold.

The change in biochemical measures for the full group was assessed between visits one and 3. All measures were significantly reduced at eight months apart from HDL, which as expected increased (see Table 32 and Figure 35).

Table 32 Change in biochemical measures (V3-V1).

	Blood Glucose 0 min mmol/l	Blood Glucose 30 min mmol/l	Total Cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Triglycerides (mg/dL)	Ratio	HbA1c %	HbA1c mmol
Mean change between V1 and V3	-0.2	-0.6	-0.2	-0.1	0.1	-0.2	-0.4	-0.12	-1.4

Mean Change in Biochemical Measures between Visit one and Visit three for the Total Cohort.

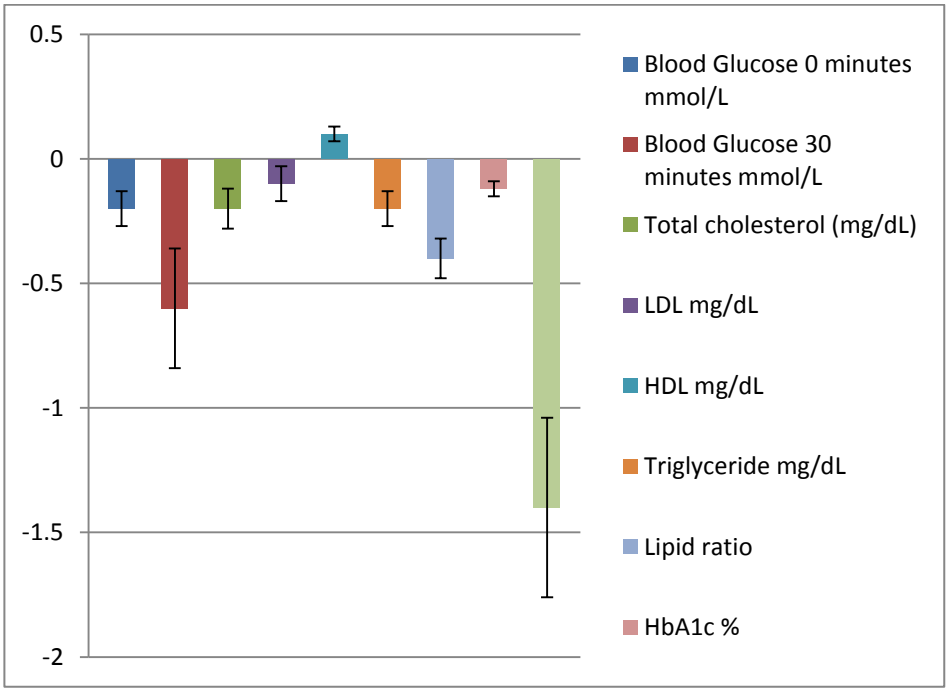


Figure 35 Bar chart showing change in blood sample measures between V1 and V3.

Changes in serum lipid, blood glucose and HbA1c between baseline and visit three with standard error bars are shown.

12.5 Measures of HRV

12.5.1 Results of HRV Measurements: Analysis of total cohort (n=38).

Heart rate variability measurements were made at the three scheduled visits. There was a percentage rise in all but one of the Ewing parameters between baseline and visit 3 suggesting a generalised improvement in autonomic tone. Statistical analysis of the Ewing measures using analysis of variance shows significant differences in all but one of the parameters (lying/standing) (see Table 33). However, for measures of HRV during metronome guided breathing there was a percentage rise in only half of the HRV measures and there were no statistically significant differences between visits.

HR data from lifestyle study (n=38) during metronome breathing (visit 1) was compared using an unpaired t-test with data from the full group of healthy participants from the influenza study (N=71) during metronome breathing (visit 1). Although all HR values are lower for the lifestyle group, there was one parameter (SD) showing a statistical difference (p=0.048) between the two groups (see Table 34).

Table 33 HRV for the full Lifestyle cohort (n=38) (Ewing and metronome breathing parameters)

Parameter	Mean V1 (±SD)	Mean V2 (±SD)	Mean V3 (±SD)	% Diff (V1-V3)	P Value
Ewing Measures					
Resting (%)	5.6 (3.5)	6.9 (4.36)	6.5 (4.5)	16	**0.001**
Forced Breathing (%)	14.06 (5.8)	15.5 (7.8)	14.58 (5.05)	3.7	**0.001**
I/E Diff (bpm)	9.2 (3.79)	11.02 (5.28)	10.5 (3.4)	14.1	**0.001**
Valsalva (ratio)	1.26 (0.12)	1.28 (0.14)	1.29 (0.14)	2.4	**0.001**
Hand grip (ratio)	1.1 (0.08)	1.2 (0.32)	1.2 (0.14)	9.1	**0.04**
Lying/Standing (ratio)	0.12 (0.01)	1.14 (0.12)	0.12 (0.08)	0	0.82
Metronome Breathing					
SD (bpm)	0.2 (0.99)	0.13 (0.85)	0.05 (0.85)	-75	0.58
A (bpm)	0.03 (0.79)	0.06 (0.77)	0.03 (0.91)	0	0.62
LPP (unitless)	0.34 (0.91)	0.31 (0.89)	0.26 (0.87)	-23.5	0.94
CORR (unitless)	-0.81 (1.52)	-0.86 (1.86)	-0.57 (1.26)	29.6	0.22
FCORR (unitless)	0.02 (0.79)	0.02 (0.77)	0.04 (0.77)	50	0.93
DEV	-0.7 (0.92)	-0.10 (1.01)	-0.01 (0.91)	85.7	0.74

Mean and standard deviation figures for HRV parameters (Ewing measures and metronome guide breathing) for total cohort. Results of analysis of variance (ANOVA) of paired data where p=<0.05 are shown in bold. Metronome breathing Parameters are described in section 3.10.6.

Table 34 HRV data for the full lifestyle study cohort during metronome breathing parameters (n=38) compared with normal range HRV data from visit 1 influenza study (N=71)

	Lifestyle full cohort N=38 Mean V1 (±SD)	Influenza Study Normal volunteers Mean V1 (±SD)	P Value (t value)
SD (bpm)	0.2 (0.99)	0.63 (1.0)	**0.048** (-2.0)
A (bpm)	0.03 (0.79)	0.33 (1.0)	0.117 (-1.58)
LPP (unitless)	0.34 (0.91)	0.65 (0.8)	0.068 (-1.8)
CORR (unitless)	-0.81 (1.52)	-0.28 (1.8)	0.230 (-1.2)
FCORR (unitless)	0.02 (0.79)	-0.1 (1.2)	0.618 (0.5)
DEV	-0.7 (0.92)	0.27 (1.2)	0.106 (-1.63)

Mean and SD for each set of HRV data during metronome guided breathing from the Lifestyle group visit 1 (n=38) and the influenza study, visit 1 (n=71). Analysis was performed using an unpaired t-test. Metronome breathing Parameters shown are described in section 3.10.6.

12.6 Correlational studies for the full lifestyle cohort (N=38)

12.6.1 Weight loss (kg) between baseline and eight months correlated against change in glycaemic indices and serum lipids over eight months.

The change in weight in kg between baseline and eight months was correlated against the change in serum lipids and glycaemic indices for the full cohort between baseline and eight months, using Pearson's correlation. There was no significant correlation with any of the parameters except for HbA1c (%) (P=0.032, r=0.35) see (figure 36).

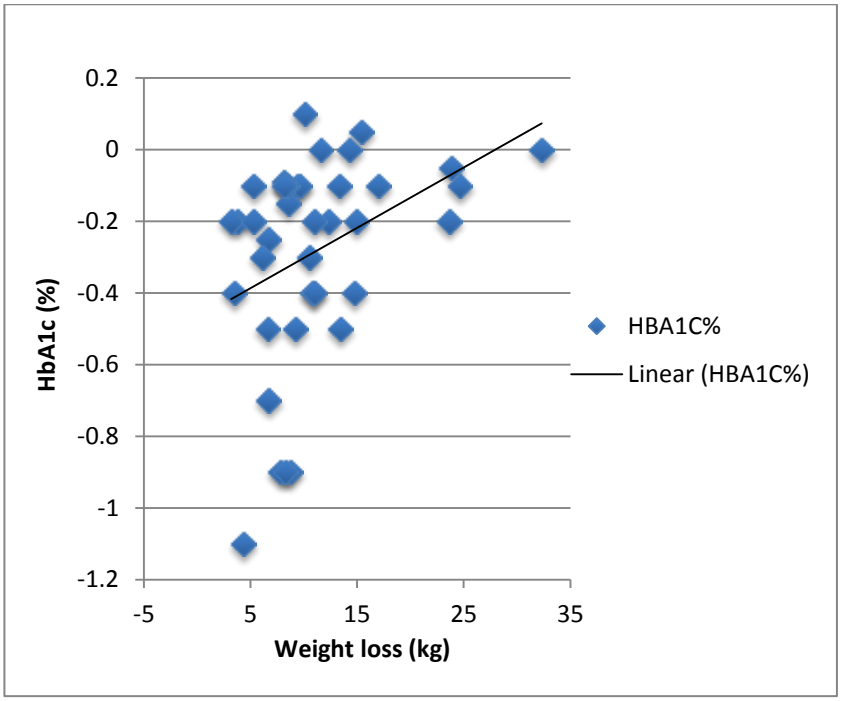


Figure 36 Correlation between weight loss (kg) and change in HbA1c (P=0.032). *Change in data from baseline and eight months.*

12.7 Pupillometry. Iris response to dark.

Due to technical problems with the goggles a number of patients did not have pupilometry performed at every visit. The majority of missing data are from visit one. Comparison of paired data has been made where data for each visit is available.

Analysis of variance (ANOVA) does not show any statistical difference in pupillary response between visits (see Table 35).

Table 35 Pupillometry Iris response to dark

Mean Pupillary response (SD) V1 (±SD)	Mean Pupillary response (SD) V2 (±SD)	Mean Pupillary response (SD) V3 (±SD)	% Diff V1-V3	P Val
-0.75 (1.04)	-0.33 (1.14)	-0.12 (1.13)	-84	0.84

Mean and standard deviation values for the complete cohort at each visit. Analysis of variance significance p value (<0.05).

12.8 Subgroup Analysis:
Changes in Autonomic Function According to Participant Weight Loss.

In this section participants were sub-grouped according to the percentage weight loss at the end of the eight month programme. Participant weight loss was measured at each visit during the eight-month period. For each participant the weight loss achieved was different. Previous analysis of HRV for the total cohort did not take weight loss into account. Some participants exceeded weight loss goals and others did not. The percentage weight loss at the eight-month visit was used to subdivide the total cohort into two subgroups according to the percentage of weight (kg) lost in the period. The following tables show results for two subgroups using a threshold of <12% weight loss (moderate) and ≥12% weight loss (large) weight loss.

12.8.1 Autonomic Function in the Subgroup (<12% weight loss) (N=28) HRV data from Ewing measures and Metronome guided breathing

Autonomic function was assessed for the subgroup of those with <12% weight loss. Statistical analysis, using analysis of variance (ANOVA) was performed on the HR data from the Ewing tests and for the 2 minutes of metronome guided breathing for this subgroup between baseline and at eight months. There were significant statistical

differences between most of the Ewing test provocations apart from handgrip and lying/standing, (the results are shown in Table 36). Although there appears to be an improvement in some HRV parameters with a percentage change increase between visit one and three, there were no statistically significant differences between the metronome guided breathing HRV data over the eight months (see Table 36), which was the same as for the full cohort.

Table 36 Assessment of autonomic function in the subgroup with the threshold of <12%

	V1 Mean (±SD)	**V2 Mean (±SD)**	**V3 Mean (±SD)**	**% Diff**	**P**
Ewing Measures					
Resting (%)	5.01 (3.9)	8.00 (4.87)	8.86 (4.42)	77	**0.001**
Forced Breathing (%)	13.80 (4.85)	19.36 (9.19)	19.44 (7.5)	41	**0.001**
I/E Diff (bpm)	9.01 (2.8)	11.72 (5.64)	11.85 (4.15)	24	**0.001**
Valsalva (ratio)	1.26 (0.13)	1.36 (0.22)	1.36 (0.13)	7.9	**0.008**
Hand grip (ratio)	1.09 (0.09)	1.14 (0.16)	1.17 (0.12)	6.8	0.079
Lying/Standing (ratio)	1.13 (0.12)	1.12 (0.12)	1.14 (0.08)	0.88	0.89
Metronome Breathing					
SD (bpm)	0.22 (0.98)	0.21 (0.86)	0.18 (0.83)	18.2	0.99
A (bpm)	0.077 (0.90)	0.078 (0.79)	0.004 (0.75)	-43	0.82
LPP (unitless)	0.35 (0.90)	0.44 (0.83)	0.37 (0.81)	5.7	0.93
CORR (unitless)	-0.62 (1.14)	-0.55 (1.1)	-0.50 (1.3)	19.4	0.35
FCORR (unitless)	0.02 (0.74)	0.18 (0.31)	0.03 (0.76)	50	0.66
DEV	-0.004 (0.91)	0.008 (0.76)	0.002 (0.88)	150	0.71

Mean (SD) and % difference (between v1-v3) HRV parameters for the subgroup with BMI<12%. Parameters shown are described in section 3.10.6.

12.8.2 Assessment of Autonomic Function using Ewing assessment and during metronome guided breathing (6 breaths per minute) for the Subgroup with >12% Weight Loss (N=10).

Statistical analysis was also performed on the heart rate data for the Ewing tests and for metronome guided breathing for a subgroup of 10 participants that lost >12% in weight over the eight month period. There is an overall tendency towards an improvement in autonomic tone with an increase in percentage difference between baseline and eight months for both Ewing measures and metronome guided breathing. Using analysis of variance there was a statistically significant difference between the resting data (p=0.001), LPP (p=0.038), FCORR (p=0.022), and DEV (p=0.005) (see Table 37).

Table 37 Assessment of autonomic function in the subgroup with the threshold of >12% weight loss.

	V1 Mean (±SD)	**V2 Mean (±SD)**	**V3 Mean (±SD)**	**% Diff V1-V3**	**P Val**
Ewing Measures					
Resting (%)	6.7 (4.4)	8.0 (4.9)	14.1 (6.4)	110.5	**0.001**
Forced Breathing (%)	14.5 (8.3)	19.3 (9.2)	21.7 (8.8)	49.6	0.067
I/E Diff (bpm)	9.5 (5.4)	11.7 (5.6)	12.9 (4.6)	35.8	0.21
Valsalva (ratio)	1.3 (0.1)	1.4 (0.22)	1.4 (0.17)	7.8	0.149
Hand grip (ratio)	1.1 (0.1)	1.14 (0.2)	1.3 (0.2)	18.2	0.203
Lying/Standing (ratio)	1.1 (0.1)	1.12 (0.11)	1.1 (0.1)	0	0.93
Metronome Breathing					
SD (bpm)	-0.016 (0.62)	0.013 (0.77)	-0.029 (0.79)	-81.3	0.322
A (bpm)	-0.2 (0.63)	-0.06 (0.67)	0.09 (0.7)	145	0.23
LPP (unitless)	0.4 (0.95)	0.41 (0.73)	0.52 (0.82)	30	**0.038**
CORR (unitless)	-0.82 (1.55)	-0.72 (0.87)	-0.51 (1.38)	37.8	0.860
FCORR (unitless)	-0.21 (0.55)	0.019 (0.35)	0.026 (0.23)	112	**0.022**
DEV	-0.39 (0.98)	-0.04 (0.62)	0.17 (0.89)	143	**0.005**

Mean and SD values for each HR parameter at each visit. Analysis performed using analysis of variance (p=<0.05) significant results are shown in bold. Metronome breathing parameters shown described in section 3.10.6.

12.9 Further Subgroup Analysis:

The total cohort was further subdivided into three subgroups:

- Subgroup A containing those participants with a BMI ≥36 (N=14)
- Subgroup B containing those participants with a BMI <36 (N=24)
- Subgroup C containing those participants who were pre-diabetic (N=5).

12.9.1 Subgroup A: Participants with Body Mass Index (BMI) ≥36) (N=14)

A threshold of BMI ≥36 was used to identify individuals with the highest BMI in the cohort at baseline. There were 14 people in this subgroup; all were female with a mean BMI of 38.2 (+/-1.04), 2 of the 14 participants were in the group identified as pre-diabetic. Calculating the percentage change between the starting point and the end point of the study there is a marked percentage decrease in all weight related parameters. Percentage weight loss dropped 12.5% equating to 13.2kg overall weight loss with a mean loss of -19.7% loss in body fat. BMI fell from 38.1 (+/-1.04) to 33.5 (+/-3.3) equating to a 12.07% reduction and waist circumference reduced by an average 16.2 cm (-13.8%).

Analysis using analysis of variance for paired data shows that all weight related parameters were markedly changed between visits for the subgroup with the highest BMI ≥36 (see Table 38 and Figure 37). There is a significant difference between visits for all weight related parameters with a fall in both systolic and diastolic pressure coinciding with the concurrent weight loss. Figure 37 (A-E) shows the changes in weight related parameters in subgroup A, between visit one and visit three as box and whisker plots.

Table 38 Table showing mean and (±SD) weight related parameters and blood pressure (mmHg) for subgroup A with BMI≥36.

Parameter	Mean Visit 1 (±SD)	Mean Visit 2 (±SD)	Mean Visit 3 (±SD)	% Diff V1-V3	P Val
Weight kg	105.6 (7.0)	95.7 (7.1)	92.4 (9.56)	-12.5	**0.001**
BMI	38.1(1.04)	34.7 (1.97)	33.5 (3.3)	-12.07	**0.001**
% body fat	48.9 (3.3)	46.18 (2.65)	44.4 (4.7)	-9.2	**0.001**
Kg body fat	51.7 (5.7)	44.26 (4.69)	41.5 (7.9)	-19.7	**0.001**
Waist Circumference (cm)	117.2 (7.2)	104.1 (10.02)	101 (13.4)	-13.8	**0.001**
Systolic BP mmHg	139 (11)	132 (11.72)	127 (17)	-8.6	**0.002**
Diastolic BP mmHg	83 (6.6)	80 (8.64)	74 (9.1)	-10.8	**0.001**

Mean, SD and percentage difference (v1-v3) are shown. Analysis of variance was performed (p=<0.05). Significant results are shown in bold.

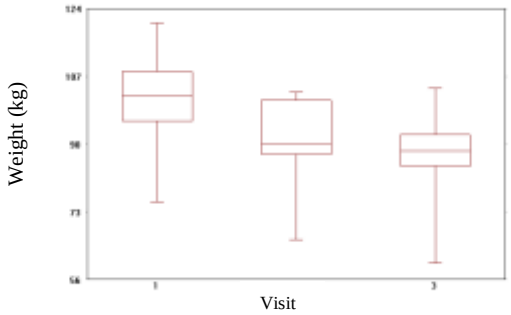


Figure 37 (A) Graph showing weight related parameter (weight kg) for the cohort with BMI≥36

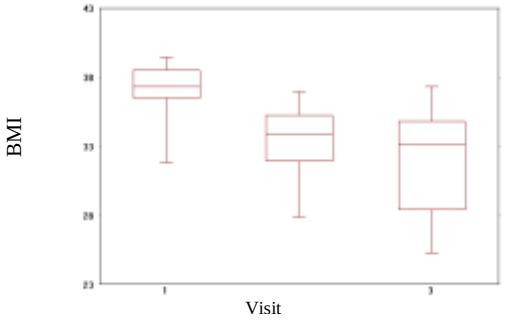


Figure 37 (B) Graph showing weight related parameter (BMI) for the cohort with BMI≥36

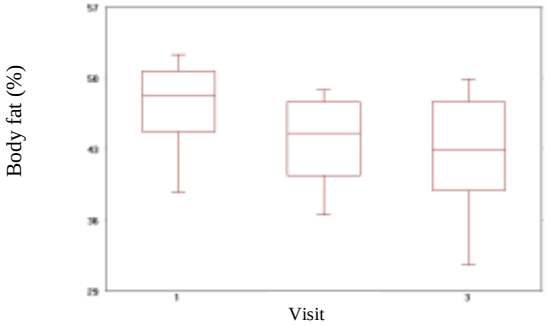


Figure 37 (C) Graph showing weight related parameter (% body fat) for the cohort with BMI≥36

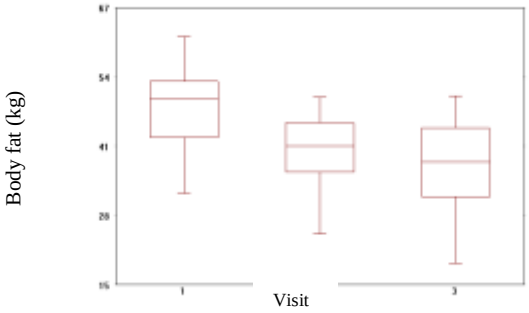


Figure 37 (D) Graph showing weight related parameter (body fat kg) for the cohort with BMI≥36

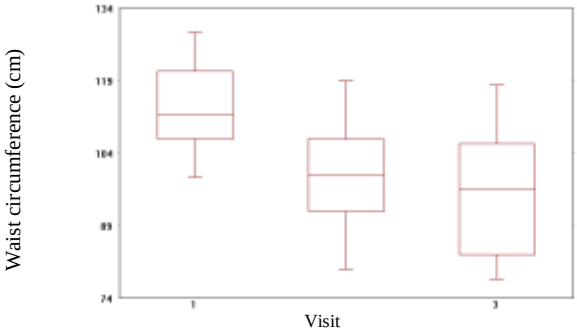


Figure 37 (E) Graph showing weight related parameter (waist circumference cm) for the cohort with BMI≥36

Figure 37 Changes in mean weight related parameters in subgroup A, over the 3 scheduled visits. *(A-E) Box and whiskers plots showing minimum, first quartile, median, third quartile and maximum values*

12.9.1.1 Analysis of Biochemical Data for Subgroup A (BMI ≥36, N=14)

There were marked changes in the biochemical data for this subgroup with a percentage drop noted for all parameters. The largest reduction was seen in GLP-1 representing a 9.6% drop in blood glucose values after the 30 minute repeat test for the group. The smallest percentage reduction was for LDL, which fell 2.8%

After statistical analysis of paired data of the blood samples for this subgroup using analysis of variance there were significant differences representing an improvement in all but blood glucose (0 min) and two of the lipid values (HDL and Triglyceride), (see Table 39). This improvement may be associated with increased exercise and the significant weight loss that occurred during the intensive eight month programme (See Table 38).

Table 39 Analysis of Biochemical Data for Subgroup A (BMI ≥36)

Parameter	**Mean Visit 1 (±SD)**	**Mean Visit 2 (±SD)**	**Mean Visit 3 (±SD)**	**% Diff v1-v3**	**P Val**
Blood Glucose (0min) (GLP-1)	5.1 (0.40	4.86 (0.43)	4.85 (0.42)	-4.9	0.073
Blood Glucose (30min) (GLP-1)	8.3 (1.18)	7.66 (1.08)	7.5 (1.85)	-9.6	**0.012**
Total Cholesterol mg/dL	5.77 (0.87)	5.55 (0.71)	5.5 (0.87)	-4.7	**0.026**
Low Density Lipid (mg/dL)	3.6 (0.89)	3.49 (0.76)	3.5 (0.81)	-2.8	**0.05**
High Density Lipid (mg/dL)	1.56 (0.45)	1.53 (0.40)	1.51 (0.48)	-3.21	0.83
Triglycerides (mg/dL)	1.32 (0.43)	1.16 (0.35)	1.22 (0.56)	-7.6	0.24
Lipid Ratio	4.14 (1.36)	3.90 (1.22)	4.0 (1.28)	-3.4	**0.001**
HbA1c (%)	5.9 (0.36)	5.74 (0.36)	5.7 (0.33)	-3.4	**0.011**
HbA1c (mmol)	40.7 (3.87)	39.29 (4.03)	38.4 (3.43)	-5.7	**0.01**

Table showing mean (±SD) and percentage difference for biochemical measures for subgroup A (BMI≥36) at each visit. Results of analysis of variance (p=<0.05) are shown in bold.

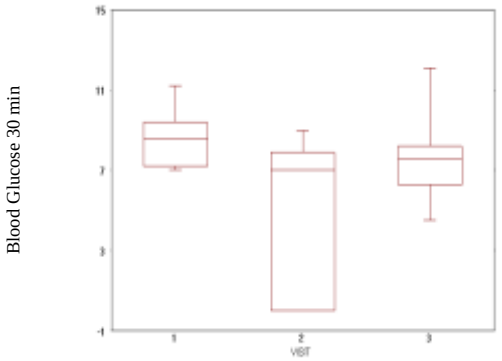


Figure 38 (A) Analysis of biochemical data (Blood Glucose 30 minutes) for subgroup A (BMI≥36) at each visit.

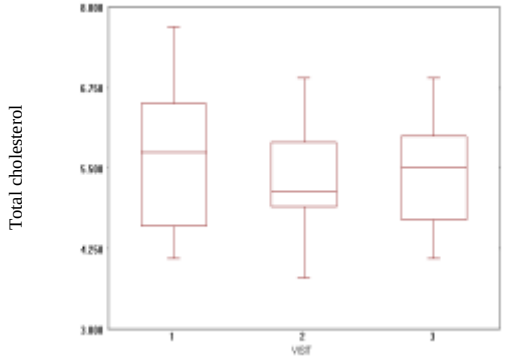


Figure 38 (B) Analysis of biochemical data (Total cholesterol) for subgroup A (BMI≥36) in the cohort at baseline and

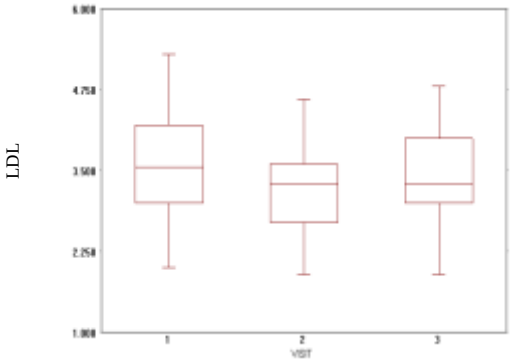


Figure 38 (C) Analysis of biochemical data (low density lipids) for subgroup A (BMI≥36) in the cohort at baseline and

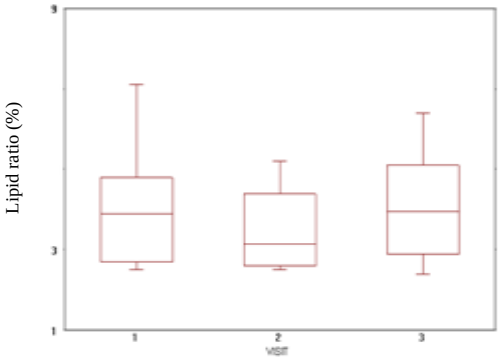


Figure 38 (D) Analysis of biochemical data (ratio) for subgroup A (BMI≥36) at each visit.

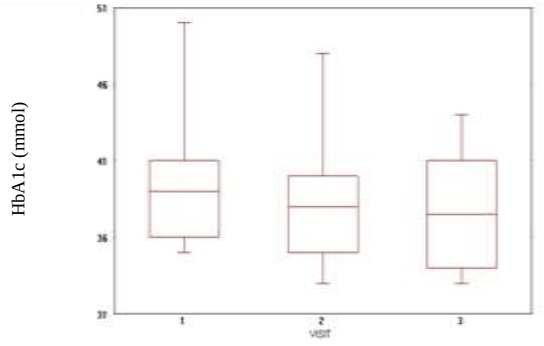


Figure 38 (E) Analysis of biochemical data (HbA1c mmol) for subgroup A (BMI≥36) at each visit.

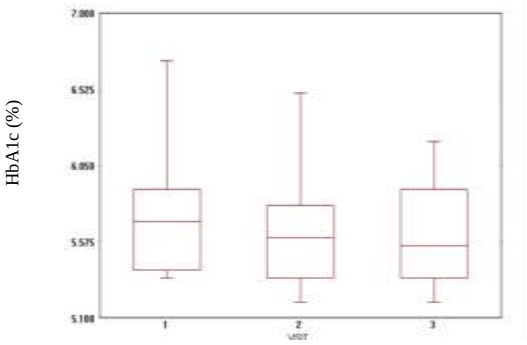


Figure 38 (F) Analysis of biochemical data (HbA1c %) for subgroup A (BMI≥36) at each visit.

Figure 38 (A-F) Box and whiskers graphs showing biochemical measures for statistically significant results for subgroup A (BMI≥36) at visit 1, 2 and 3. (*A) shows blood glucose (30 minutes). (B) shows Total cholesterol. (C) shows LDL. (D) shows Lipid ratio. (E) shows HbA1Cmmol. (F) shows HbA1c%. Box and whiskers plots show minimum, first quartile, median, third quartile and maximum values*.

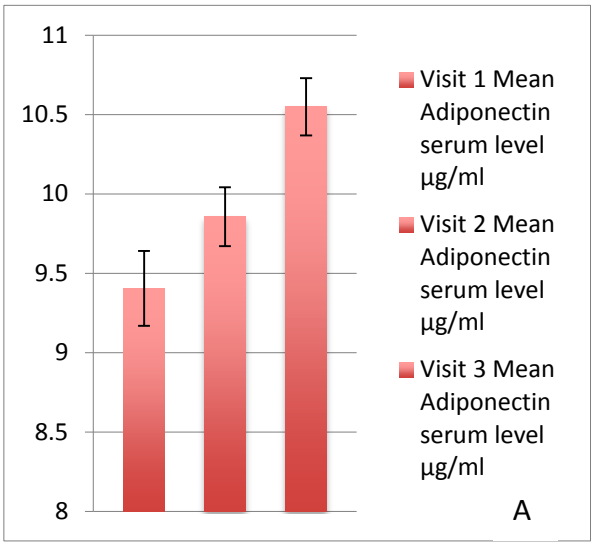
12.9.2 Changes in Serum Adiponectin and Leptin Levels for Sub-group A (BMI ≥36) (N=14)

There was a rise in serum adiponectin between baseline and visit three (24.3%) and a modest decrease in serum leptin (-5.6%). This would be expected with significant weight loss (Vinik 2013). Serum levels of adiponectin and leptin for subgroup A were analysed using analysis of variance for data over eight months. There is a statistically significant difference (increase) between the serum adiponectin levels values, (p=0.001) from baseline and visit three. We also found after statistical analysis, mean leptin levels were higher at baseline associated with the higher BMI in this obese group and this was significantly lower (p=0.001) at eight months after considerable weight loss (see Table 40 and Figure 39**).** The results indicate an inverse relationship between obesity and low adiponectin and a rise in serum adiponectin levels is associated with significant weight loss. Correlational studies in section 12.10 also support this.

Table 40 Serum Adiponectin and Leptin levels for each visit for sub-group A (BMI ≥36) (N=14)

	Visit 1	**Visit 2**	**Visit 3**	**% Diff (v1-v3)**	**P Value**
Mean Adiponectin serum level µg/ml (±SD)	9.21 (0.68)	9.85 (0.69)	11.45 (10.87)	24.3	**0.001**
Mean Leptin serum level ng/ml (±SD)	59.5 (1.16)	58.0 (0.76)	56.17 (1.52)	-5.6	**0.001**

Mean (±SD) and Percentage difference between visit 1 and visit 3 Results of analysis of variance (ANOVA) are shown in table below. Statistically significant values (<0.05) are shown in bold.



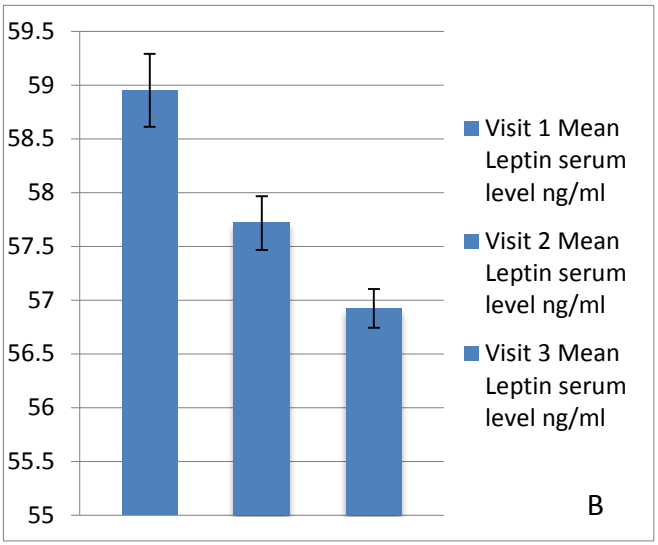


Figure 39 Bar charts showing mean serum adiponectin (A) and Leptin (B) for each visit for subgroup A (BMI≥36).

Standard error bars are shown. Analysis of variance shows a statistically significant difference (p=0.001) between visits with an increase in adiponectin and a decrease in leptin in association with concomitant weight loss

12.9.3 Assessment of Heart Rate Variability for Subgroup A (N=14) (BMI ≥36).

For subgroup A, percentage change was calculated between visit one and visit 3. There is an overall increase for most HRV parameters, apart from LPP (-6.7%) and lying/standing (0%) see (Table 41). Analysis of the HRV data for the three visits using analysis of variance there was a statistically significant difference in the Ewing parameters, resting (p=0.001), forced breathing (p=0.013), I/E diff (p=0.021) and Valsalva (p=0.002) (see Table 41 and Figure 40). Comparison of HRV data during metronome guided breathing using analysis of variance, showed there were no statistically significant differences between data, despite percentage increases between most parameters at baseline and visit three. This percentage increase in HR data does however suggest a trend towards an increase in the HRV parameters over the eight months; these are shown in Table 41.

Table 41 Assessment of Heart Rate Variability for Subgroup A (N=14) with a BMI ≥36.

	V1 Mean (±SD)	V2 Mean (±SD)	V3 Mean (±SD)	% Diff v1-v3	P Value
Ewing Measures of HRV					
Resting (%)	4.84 (2.9)	7.35 (5.6)	7.6 (3.1)	58	**0.001**
Forced Breathing (%)	13.2 (5.4)	18.5 (10.2)	22.03 (9.2)	66.9	**0.013**
I/E Diff (bpm)	8.3 (3.1)	11.15 (6.2)	11.9 (4.9)	43.4	**0.021**
Valsalva (ratio)	1.21 (0.1)	1.37 (0.18)	1.37 (0.15)	7.4	**0.002**
Hand grip (ratio)	1.08 (0.05)	1.25 (0.50)	1.23 (0.21)	13.9	0.291
Lying/Standing (ratio)	1.13 (0.11)	1.16 (0.14)	1.13 (0.07)	0	0.734
Metronome Breathing					
SD (bpm)	0.02 (0.67)	0.04 (0.93)	0.05 (0.68)	150	0.064
A (bpm)	-0.34 (0.53)	-0.04 (0.89)	-0.02 (0.86)	94	0.424
LPP (unitless)	0.15 (0.71)	0.13 (1.03)	0.14 (0.96)	-6.7	0.242
CORR (unitless)	-0.12 (1.2)	-0.12 (1.08)	-0.041 (0.85)	65	0.197
FCORR (unitless)	-0.26 (0.88)	-0.06 (0.34)	-0.26 (0.59)	0	0.242
DEV	-0.52 (0.73)	-0.03 (1.01)	-0.04 (1.0)	92	0.077

HRV was assessed using Ewing measurements and Metronome guided breathing during two minutes at 6 breaths per minute. Mean, SD and percentage difference are shown. Metronome breathing parameters described in section 3.10.6. HR data was analysed using ANOVA. Significant results are shown in bold.

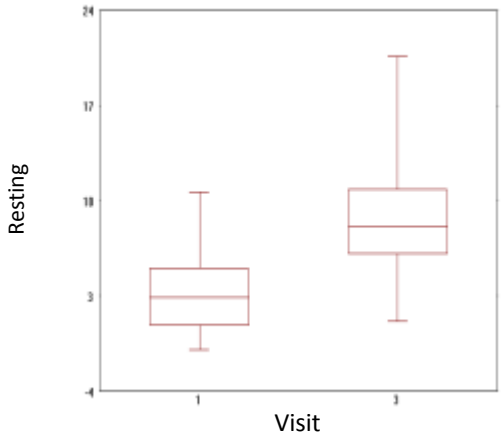


Figure 40 (A) Graph showing HRV values for cohort with a BMI ≥36. Assessment of HRV at visit 1 and visit 3 for Ewing measurement (resting parameter)

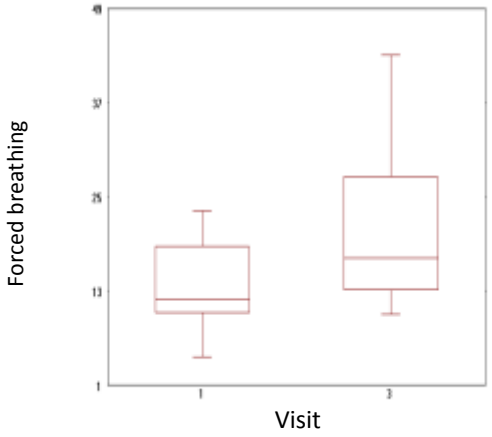


Figure 40 (B) Graph showing HRV values for cohort with a BMI ≥36. Assessment of HRV at visit 1 and visit 3 for Ewing measurement (forced breathing parameter).

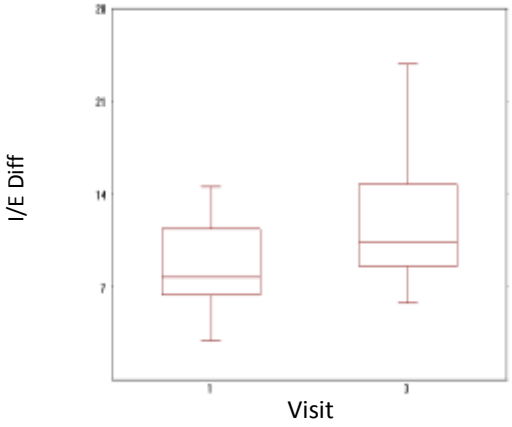


Figure 40 (C) Graph showing HRV values for cohort with a BMI ≥36. Assessment of HRV at visit 1 and visit 3 for Ewing measurement (I/E Diff parameter).

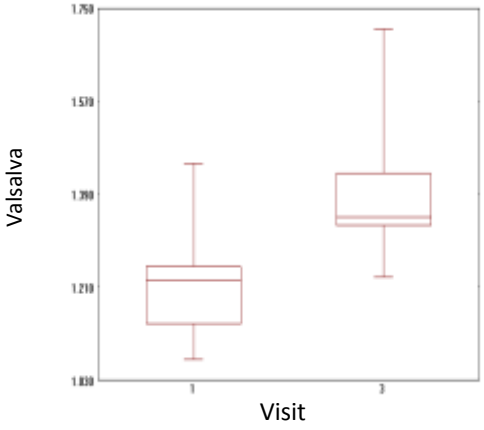


Figure 40 (D) Graph showing HRV values for cohort with a BMI ≥36. Assessment of HRV at visit 1 and visit 3 for Ewing measurement (Valsalva parameter).

Figure 40 HRV parameters for Subgroup A (BMI≥36) during Ewing assessment

(A-D) show *(A) Resting, (B) Forced Breathing, (C) I/E Diff, (D) Valsalva). Box and whisker plots represent the parameters with statistically significant differences after analysis of variance, showing minimum, first quartile, median, third quartile and maximum values.*

12.9.3.1 Analysis of HRV measures at baseline and at eight months using a paired t-test

Due to the percentage increase in heart rate data noted between visit 1 and 3 for the metronome breathing data in Table 41, the data for subgroup A from baseline and at the end of the study (eight months) was re-analysed using a paired t-test. Results show a significant difference in all Ewing measures and metronome guided breathing HRV parameters apart from LPP and FCORR (see Table 42).

Table 42 Analysis of HRV measures at baseline and at eight months using a paired t-test

	V1 Mean (±SD)	**V3 Mean (±SD)**	**% Diff V1-V3**	**P Value**
Ewing Parameters				
Resting (%)	4.84 (2.9)	7.6 (3.1)	58	**0.005**
Forced Breathing (%)	13.2 (5.4)	22.03 (9.2)	66.9	**0.01**
I/E Diff (bpm)	8.3 (3.1)	11.9 (4.9)	43.4	**0.01**
Valsalva (ratio)	1.21 (0.1)	1.37 (0.15)	7.4	**0.004**
Hand grip (ratio)	1.08 (0.05)	1.23 (0.21)	13.9	**0.02**
Lying/Standing (ratio)	1.13 (0.12)	1.13 (0.08)	0	**0.05**
Metronome Breathing				
SD (bpm)	0.02 (0.67)	0.05 (0.68)	150	**0.02**
A (bpm)	-0.34 (0.53)	-0.02 (0.86)	94	**0.04**
LPP (unitless)	0.15 (0.71)	0.14 (0.96)	-6.7	0.11
CORR (unitless)	-0.12 (1.2)	-0.041 (0.85)	65	**0.05**
FCORR (unitless)	-0.26 (0.88)	-0.26 (0.59)	0	*0.07*
DEV	-0.52 (0.73)	-0.04 (1.0)	92	**0.05**

Mean (±SD) measures of HRV for subgroup A. Analysis using a paired t test for data from baseline visit and at eight months, statistical significance at p=<0.05. Significant results are shown in bold. Metronome guided breathing parameters shown are shown in section 3.10.6.

12.9.3.2 Comparison of Heart Rate Variability for Subgroup A (BMI ≥36) and the Cohort of healthy volunteers from the Influenza Study (N=71).

Analysis was performed using an unpaired t-test to compare baseline HRV data for subgroup A (BMI ≥36) (during metronome guided breathing) and the baseline HRV data for the cohort of healthy volunteers from the Influenza study (during metronome guided breathing). Results show a significant difference in heart rate variability parameters (SD, A, LPP and DEV), see Table 43. Heart rate variability is attenuated in Subgroup A at baseline compared to a normal range, which is likely to be linked to the level of obesity and raised BMI in this group, we note an improvement in autonomic tone at eight months after significant weight loss.

Table 43 Comparison of Subgroup A (BMI≥36) (N=14) HRV data from visit one with the Healthy Volunteers from the influenza study HRV data from visit one (N=71)

	V1 Mean (±SD) Subgroup A BMI>36 (N=14)	**V1 Mean (±SD) Influenza Study Healthy Volunteers (N=71)**	**P Value (t val)**
SD (bpm)	0.02 (0.67)	0.63 (1.0)	**0.011 (-3.76)**
A (bpm)	-0.34 (0.53)	0.33 (1.0)	**0.001 (-2.71)**
LPP (unitless)	0.15 (0.71)	0.65 (0.8)	**0.001 (-3.37)**
CORR (unitless)	-0.12 (1.2)	-0.28 (1.8)	0.640 (0.47)
FCORR (unitless)	-0.26 (0.88)	-0.1 (1.2)	0.649 (-0.46)
DEV	-0.52 (0.73)	0.27 (1.2)	**0.013 (-2.55)**

HRV was assessed during two minutes of Metronome guided breathing at 6 breaths per minute. Mean and are shown. Metronome breathing parameters described in section 3.10.6. HR data was analysed using an unpaired t-test. Significant results are shown in bold.

12.9.3.3 Summary of Resting Heart Rate Variability for Subgroup A (BMI≥36) during Eight Month Lifestyle Programme

Analysis of resting HRV was performed using analysis of variance. A table of results (Table 44) summarises the differences between measures of resting heart rate variability. Other measures include Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. Assessment with analysis of variance showed there were no statistically significant differences between any heart rate variability measures during the eight month study period.

Table 44 Analysis of extended resting heart rate data for Subgroup A (BMI ≥36) N=14

Parameter	Visit 1 Mean (±SD)	Visit 2 Mean (±SD)	Visit 3 Mean (±SD)	P value
***Resting Heart Rate (bpm)**	73.79 (9.83)	73.57 (10.11)	74.18 (11.14)	0.481
Fractal Dimension	-1.24 (0.04)	-1.25 (0.04)	-1.25 (0.06)	0.233
SDNN (ms)	59.03 (36.42)	86.09 (76.2)	80.3 (52.30)	0.237
PNN50 (%)	11.09 (17.07)	13.08 (14.9)	17.39 (18.08)	0.384
LF/HF Ratio	2.84 (2.05)	3.36 (1.98)	2.94 (2.40)	0.525

*Mean and Standard deviation for each visit. Fractal dimension as defined by Higuchi's algorithm. SDNN = Standard deviation of RR interval. PNN50 = percentage of RR intervals where the gap between adjacent intervals is greater than 50ms. LF/HF ratio = ratio of integrated spectral power of the portions of the heart rate frequency plot in the low frequency (0.04-0.15 Hz) and high frequency (0.15 – 0.4 Hz) bands. *Extended resting period. ANOVA performed. P values are shown as significant at p=<0.05.*

12.10 Correlational Studies: Change in Serum lipids and glycaemic indices between baseline and eight months against change in weight (kg) between baseline and eight months for subgroup A.

Correlational analyses were performed using Pearson's product moment correlation coefficient to ascertain the degree of correlation between change in weight in kilogrammes for subgroup A between visit 1 and visit 3 and serum lipids and glycaemic indices. There was a significant correlation between weight loss and the change in triglyceride (p=0.05) and HbA1c levels (p=0.029) between baseline and visit 3 (see Figure 41 (A) and Figure 41 (B))

Table 45 Correlational Studies: Change in Serum lipids and glycaemic indices between baseline and eight months against change in weight (kg) between baseline and eight months for subgroup A.

Parameter	Pearson's r	T value	P value
Blood Glucose (0min) (GLP-1)	-0.24	-0.84	0.414
Blood Glucose (30min) (GLP-1)	-0.40	-1.5	0.15
Total Cholesterol mg/dL	-0.35	-1.28	0.22
Low Density Lipid (mg/dL)	-0.24	-0.89	0.39
High Density Lipid (mg/dL)	0.19	0.6	0.51
Triglycerides (mg/dL)	0.53 (gradient = 0.0236 mg/dL/Kg weight loss)	2.4	**0.05**
Lipid Ratio	-0.3	2.08	0.29
HbA1c (%)	0.58 (gradient = 0.0198 %/kg weight loss)	2.4	**0.029**
HbA1c (mmol)	0.46	1.8	0.08

Results using Pearson's correlation coefficient for the change in Serum lipids and glycaemic indices between baseline and eight months against change in weight (kg) between baseline and eight months for subgroup A. Significant results (p=<0.05) are shown in bold

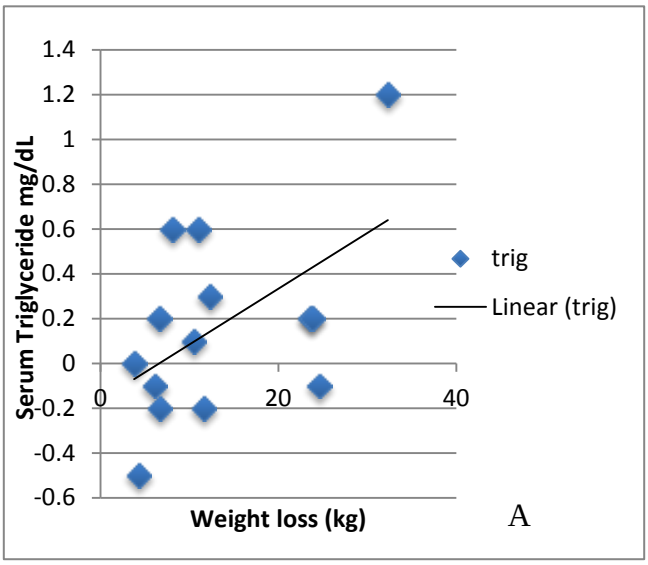


Figure 41 (A)

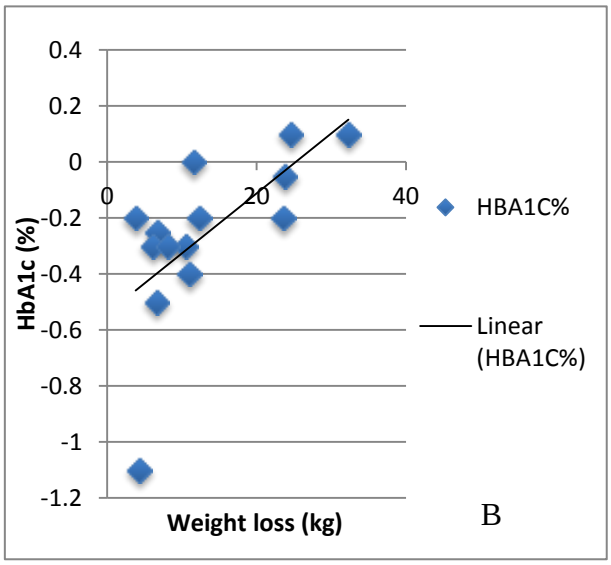


Figure 41 (B)

Figure 41 Correlation of change in weight and change in HbA1c and triglyceride.

Graphs showing correlation between (A) Change in Serum triglyceride correlated against change in weight (kg) over eight months for subgroup A and (B) Change in HbA1c correlated against change in weight (kg) over eight months for subgroup A (BMI ≥36)

12.11 Correlational Studies: Change in HRV parameters correlated against weight change (kg) between baseline and eight months for Subgroup A (BMI≥36)

Correlational analyses were performed using Pearson's product moment correlation coefficient to ascertain the degree of correlation between the change weight (kg) in subgroup A between visit 1 and visit 3 and HRV parameters during 2 minutes of metronome guided breathing and for Ewing measures.

Statistical analysis shows a significant correlation between HRV parameters (forced breathing (p=0.03), handgrip (p-0.04), LPP (p=0.028), CORR (p=0.03), DEV (p=0.02)) and change in weight (kg). As weight decreased these HRV parameters increased

representing an improvement in autonomic tone (see Table 46, Figure 42 and Figure 43).

Table 46 Change in HRV parameters correlated against weight change (kg) between baseline and eight months for Subgroup A (BMI≥36)

HRV correlated against weight change (kg)	Pearson's R	T Value	P Value
Ewing Parameters			
Resting (%)	0.356	1.3	0.2
Forced Breathing (%)	0.58 (gradient = 0.447%/kg weight loss)	2.4	**0.03**
I/E Diff (bpm)	0.504	2.5	0.06
Valsalva (ratio)	0.59	2.5	**0.04**
Hand grip (ratio)	0.21 (gradient = 0.014/kg weight loss)	0.7	0.4
Metronome Breathing			
SD (bpm)	0.44	1.5	0.06
A (bpm)	0.45	1.15	0.08
LPP (unitless)	0.58 (gradient = 0.0428/kg weight loss)	2.5	**0.028**
CORR (unitless)	0.36 (gradient = 0.0428/kg weight loss)	2.4	**0.03**
FCORR (unitless)	0.04 (gradient = 0.0187/kg weight loss)	0.17	0.86
DEV	0.31 (gradient = 0.0296/kg weight loss)	2.15	**0.02**

Results using Pearson's correlation coefficient for the change in Ewing and metronome guided breathing HRV parameters and weight change (kg). Correlation between data from baseline and visit 3 for subgroup A (BMI ≥36.) Metronome breathing Parameters shown are described in section 3.10.6.

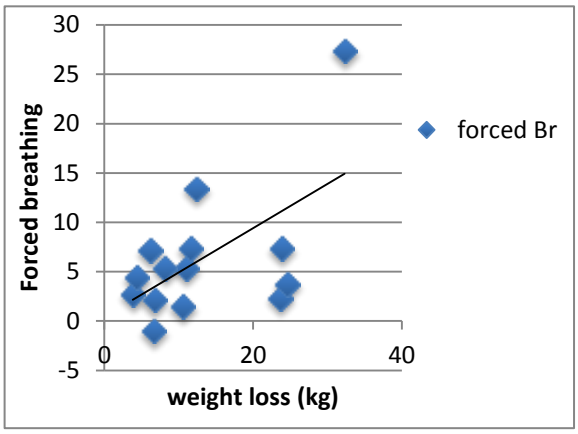


Figure 42 (A)

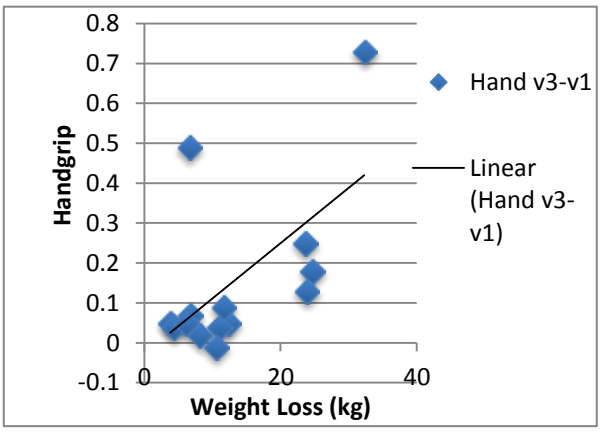


Figure 42 (B)

Figure 42 Scatter graphs showing significant correlational data for Ewing measures of HRV

(A) Forced breathing, (B) Handgrip correlated against a change in weight from baseline and visit 3 for subgroup A

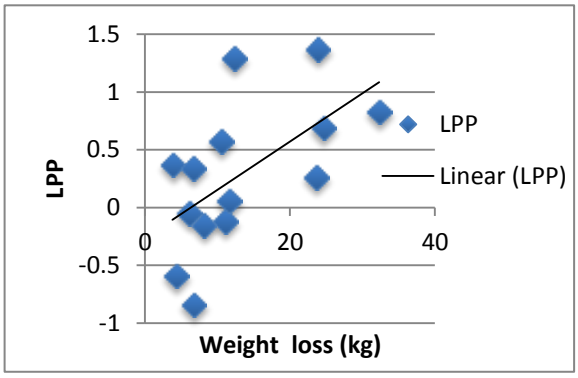


Figure 43 (A)

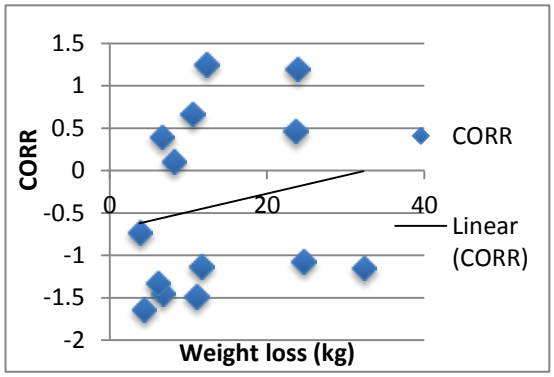


Figure 43 (B)

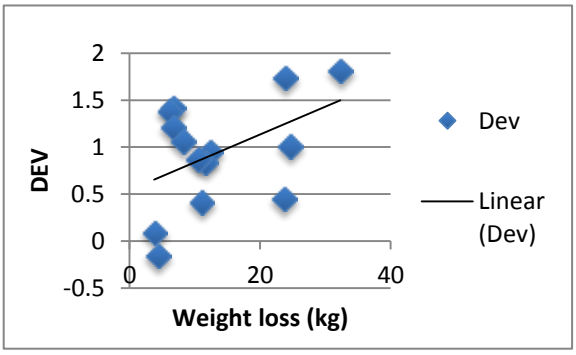


Figure 43 (C)

Figure 43 Scatter graphs showing significant correlation for measures of HRV during metronome guided breathing. *(A) LPP (B) CORR and (C) DEV correlated against a change in weight (kg) from baseline and visit 3 for subgroup A.*

12.12 Correlational Studies: Change in Serum levels of Adiponectin and Leptin between baseline and visit 3 correlated against changes in weight (kg) between baseline and visit 3 for Subgroup A (BMI≥36）(N=14).

Correlational studies were performed on data from subgroup A (N=14) using Pearson's correlation. Change in weight (kg) between visit one and visit three was correlated with the change in adiponectin and leptin levels between visit one and visit 3. There was a significant inverse correlation between a reduction in weight and a rise in serum adiponectin (p=<0.01) and a significant correlation with a fall in weight and a fall in serum leptin levels (p=<0.01) (see Table 47 and Figure 44). Analysis of variance shows levels of adiponectin and leptin both changed significantly over the course of the eight month study (see Table 40 and Figure 39).

Table 47 Change in Serum Adiponectin and Leptin correlated against change in weight (kg).

	Pearson's R	**T Val**	**P Val**
Weight loss correlated against Adiponectin (V3-V1)	**0.839 (gradient = 0.0139/kg weight loss)**	5.34	**<0.01**
Weight loss correlated against Leptin (V3-V1)	-0.917 (gradient = 0.0454/kg weight loss)	-7.97	**<0.01**

Data from Subgroup A. Results of Pearson's r correlation where (p=<0.05). significant figures are shown in bold.

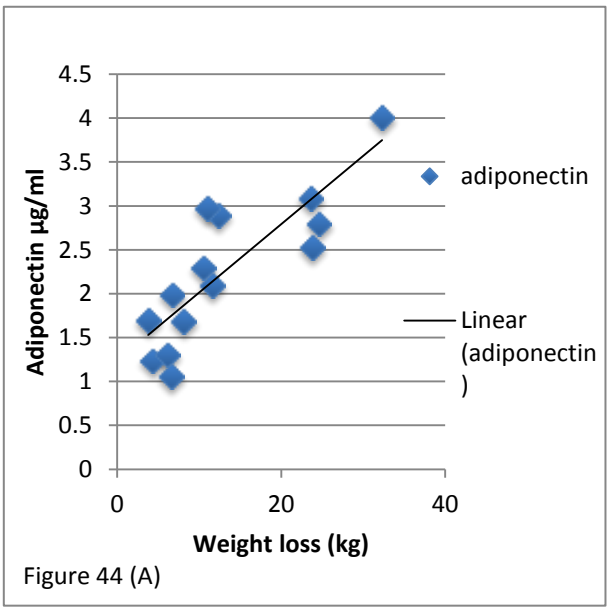


Figure 44 (A)

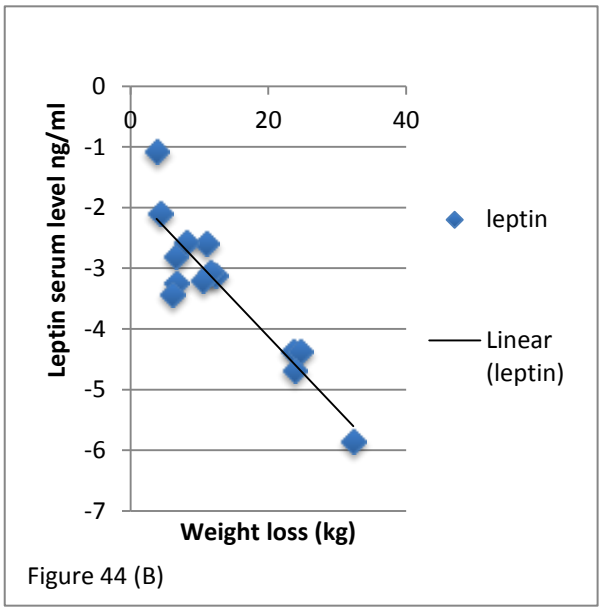


Figure 44 (B)

Figure 44 Graphs showing the significant results of correlational studies for subgroup A:
Changes in (A) Serum Adiponectin and (B) Serum Leptin between baseline and visit 3 were correlated against the change in weight (kg) between baseline and visit 3. Lines of best fit have been added.

12.13 Correlational Studies: Change in Serum levels of Adiponectin and Leptin between baseline and visit 3 correlated against changes in Heart Rate Variability Parameters for subgroup A

The change in serum adiponectin and leptin levels between baseline and visit 3 were also correlated with changes in HRV parameters between baseline and visit 3. There was no significant correlation between HRV measures and serum adiponectin or serum leptin levels apart from handgrip and leptin ($p=0.02$, $r=-0.58$).

12.14 Pupillometry: Iris response to dark. In Subgroup A (N=14) with a BMI ≥36

Pupillometry was performed and analysed for subgroup A for visits two and three. The reason for this was that we had technical difficulties with the infrared goggles during the first visit for a number of the participants making this subgroup too small to perform paired analysis with the other visits.

Although there was a 10.5% increase in pupil: iris ratio noted between visit 2 and visit 3, statistical analysis did not show any differences (p=0.63) between the pupilometry data for visit 2 and visit 3.

Table 48 Pupillometry for Subgroup A.

	Mean V2	**SD V2**	**Mean V3**	**SD V3**	**% Diff V2-V3**	**P Val**
V2 vs. V3	-0.86	1.2	-0.77	1.2	10.5	0.63

A Comparison of Standard Deviation from the age matched normal range for a Subgroup A (N=14) with a BMI ≥36. No statistically significant differences.

12.15 Further Subgroup Analysis: Subgroup B: Participants with Body Mass Index (BMI) <36 (N=24)

12.15.1 Changes in Anthropometric Measurement for Subgroup B Over Eight Month Period

Individuals with a BMI <36 were identified from the total cohort. There were 24 subjects in this subgroup. This subgroup lost a significant amount of weight over the eight month period equating to a mean percentage loss of 10.9% of their original weight and a mean difference of -18.2% in body fat. The average BMI of the subgroup at the end of the programme was 29.2 (+/-2.3) a reduction of 11% and waist size dropped a significant 13.5%.

Weight related data for subgroup B were analysed using analysis of variance. Statistical analysis shows all parameters were significantly different between visits. The results are shown in Table 49. Blood pressure was also measured at each visit and the percentage difference fell between the start and end of the study, however using analysis of variance only the diastolic blood pressure was significantly different with a mean fall from 85mmHg to 78 mmHg

Table 49 Table showing anthropometric parameters for subgroup B (BMI<36)

Parameter	Mean Visit 1 (±SD)	Mean Visit 2 (±SD)	Mean Visit 3 (±SD)	% Diff (v1-V3)	P Value
Weight kg	91.1 (12.1)	83.5 (12.1)	81.2 (12.6)	-10.9	**0.001**
BMI	32.8 (1.7)	30.1 (1.8)	29.2 (2.3)	-11.0	**0.001**
% body fat	40.8 (6.2)	38 (6.7)	37.1 (7.4)	-9.1	**0.001**
Kg body fat	36.9 (6.2)	31.5 (6.8)	30.2 (7.8)	-18.2	**0.001**
Waist Circumference (cm)	104.2 (8.6)	94.6 (8.7)	90.14 (8.7)	-13.5	**0.001**
Systolic BP (mmHg)	129 (13.1)	126 (12.1)	125 (10.4)	-4.6	0.09
Diastolic BP (mmHg)	85 (7.5)	79 (6.8)	78 (6.7)	-8.2	**0.002**

Mean (±SD) values for anthropometric measures are shown. Analysis using ANOVA was performed with significant results shown in bold.

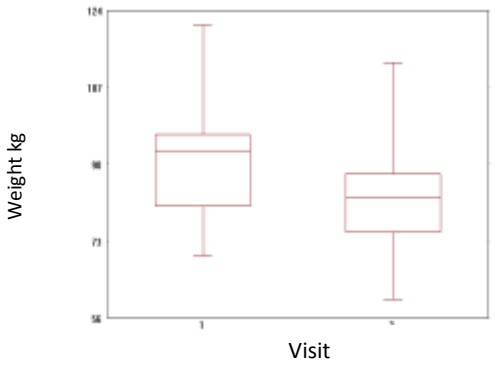


Figure 45 (A) Graph showing weight related parameter (weight kg) for the cohort with BMI<36

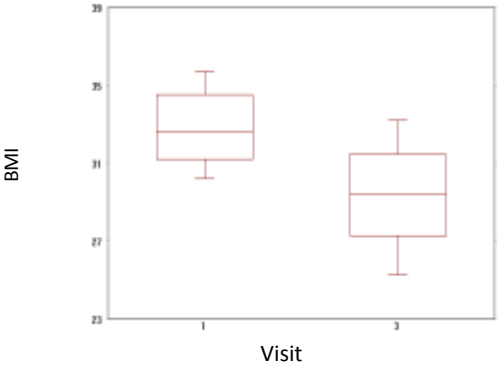


Figure 45 (B) Graph showing weight related parameter (BMI) for the cohort with BMI<36

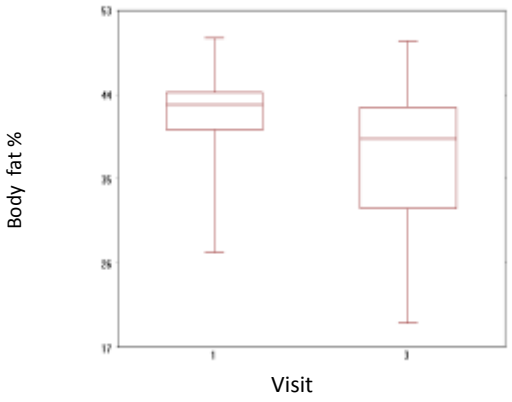


Figure 45 (C) Graph showing weight related parameter (% body fat) for the cohort with BMI<36

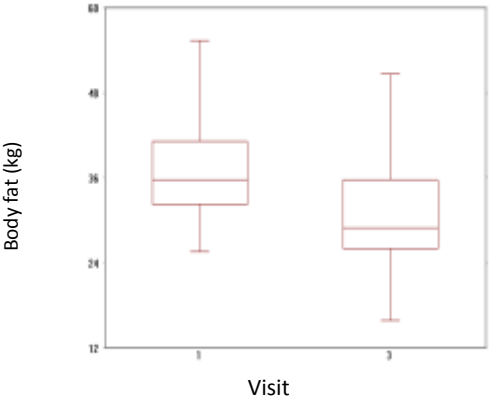


Figure 45 (D) Graph showing weight related parameter (body fat kg) for the cohort with BMI<36

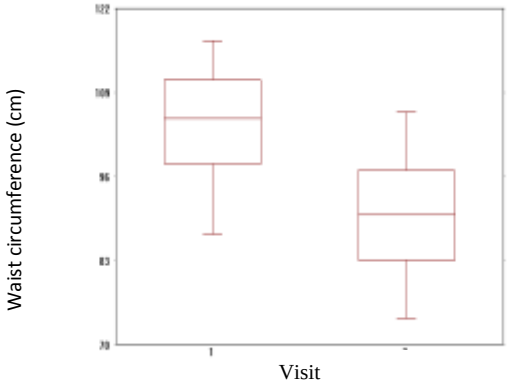


Figure 45 (E) Graph showing weight related parameter (waist circumference cm) for the cohort with BMI<36

Figure 45 Anthropometric parameters at visit 1 and visit 3 for subgroup B the cohort with BMI<36

(*A-E) show box and whiskers plots showing minimum, first quartile, median, third quartile and maximum values.*

12.15.2 Statistical analysis of biochemical data for subgroup B (BMI <36)

All biochemical measures reduced between baseline and visit 3. After statistical analysis of the blood samples for this subgroup using a repeated measures analysis of variance (ANOVA) there were significant differences in blood glucose (p=0.01), HDL (P0.012), Triglyceride (p=0.021) and HbA1c (p=0.05) values (see Table 50).

Table 50 Statistical analysis of biochemical data for participants with BMI <36 baseline and eight months

Parameter	**Mean Visit 1 (±SD)**	**Mean Visit 2 (±SD)**	**Mean Visit 3 (±SD)**	**% Diff (v1-v3)**	**P Val**
Blood Glucose (0min) (GLP-1)	5.2 (0.5)	4.8 (1.2)	5.0 (0.5)	-3.8	0.325
Blood Glucose (30min) (GLP-1)	8.6 (1.7)	8.0 (1.5)	8.3 (1.7)	-3.5	**0.001**
Total Cholesterol mg/dL	5.6 (1.1)	5.5 (1.04)	5.4 (0.98)	-3.6	0.092
Low Density Lipid (mg/dL)	3.6 (0.88)	3.5 (0.84)	3.4 (0.86)	-5.6	0.069
High Density Lipid (mg/dL)	1.4 (0.4)	1.4 (0.39)	1.37 (0.32)	-19	**0.012**
Triglycerides (mg/dL)	1.4 (0.67)	1.3 (0.8)	1.1 (0.64)	-21.4	**0.021**
Lipid Ratio	4.3 (1.1)	4.1 (1.2)	3.8 (0.96)	-11.6	**0.001**
HbA1c (%)	5.8 (0.4)	5.7 (0.4)	5.7 (0.35)	-1.7	0.09
HbA1c (mmol)	39.6 (4.5)	38.9 (4.6)	38.5 (3.89)	2.78	**0.05**

Mean, SD and percentage difference are shown for all measures. Results of repeated measures ANOVA are shown in bold where p=0.05

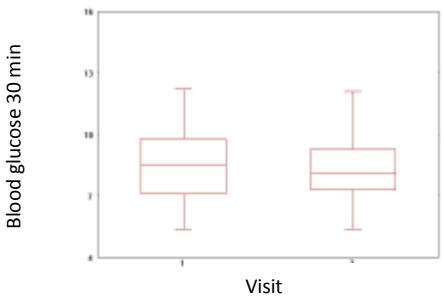


Figure 46 (A) Graph showing blood glucose 30 minutes for the cohort with BMI<36 at visit 1 and 3

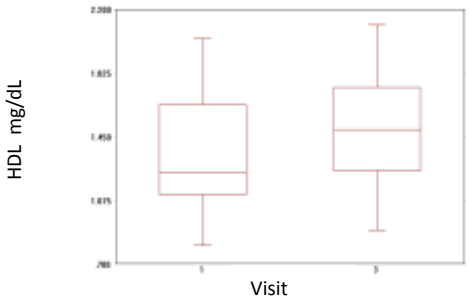


Figure 46 (B) Graph showing HDL for the cohort with BMI<36 at visit 1 and 3

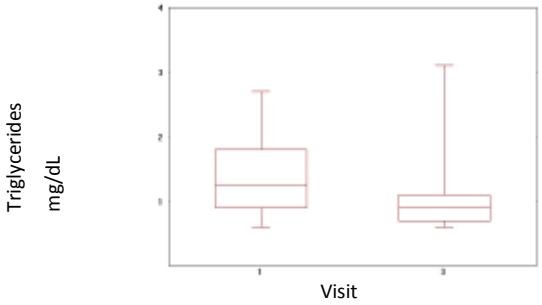


Figure 46 (C) Graph showing triglycerides for the cohort with BMI<36 at visit 1 and 3

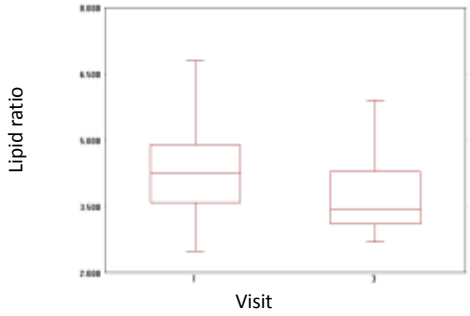


Figure 46 (D) Graph showing ratio for the cohort with BMI<36 at visit 1 and 3

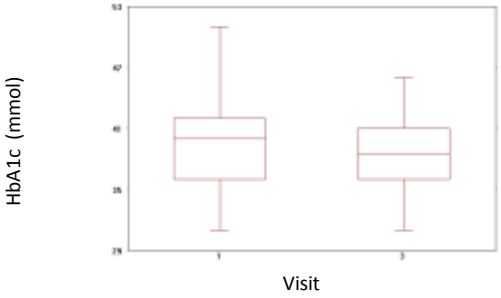


Figure 46 (E) Graph showing HbA1c mmol for the cohort with BMI<36 at visit 1 and 3

Figure 46 Box and whiskers plots show the biochemical data for subgroup B, (BMI<36) for visit one and visit three.

Statistically significant differences were seen for all parameters shown above

12.15.3 Assessment of Heart Rate Variability for Subgroup B (BMI <36)

For subgroup B (BMI <36), the percentage change in HR data between visits one and 3 was calculated. For the Ewing measures there was a percentage increase in all but the lying/standing parameter. For the metronome breathing data the result was more mixed with a percentage rise seen in HR data in only the (A) and (FCORR) parameter.

Analysis was performed using a paired ANOVA; there were statistical differences in all Ewing measures apart from the lying/standing parameter (see Table 51). There were no statistically significant differences in HRV for the same subgroup during metronome guided breathing.

Table 51 HRV for Ewing parameters and metronome guided breathing parameters for subgroup B (BMI <36).

	V1 Mean (±SD)	**V2 Mean (±SD)**	**V3 Mean (±SD)**	**% Diff (V1-V3**	**P Value**
Ewing Measures					
Resting (%)	6.6 (4.3)	8.6 (4.4)	11.8 (5.8)	78.8	**0.001**
Forced breathing (bpm)	14.0 (6.8)	19.5 (8.56)	19.6 (7.5)	40	**0.001**
I/E Diff (bpm)	9.4 (4.3)	12.0 (5.5)	12.1 (4.1)	28.7	**0.004**
Valsalva (ratio)	1.30 (0.1)	1.34 (0.13)	1.35 (0.1)	3.85	**0.237**
Handgrip (ratio)	1.18 (0.09)	1.2 (0.14)	1.12 (0.11)	1.7	**0.014**
Lying/standing (ratio)	1.12 (0.11)	1.14 (0.1)	1.12 (0.08)	0	0.758
Metronome Breathing					
SD (bpm)	0.4 (1.0)	0.35 (0.95)	0.39 (0.8)	-2.5	0.464
A (bpm)	0.12 (0.8)	0.15 (0.93)	0.14 (0.75)	16.7	3.438
LPP (unitless)	0.7 (0.85)	0.44 (0.93)	0.43 (0.7)	-38.6	0.364
CORR (unitless)	-0.07 (1.6)	-0.97 (2.2)	-0.09 (0.9)	-28.6	0.254
FCORR (unitless)	-0.06 (0.68)	-0.05 (0.97)	-0.04 (0.57)	33.3	0.491
DEV (unitless)	0.23 (0.83)	0.12 (1.19)	0.21 (0.78)	-8.7	0.391

Mean (±SD) values for HRV parameters for each visit. Analysis of variance for repeated data. Significant results are shown in bold.

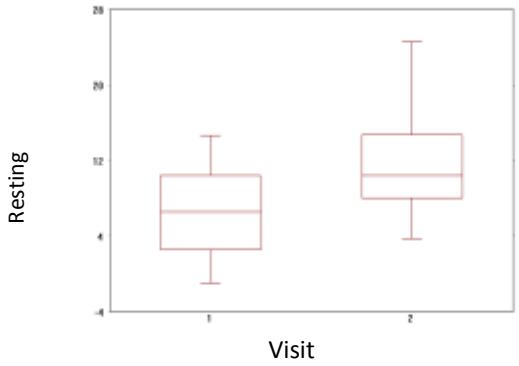


Figure 47 (A) HRV during Ewing assessment (resting) for the subgroup with the BMI <36 for visit one and visit three.

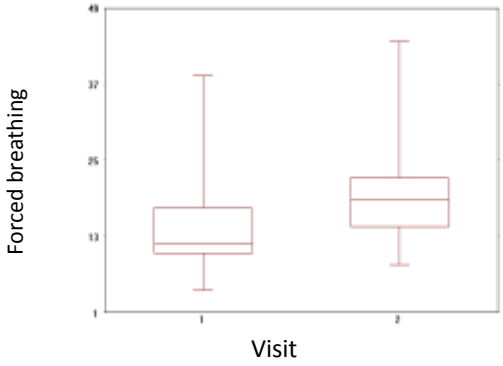


Figure 47 (B) HRV during Ewing assessment (forced breathing) for the subgroup with the BMI <36 for visit one and visit three.

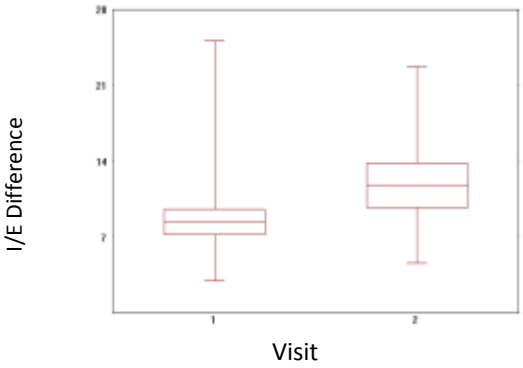


Figure 47 (C) HRV during Ewing assessment (I/E Diff) for the subgroup with the BMI <36 for visit one and visit three.

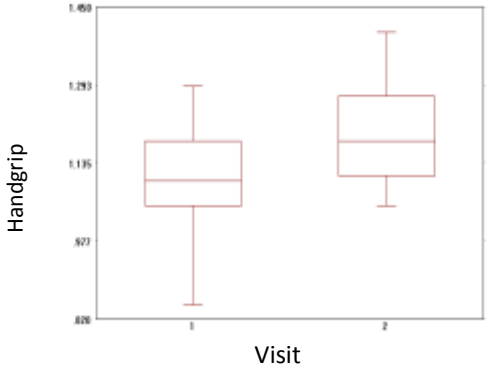


Figure 47 (D) HRV during Ewing assessment (handgrip) for the subgroup with the BMI <36 for visit one and visit three.

Figure 47 Box and whisker plots show statistically significant HRV measures from Ewing assessments for subgroup B with BMI<36.

12.16 Further Subgroup Analysis: Subgroup C (N=5)

12.16.1 Subgroup C: Pre-diabetic Heart Rate Variability Measures

A small subgroup (n=5) of patients was identified by the specialist diabetes nurse as being in the "pre-diabetic range" according to their (IGT) blood results at visit one.

These results still put them within a normal range and they were not classified as diabetic. They were able to continue with the trial as per the normal protocol.

Overall there was a percentage increase in HRV data between visit one and three for both Ewing measures and metronome guided breathing, only handgrip (-3.3%) SD (-62%) and A (-33%) reduced. Statistical analysis was performed using analysis of variance on the HRV data from subgroup C. Despite the increase in percentage change between baseline and eight months there was no statistical difference between the data (see Table 52).

Table 52 Subgroup C: Pre-diabetic Group HRV Measures

Parameter	**Mean V1 (±SD)**	**Mean V2 (±SD)**	**Mean V3 (±SD)**	**% Diff (V1-V3)**	**P Value**
Ewing Measures					
Resting (%)	2.52 (2.49)	6.65 (5.47)	6.83 (1.81)	170	0.052
Forced breathing (bpm)	10.85 (3.02)	19.9 (9.33)	18.4 (5.65)	70	0.051
I/E Diff (bpm)	7.67 (1.71)	12.65 (6.13)	11.32 (2.00)	47.6	0.124
Valsalva (ratio)	1.28 (0.13)	1.33 (0.19)	1.32 (0.05)	3.1	0.787
Handgrip (ratio)	1.1 (0.05)	1.05 (0.27)	1.16 (0.08)	-3.3	0.525
Lying/standing (ratio)	1.17 (0.12)	1.11 (0.11)	1.19 (0.06)	1.7	0.590
Metronome Breathing					
SD (bpm)	0.52 (1.08)	0.51 (0.61)	0.2 (0.57)	-62	0.701
A (bpm)	0.06 (0.84)	0.06 (0.62)	0.04 (0.59)	-33	0.751
LPP (unitless)	0.2 (0.45)	0.67 (0.49)	0.48(0.51)	140	0.613
CORR (unitless)	-0.6 (0.89)	-0.2 (0.44)	0.09 (0.47)	115	0.067
FCORR (unitless)	-0.55 (1.32)	0.08 (0.7)	0.03 (0.07)	105	0.296
DEV (unitless)	0.2 (0.83)	0.3 (0.43)	0.3 (0.39)	50	0.173

Mean and standard deviation figures for HRV measures for Ewing assessment and metronome guided breathing for subgroup C. Analysis of variance was performed. No result was below the p= <0.05 threshold.

12.16.2 Pre-diabetic (N=5) Autonomic Measures Compared With the Rest of the Lifestyle Cohort (N=33) on the First Baseline Visit

It was decided to further analyse data from subgroup C in order to ascertain whether it would have a confounding effect if included with the full data from the rest of the lifestyle cohort.

Baseline data from the remaining lifestyle cohort (n=33) were compared with baseline data from a small subgroup of patients (N=5) who were identified as "pre-diabetic" using an unpaired t-test. Despite being pre-diabetic, this group of participants did not show any significant differences in HRV compared to the rest of the group (see Table 53).

Table 53 Pre-diabetic HRV Data Compared with the rest of the Lifestyle Cohort (N=33) for visit 1.

Parameter	Mean total group (±SD)	Mean pre diabetics (±SD)	P value
Ewing measures			
Resting (%)	6.17 (4.14)	2.5 (2.49)	0.06
Forced breathing (bpm)	9.5 (4.0)	7.6 (1.71)	0.33
I/E Diff (bpm)	1.2 (0.12)	1.2 (0.13)	0.74
Valsalva (ratio)	1.1 (0.08)	1.1 (0.05)	0.92
Handgrip (ratio)	1.11 (0.12)	1.17 (0.12)	0.31
Metronome Breathing			
SD (bpm)	0.23 (0.95)	0.448 (1.13)	0.65
A (bpm)	0.10 (0.83)	-0.14 (0.9)	0.77
LPP (unitless)	0.42 (0.89)	0.32 (1.1)	0.83
CORR (unitless)	-0.88 (1.63)	-1.03 (1.06)	0.84
FCORR (unitless)	0.09 (0.59)	-0.55 (1.32)	0.35
DEV	-0.03 (0.89	0.3 (0.39)	0.42

Mean and SD heart rate variability measures for the total group and for subgroup C at visit 1. Analysis performed using an unpaired t-test (p=<0.05) There were no significant differences between the groups.

Chapter 13: Discussion: (Lifestyle Project)

13.1 Demographic Factors and their Effects on HRV

Recruitment for this study was good with participant retention remaining high (see Table 26). 51 participants were initially recruited; however 38 participants completed the full eight month study. The drop-out rate was 19%. The participants were a mixed gender group with an average age of 52 years (+/- 9.39). All took part in the eight-month intensive lifestyle programme. All subjects all had a BMI 30.00 – 39.99kg/$m^2$with a self -reported family history of Diabetes of 1^{st} degree relatives. 3 participants were identified as diabetic on the blood analysis and did not continue with the study, (see Table 26). Six participants were established as pre-diabetic but continued with the study, one of these participants dropped out of the study before completion.

13.2 Diversity of the Study Cohort

The study group was not ethnically diverse. There was a significant bias, as greater than 95% of volunteers were Caucasian. A literature search does not suggest any evidence relating ethnicity with heart rate variability. There was a bias towards female participants and all came from the local area.

13.3 Study Protocol

At baseline, 4 months and at the end of the 8-months programme participants were required to attend for measurements. There were 7 additional group sessions in the first 4 month period and four additional group sessions and one individual session in the second four month period. There was a focus on increasing activity and exercise, which all participants managed to achieve, and many reported a wish to maintain new healthier lifestyle.

13.4 Anthropometric Parameters

Anthropometric measures were made at the baseline and at visit 2 and 3; these measurements are shown in Table 28. Participants lost a statistically significant amount of weight and body fat and changed shape in terms of a reduction in waist circumference. All of the anthropometric parameters were significantly different between each visit.

13.5 Biochemical Measurement

Biochemical measures taken during the study improved over the eight months of intensive lifestyle coaching and exercise with a significant percentage reduction in values for all parameters apart from HDL, which increased 2.04% (see Table 32). This is in keeping with research by Horton et al (2010) who found blood sample analysis for glycaemic control, and lipids all improved in association with weight loss apart from HDL, which rose. Statistical analysis shows a significant difference in many of the measures particularly those relating to glycaemic control. Change in weight over eight months was correlated against change in HbA1c levels and a significant correlation ($p=0.032$) was demonstrated (see Figure 36)

We are confident that the intensive lifestyle programme, which was responsible for the weight loss and increased exercise, is the reason for these findings. This is in keeping with research by Stanford et al (2012) who found fasting blood glucose correlated with a change in weight and that as with this study diet and exercise is effective in reducing HbA1c and fasting blood sugars in both diabetics and non-diabetics.

13.6 Blood Pressure Measures

Blood pressure was also measured but did not change significantly between the visits for the full group despite the significant weight loss, (see Table 30) whereas Horton et

al (2010) found a significant correlation between weight loss and blood pressure. We did however see a significant difference in blood pressure between visits for subgroup A (BMI≥36). Systolic and diastolic blood pressure decreased at the same time weight in this group significantly reduced (see Table 38).

13.7 Heart Rate Variability (Ewing Measures) for the full cohort.

During analysis of the lifestyle study data statistically significant differences were seen between the data for all Ewing parameters apart from lying/standing, (see Table 33). The reasons for seeing such changes are not entirely clear but may be in part due to attenuation in inflammation resulting from weight loss, and increased levels of activity, as vigorous exercise is known to be implicated in increasing heart rate variability. It is also possible that during HRV assessment on subsequent visits there was better participant compliance with the test provocations resulting from familiarity with the protocol rather than true increased heart rate variability. True changes in HRV at rest are sometimes difficult to accurately identify, as there is no specific provocation that results in large changes in heart rate (as is the case during the Valsalva manoeuvre or during deep inspiration)..

13.7.1 Heart Rate Variability in the full cohort (2 minute metronome guided breathing measures)

Results for frequency analysis of heart rate data collected during 2 minutes of metronome guided breathing for the full cohort suggest there were no statistically significant differences in autonomic tone between visits (see Table 33) This is surprising given the results of the Ewing tests which are statistically significant (see section 13.7), the reason for this is unclear although this is similar to the previous findings in the influenza study where we did not see heart rate changes relating to the metronome breathing provocation, (see section 4.4). This was either due to changes in HRV being subtle and temporary or could be due to the small sample size. It is important to note that the changes occurring between visit one and visit three suggest

that more time is needed to see subtle changes in HRV than for biochemical measures which occur from four months. However the heterogeneity of the group may have been an issue in that as a cohort they were all obese but with a range of BMI's over 30-39.9 with a wide range of percentage weight lost over the eight months.

The baseline HR data for the full cohort (n=38) during metronome breathing was also compared with baseline HR data for the cohort of healthy volunteers during metronome breathing from the Influenza study (n=71). There were no statistical differences in heart rate variability between the groups apart from the SD parameter (see Table 34). The results show although mean parameter values are lower for the lifestyle cohort than the healthy group, although obese; the lifestyle group is not statistically dissimilar to the healthy normal range.

13.8 Subgroup Analysis

13.8.1 Analysis of Heart Rate Data According to the Percentage Weight Loss between Baseline and Eight Months

During the eight month study period, the study group as a whole lost a significant amount of weight through implementing lifestyle change, diet and exercise. The total cohort was subdivided for analytical purposes using two different approaches. The first approach uses arbitrary thresholds of percentage weight loss for each individual between, visit one and visit three. The percentage thresholds were <12% (moderate) weight loss and >12% (large) weight loss. Analysis was performed on each set of heart rate variability data for each subgroup.

For the subgroup of <12% weight loss, statistical analysis showed significant differences between all Ewing parameters except for the handgrip and lying/standing provocation. Metronome breathing parameters were not statistically different between visits (see Table 36).

For the subgroup with >12% weight loss, there was a reversal in which set of HRV measures recorded statistical differences. The Ewing assessment parameters showed a

significant statistical difference for only the resting parameter, (see Table 37), but the metronome guided breathing assessment of HRV showed that LPP, FCORR and DEV parameters were statistically different (Table 37). Our findings are very similar to those of Sjoberg et al (2011) in which moderate weight loss (10%) in type two diabetics was enough to elicit changes in HRV and were correlated with a change in BMI alongside the same physiological, biochemical and anthropometric measures. However Tompkins et al (2011) found physical activity induced improvement in insulin dynamics without changes in body fat in obese type two diabetics which may suggest "increased physical activity alone and not weight loss may be the key to promoting cardiovascular autonomic function." Results of our lifestyle programme are in agreement with research by Carnethon et al (2006) who found that indexes reflecting autonomic function and fitness improved in the lifestyle modification arm of their study where 74% of participants met the required amount of physical activity a week.

13.8.2 Analysis of Heart Rate Data According to BMI at Baseline

The second approach was to subdivide the cohort according to baseline BMI. The total cohort was subdivided into three subgroups, those in Subgroup A (N=14) had a BMI ≥36, those in Subgroup B (N=24) had a BMI<36 and Subgroup C (N=5) were a small group identified as pre-diabetic on blood sampling.

13.8.2.1 Analysis of HRV Data for Subgroup A (BMI≥36) N=14

Subgroup A contained participants with the highest BMI (≥36). Anthropometric measures were assessed by analysis of variance and were all significantly different between visits. The percentage change in data between the starting point and the end point of the study shows there is a marked decrease in all weight related parameters. Percentage weight loss dropped 12.5% equating to 13.2kg overall weight loss with a mean loss of -19.7% loss in body fat. BMI fell from 38.1 (+/-1.04) to 33.5 (+/-3.3) equating to a 12.07% change and waist circumference reduced by an average 16.2 cm (-13.8%), see Table 38. There were marked changes in the biochemical data for this

subgroup with a percentage drop noted for all parameters. The largest reduction was seen in GLP-1 representing 9.6% drop in blood glucose values after the 30 minute repeat test for the group. The smallest percentage reduction was for LDL, which fell only 2.8%. Biochemical data for this group was analysed using analysis of variance for repeated measures. Many of the lipid and glycaemic values were statistically different, however triglyceride, HDL and blood glucose (0min) were not statistically different (see Table 39). For the participants in subgroup A, we would expect lower levels of adiponectin at baseline with a subsequent rise following significant weight loss. Associated with this subgroup there may be obesity-associated inflammation (Wolfson et al 2012). We saw lower levels of serum adiponectin at baseline and a percentage increase of 24% between the start and end of the study with significant statistical difference between the baseline and eight month data, (see Table 40 and Figure 39 (a)) We also saw a significant drop (-5.6%) in serum leptin levels in association with concomitant weight loss over eight months (see Figure 39 (b)).

The heart rate data for these participants were analysed using analysis of variance and there were significant statistical differences between the data collected for most of the Ewing parameters, (resting, forced breathing, Insp/Exp and Valsalva) (see Table 41). The heart rate data collected during two minutes of metronome guided breathing at 6 breaths per minute was also analysed using ANOVA, no significant differences were seen in any of the parameters measured comparing visit data (see Table 41), there is however a trend in the data with an increase in all parameters apart from CORR.

HRV data for subgroup A was compared using an unpaired t-test with the full cohort of healthy volunteers from the influenza group during metronome breathing (visit1), There were statistical differences in SD, A, LPP and DEV parameters (see Table 43). All HRV parameter values were lower in the lifestyle subgroup A compared with the influenza group apart from the CORR parameter, which was higher. These findings are in direct contrast to those in Table 34 where the full lifestyle group was compared to the full influenza cohort and no differences were seen apart from the SD parameter. We can infer from this result that the increased adiposity and associated inflammation in subgroup A is marked enough to show a statistical difference in heart rate variability,

which was reduced compared to a normal range and improved after significant weight loss and increased physical activity.

HRV data from baseline and visit 3 of the lifestyle study was also compared using a paired t-test to establish differences between the start and end point of the study. The results are all significantly different apart from the FCORR parameter and it is potentially the resulting inflammation from increased adiposity that is the confounding factor in a change in HRV. We can say that in this particular subgroup (BMI≥36) there is a significant difference in HRV that may be due to the intensive lifestyle programme which includes significant increase in exercise, dietary control, weight loss and other lifestyle modifications. Associated with these lifestyle changes we would also expect to see a reduction in inflammation related to a reduction in adiposity. We cannot comment on this directly as inflammatory markers were not measured, however we can infer from this result that in a subgroup of obese individuals (where significant weight loss occurred with correlated changes in HbA1c, lipids and serum adiponectin and leptin (see below)) the significant changes in heart rate variability are related to the reduction in adipose tissue which determines the associated levels of inflammation present.

Analysis of an extended period of resting heart rate using analysis of variance was performed for subgroup A. Assessment of resting heart rate, fractal dimension, SDNN, PNN50 and LF/HF ratio during the eight months lifestyle study was made. There were no statistically significant differences in measures for either group see (Table 41). This was the same finding for the analysis performed on resting heart rate data from the influenza study where no significant differences were found.

13.8.3 Correlational studies for Subgroup A (BMI≥36)

Correlational analyses were performed using Pearson's product moment correlation coefficient to ascertain the degree of correlation between change in weight in kilogrammes for subgroup A between baseline and visit 3 and serum lipids and

glycaemic indices. There was a significant correlation between weight loss and the change in triglyceride and HbA1c levels between baseline and visit 3, (see Figure 41 (A), Figure 41 (B) and Table 45). This is particularly relevant to this group as they have the highest BMI and are most likely to go on to develop diabetes. A significant drop in HbA1c is directly proportional to a drop in blood sugar levels, suggesting a reversal in the predisposition toward disease.

Correlational analyses were performed using Pearson's product moment correlation coefficient to ascertain the degree of correlation between the change in weight (kg) in subgroup A between baseline and visit 3 and the change in HRV parameters between baseline and visit 3 during 2 minutes of metronome guided breathing and for Ewing measures. Statistical analysis shows a significant correlation between changes in some HRV parameters and change in weight over eight month period. As weight decreased some HRV parameters increased representing an improvement in autonomic tone (see Table 46).

Correlational studies were also performed on data from subgroup A for the change in weight (kg) between baseline and visit three and the change in adiponectin and leptin levels between baseline and visit 3. There was a significant inverse correlation between weight loss and a rise in serum adiponectin (P=<0.001) and a significant correlation with a reduction in weight a fall in leptin levels (P=<0.001) (see Table 47 and Figure 44), Levels of adiponectin and leptin both changed significantly over the course of the eight month study (see Table 40 and Figure 39). In this obese subgroup with the highest BMI who lost the highest percentage of weight over the eight month study period, we saw a significant correlation with weight loss and serum adiponectin and leptin levels. This was not replicated in the total cohort and may be due to the variation in weight loss where some participants did not lose as much weight as others.

The changes in serum adiponectin and leptin levels between baseline and visit 3 were also correlated with changes in HRV parameters. There was no significant correlation

between HRV measures and adiponectin and leptin levels apart from for handgrip and leptin (p=0.02).

13.8.4 Analysis of Heart Rate Data for a Subgroup (B) BMI <36 (N=24)

Statistical analysis of anthropometric data for this subgroup showed that all parameters changed significantly between visits (p=0.001). Systolic and diastolic blood pressure also decreased between visits but only diastolic blood pressure was statistically different (p=0.002) (see Table 49). Analysis of biochemical data for this sub group between visits showed statistical differences in five out of nine of the parameters measured (see Table 50). However there are fewer statistically different changes in biochemical measures in this cohort compared with subgroup A.

Heart rate variability parameters were assessed for this subgroup. The Ewing assessments showed statistically significant differences in all parameters apart from lying/standing (see Table 51). For HRV assessment during metronome guided breathing there was no statistically different differences (see Table 51). There were more participants in this subgroup (N=24) than for subgroup A (N=14), with greater heterogeneity (BMI of >30 - <36). Although we did see some heart rate changes in this group they were not the group with the highest BMI (with assumed higher levels of inflammation) nor the group with the greatest mean weight loss (with associated rise in adiponectin and drop in leptin) and therefore we would not have expected HRV in this group to change as significantly as for subgroup A.

13.8.5 Comparison of Subgroup (C) Pre-diabetic Data against the rest of the Lifestyle Cohort.

The cohort was also subdivided into those identified on blood measurements as being pre-diabetic (N=5) although still within the normal range. Heart rate data for this subgroup was compared with the rest of the group (N=33) using an unpaired t test. For the Ewing measures and for the metronome guided breathing frequency analysis there

were no differences in HRV between the groups. This meant that including the pre-diabetic data with the rest of the cohort was valid and would not affect the results of the rest of the group. Wu et al (2007) found in pre-diabetics there was a move towards increased sympathetic tone and attenuation in parasympathetic modulation in association with the development of impaired glucose tolerance leading towards diabetes. We did not see these changes in the small cohort of pre-diabetics identified by an impaired glucose tolerance test. Our findings from this research are in keeping with research by Lee et al (2010) who assessed pre-diabetic obese adolescents. Their findings did not correlate obesity with attenuation in HRV and these HRV measures were not significantly different from those with normal glucose levels. Schroeder et al (2005) also suggested only a weak link between adult HRV measures and pre-diabetes.

13.9 Obesity, Inflammation and Heart Rate Variability

Vinik et al (2012) found that a modest increase in weight can have a negative effect on HRV, we did not see this in the full cohort and there was no significant difference on statistical analysis. We did however; see an improvement in HRV in subgroup A (BMI ≥36) relating to weight loss, (see Table 41). We also saw concomitant changes in serum lipids, leptin, adiponectin and HbA1c. Freedman et al (1995) and Windham (2012) suggest one explanation for obesity-related autonomic dysfunction is that the distribution of body fat may be more important than a measure of overall obesity, such as BMI, due to the metabolic activity of visceral or abdominal adipose tissue.

13.9.1 Obesity

Obesity has reached unprecedented levels worldwide in adults and children with one in ten of the world's population obese (WHO 2013). It is the fifth leading risk for global deaths and is implicated in the development of many health related problems. In the cohort that lost >12% in weight we saw significant differences in the frequency parameters between visits and in the full group and sub group A (BMI≥36), there were significant differences between Ewing measures of heart rate variability over the eight

month study period (see Table 37 and Table 41). Small sample size may account for the lack of statistical difference between visits for the metronome guided breathing frequency parameters of HRV. Assessing the differences between measures of HRV for subgroup A at the baseline visit and at eight months using a paired t test there were significant differences in all of the Ewing and Frequency parameters (see Table 42).

Long-term exercise is associated with enhanced cutaneous blood flow (Colberg et al 2003), restoration of baroreflex sensitivity, (Colberg et al 2003) and improved vagal activity in early cardiac autonomic neuropathy (Michalsen et al 2006). This study group underwent a programme of lifestyle modification including exercise, diet and nutrition in a bid to slow or reverse the progression towards disease states such as diabetes and cardiovascular disease with associated dysautonomia.

13.9.2 Adipose Tissue

In addition to containing adipocytes, adipose tissue is involved in maintaining homeostasis through secretion of leptin, adiponectin, resistin, TNF-α, Interleukin-6 angiotensinogen and others. In our research all subjects were considered obese, all had struggled to lose and maintain weight loss throughout adulthood and all had a strong family history of diabetes. There is evidence that dietary excess and obesity may both activate inflammatory signalling pathways in cells, leading to sub-acute chronic inflammation. This obesity-induced inflammation can in turn play a part in the development and progression of diseases such as diabetes (Shoelson 2007).

We saw significant differences in HRV parameters in the full cohort and for some measures in the subgroups. This was particularly true for subgroup A, we saw a significant statistical difference between all heart rate variability measures before and after the occurrence of significant weight loss, when comparing baseline measures and those at eight months (see Table 42). Correlational analyses also demonstrate a significant correlation between HRV measures and weight loss (see Table 46). The significance of this suggests that in essentially healthy yet obese individuals there are some positive changes in HRV associated with a significant weight loss. However, the participants with the highest BMI who demonstrated the greatest percentage change in

weight benefit most significantly from lifestyle modification, increased physical activity and resultant weight loss with respect to improving heart rate variability.

13.9.3 Obesity Induced Inflammation

In recent years there has been increasing evidence identifying the role of inflammation in the aetiology of disease and the mechanism behind the pathogenesis of obesity related disease states (Zulet et al 2007, and Shoelson 2007). Research has revealed a "*close relationship between nutrient excess and derangements in cellular and molecular mediators of immunity and inflammation"* (Lumeng and Saltiel 2011). The third part of this PhD research aimed to assess the link between obesity / pro-inflammatory adiposity, weight loss and the resulting effect on heart rate variability. We found that in a cohort of essentially healthy yet obese individuals with a family history of diabetes there were significant differences in HRV parameters in subgroups of participants where varying amounts of weight loss had occurred, particularly in a subgroup A (identified with a BMI $\geq$36) where the greatest percentage weight loss occurred. All participants taking part in the lifestyle programme lost a significant amount of weight; all had increased levels of physical activity and had taken part in nutrition and dietary group sessions. We saw an increase in HRV between baseline and eight months in participants where we saw the greatest reduction in weight. We would argue that this increase in HRV potentially resulted from a reduction in the inflammatory response to obesity through reduced adiposity.

The inflammatory response triggered by obesity is similar to the response to pathogens including a systemic increase in inflammatory cytokines and acute phase reactants, and the recruitment of leukocytes and generation of reparative tissue responses. Obesity induced inflammation is generally chronic and produces low grade activation of the immune system and diminished vagal nerve activity. It is clear that this low grade inflammation is implicated in the link between obesity and disease. *"Obesity triggers inflammatory pathways in the brain and adipose tissue that dysregulate physiological responses that maintain insulin and leptin sensitivity. Lipid accumulates in muscle,*

liver and blood vessels that activate leukocytes contributing to tissue specific disease and exacerbates systemic insulin resistance (Lumeng and Saltiel 2011).

In subgroup A we saw a statistically significant difference in adiponectin and leptin levels (p=0.001), (see Table 40) and all other biochemical measures apart from HDL and triglycerides (see Table 39), over the eight month study period. In this subgroup there was an improvement in autonomic tone (HRV) with concurrent weight loss, increased activity, and dietary modification, (see Table 41 and Table 42). "*Since weight loss and exercise are each associated with increasing vagus nerve activity, one can consider whether enhanced activity in the cholinergic anti-inflammatory pathway might decrease cytokine production and reduce the damage and metabolic derangements mediated by chronic, low-grade systemic Inflammation that is characteristic of the metabolic syndrome."* (Tracey 2007, p294). It could be argued that the level of weight loss is an important factor in reducing the associated inflammatory process thus restoring HRV to normal levels. But it may be a combination of factors including peer support, shared learning, reduced calories and increased activity that contribute to the overall effect. The improvement in quality of life was evident within the group, with participants suggesting they felt generally better, had lost weight, and had more energy and that the programme was benefitting the other members of the family with a significant shift in attitude towards nutrition, dietary modification and exercise.

13.9.4 Measures of Serum Adiponectin in Subgroup A (BMI≥36)

Adiponectin has an important role in lipid metabolism, atherogenesis, inflammation and insulin sensitivity (Wolfson et al 2012). It circulates in several sized complexes in serum. Despite the fact it is produced in adipocytes (fat cells) serum levels are lower in obese patients, it has widely been accepted as a marker in the context of obesity and diabetes and cardiovascular disease (Scherer 2006). "*Other than weight loss the only approach to improve adiponectin serum levels is through the use of pharmacological activators of nuclear receptor peroxisome proliferator-activated receptor by*

thiazolidinediones, which are widely used as insulin sensitizers in diabetes" (Scherer 2006).

Serum adiponectin was sampled at baseline, four months and eight months after starting the programme. There has been a delay in analysis of the samples and we await the results for the total cohort. Blood samples from subgroup A were analysed separately. (The results are shown in Table 40). In this research we see a significant statistical difference (P=0.001) between the samples over the study period confirming a significant increase in adiponectin (see Table 40). Correlational studies comparing change in weight between baseline and at eight months and the change in serum adiponectin over the same period confirmed an inverse correlation (p=0.01) between weight loss and a rise in adiponectin, (see Table 47 and Figure 44). Several studies have found an inverse association between adiponectin concentration and different markers of central obesity (Kwon 2005). The results of the present study confirm this relationship between plasma adiponectin levels and markers of central obesity.

13.9.5 Measures of Serum Leptin from Subgroup A (BMI≥36)

Leptin is a hormone expressed in adipose tissue and is strictly correlated with reduced body fat content. It acts centrally, acting on the hypothalamus increasing energy expenditure and decreasing appetite (Pelleymounter et al 1995). There is an association between leptin and its impact on the autonomic nervous system namely stimulating the sympathetic branch (Paolisso et al 2000), which has been indicated in cardiovascular disease. Previous studies by Collins et al (1996) have shown that leptin may affect the autonomic nervous system, enhancing noradrenaline turnover in brown adipose tissue suggesting increased sympathetic outflow. Murialdo et al (2007) reported, "*bulimia nervosa patients have reduced heart rate variability associated with low leptin levels and apparent sympathetic insufficiency."* Results show a significant statistical difference (P=0.001) over the eight month study period (see Table 40) with a decrease in levels in association correlated with concurrent weight loss (p=<0.01) (see Table 47 and Figure 44). Our result shows a reduction in leptin at eight months, which is correlated with a reduction in weight. This is in keeping with work by Quillot et al

(2008) who found that the relationship between leptin and the autonomic nervous system is disturbed in obese subjects. This finding could strengthen the argument that weight loss and decrease in adipose related inflammation may be responsible for restoring HRV.

The immune process is regulated by neural reflexes, which comprise an afferent arm that senses inflammation and an efferent that inhibits innate immune responses "*This inflammatory reflex, now termed the cholinergic anti-inflammatory pathway* (Tracey 2002)" has been extensively studied in terms of its immunomodulating function and protective effects against a wide range of inflammation related disorders (Wang et al 2011). In obesity the cholinergic signalling pathway may already be suppressed in adipose tissue, which may contribute to increased adipose tissue inflammation typically seen in obesity (Wang et al 2011). Heart rate variability as a marker for vagal activity is significantly reduced in inflammatory conditions (Bruchfeld 2010), indicating that reduced cholinergic activity may be a common feature in chronic inflammation, including obesity (Wang et al 2011). In our research there was a significant statistical improvement in autonomic tone (HRV) after eight months of lifestyle coaching and significant weight loss from within the full cohort and particularly from subgroup A. This suggests that weight loss may be responsible for the improvement in HRV by reducing the inflammatory process. The cholinergic anti-inflammatory pathway, which regulates immunity and has an important immunoprotective effect against diverse inflammation related disorders (Bencherif et al 2010), may also be protective against obesity induced inflammation and insulin resistance (Wang et al 2011).

13.10 Weight Loss and Changes in Heart Rate Variability

It has been stated that obesity significantly increases the likelihood of developing health problems including heart disease and diabetes and is implicated with increased mortality and sudden death (Poirier et al 2000). "*In diabetes reduced cardiovascular autonomic activity is associated with a number of clinically significant manifestations including exercise intolerance, intraoperative cardiovascular lability, orthostatic*

hypotension, silent myocardial ischaemia and increased risk of mortality" (Vinik et al 2007). Obesity and the cardiac autonomic nervous system are intrinsically linked. A 10% increase in body weight is associated with a reduction in parasympathetic tone and accompanied by a rise in mean heart rate (Hirsch et al 1991). In our lifestyle study in subgroup A, where an average weight loss of 14% was seen, and for the full group who lost an average of 11% weight, our findings concur with those of Poirier et al (2003) who found "*weight loss of 10% in obese patients was associated with a significant improvement in autonomic nervous system cardiac modulation, translating into increased HRV and decreased heart rate*" (see Table 29, Table 39 and Table 41). In obesity it has been noted that parasympathetic autonomic activity is reduced with a shift towards augmented sympathetic tone. Autonomic dysfunction increases cardiovascular load, haemodynamic stress, arrhythmias and significant cardiac pathology (Maser and Lenhard 2007). Autonomic dysfunction in new onset diabetes is reversible with weight reduction. Proper diet and exercise is the first line therapy to promote weight loss and improve glycaemia (Horton et al 2010). "*Physical activity is a key element in the prevention and management of obesity and diabetes. Regular physical activity efficiently supports diet-induced weight loss, improves glycemic control, and can prevent or delay type 2 diabetes diagnosis* (Voulgari et al 2012)." Our cohort undertook an eight month exercise and lifestyle regime resulting in significant weight loss and improved autonomic tone and reversal of the likelihood of developing disease with improved HbA1c. Anthropometric measures, biochemical measures and autonomic function parameters all largely showed an improvement over eight months associated with significant weight loss, this is in keeping with similar work by Sjoberg et al (2011) who found weight loss improves heart rate variability in overweight and obese patients with diabetes.

13.11 Pharmacological Therapy in Obesity.

There are currently no effective treatments applicable to the large majority of obese people and most treatment is aimed at targeting the complications of obesity. Previous obesity medications have caused unacceptable morbidity and mortality. Currently

Sibutramine, Orlistat and Rimonabant are licensed in Europe (Pagotto et al 2008) and when used properly reduce body weight and improve cardio-metabolic risk factors (Pagotto et al 2008). Patients identified as those who may benefit from anti-obesity therapy are those with a body mass index between 27-29.9kg/M^2 with obesity related comorbidities such as diabetes and hypertension dyslipidaemia and metabolic syndrome (Pagotto et al 2008). Another non-pharmacological and more radical treatment option, which is becoming increasingly used to treat obesity, is gastric bypass, banding or ligation bariatric surgery, which will also serve to eradicate diabetes in diabetic patients. Aside from such radical steps, augmenting heart rate variability through weight loss and improving cardiovascular fitness in overweight and obese people is a simple cost effective solution to health improvement. Lifestyle studies such as ours show a marked improvement in all measures at eight months and a change in eating habits and how often they exercise, alongside nutritional education. It is the combination of approaches to changing behaviours and the group support, which appears to work, Follow up will show whether these changes are maintained in the long-term.

13.12 Targeting Inflammation

Attenuated vagal activity and the loss of the critical homeostatic balance between parasympathetic and sympathetic branches of the ANS (sympathovagal balance) has been shown to cause exaggerated pro-inflammatory responses and increased morbidity and mortality (Thayer and Fischer 2009). Early in the development of autonomic dysfunction there is an established correlation between increasing inflammatory markers and a reduction in HRV due to the cholinergic anti-inflammatory pathway. Activation of this pathway through administration of acetylcholine receptor agonist causes a decrease in pro-inflammatory cytokine production and a reduction in disease severity (Van Maanen et al 2009).

Lieb et al (2011) identified a loss of heart rate variability and sympatho-vagal balance before the advent of inflammation and Vinik (2012) suggests that "*adiponectin and leptin levels may be early predictive markers and the mechanism may be via insulin*

resistance or direct actions of leptin on the hypothalamus and the autonomic nervous system." Vinik (2012) found newly diagnosed diabetics had significantly lower levels of adiponectin and leptin ratios and correlated the adiponectin / leptin ratios with measures of increased sympathetic autonomic tone.

In subgroup C (pre-diabetic group), we did not see any changes in heart rate variability over the eight month period (see section 12.16.1), and (Table 51). There were also no significant differences between this small subgroup and the rest of the lifestyle cohort. When biochemical data are available I will analyse serum adiponectin and leptin levels in the pre-diabetic group to identify whether levels are lower as in the Vinik (2012) research but these are not available at the current time.

Ziegler et al (2011) found antioxidant therapy with alpha lipoic acid restores autonomic function towards normal and is one of the few drugs endorsed by the Toronto Consensus to target the autonomic nervous system. Pavlova et al (2007) showed that "selective muscarinic acetylcholine receptor suppresses innate immune response by increasing the firing rate of action potential in the vagus." Gaede et al (2008) showed that controlling BP, Lipids and hyperglycaemia reduces autonomic dysfunction by 68%. Vinik (2012) suggests "*the earliest changes that are detectable in the evolution of diabetes are abnormalities in autonomic balance" and implicates the hypothalamus as the "conductor of the endocrine orchestra*." Our lifestyle study shows that in an obese group of patients with a family history of diabetes there is a statistically significant difference in heart rate variability alongside a concurrent reduction in weight.

Correlational studies were performed on data from subgroup A (BMI≥ 36). Change in weight (kg) between baseline and visit three was correlated with the change in serum adiponectin and serum leptin levels between baseline and visit 3, a significant correlation was demonstrated, (see Table 45). The change in adiponectin and leptin levels over the study period was also correlated with the change in HRV parameters (see Table 46). Results show a significant correlation between leptin and handgrip parameter. Other measures of HRV did not correlate with either leptin or adiponectin.

13.13 Pupillometry

Statistical analysis was performed on the pupillometry data for the total group (see Table 35) and for the other weight related subgroups. There was no significant difference in the number of standard deviations below age matched normal values for iris:pupil ratio. It is difficult to draw conclusions from these findings, as the sample was small. The measurement technique on this occasion is insufficiently sensitive to determine changes in autonomic tone.

13.14 Limitations of the lifestyle study

Throughout this document limitations of the measurement technique or protocol have already been discussed in 5.6 and 9.9. We have encountered similar limitations during this study with regard to sample size, technical aspects and protocol limitation.

We were again involved in research where there were limitations on the amount of influence we had over the protocol as we were working in collaboration with another department. We joined this project slightly later than we would have liked and were not able to implement changes to the blood sampling regime. Ideally we would have measured inflammatory markers (CRP and Il-6) but as with the other projects we were not able to do so for practical, financial and logistical reasons. Future work in this field will allow for inflammatory markers to be measured.

The most significant limitation of this study was getting blood samples analysed. Some were analysed by the haematology department throughout the eight month period and were available on completion however the adiponectin and leptin results were not analysed or available for statistical analysis. The adiponectin and leptin results for subgroup A (n=14) BMI≥36 were analysed separately at additional cost. Unfortunately the remaining results of the full cohort will not be available until after the submission of this document.

Another issue we encountered was the repeat visits needed to commit to the study. We had a number of patients who attended for their first visit but did not continue to attend despite a follow up call. We think it was the combination of the time and effort needed to make repeated visits to the hospital. Despite this the dropout rate was relatively low and many of the participants could see immediate benefits from the sessions and had a vested interest to continue in the study. Many of the participants had experienced lifelong problems with their weight and found this lifestyle study had a positive effect on many aspects of their lives resulting in improved fitness, significant weight loss and the associated positive health benefits which accompany this.

13.14.1 Limitations of HRV Testing

The study group chosen did not have pre-existing heart disease, diabetes or other co-morbidities and were an essentially healthy group apart from a high BMI putting them into an obese category. The selection of healthy participants for this study is in itself a limitation, as we would not expect heart rate variability to be much different to that of the healthy normal range even in an obese cohort. We would therefore also not expect a dramatic change in autonomic function (HRV) comparing baseline measures and those at eight months. The changes in HRV anticipated, were expected to be subtle and potentially relating to obesity and inflammation associated with obesity and the family history of diabetes.

Care needs to be taken when interpreting the statistical significance of multiple parameters as the likelihood of one reaching the threshold for significance is additive. However, in this case multiple parameters display significant difference between baseline and eight months and we are confident that this is not due to chance.

Contrary to the Influenza and GORD research projects, results from this lifestyle study suggest that the “Gold Standard” Ewing measures are comparable with other measures of heart rate variability. Therefore they remain an important part of the global assessment of autonomic function and will continue to be used in our routine clinical assessments.

There is usually a bias towards an older patient for assessment for autonomic function. With increasing age often comes concomitant disease, which may preclude the patient from performing some of the test provocations. It is therefore advantageous to have a range of tests available to assess the different branches of the ANS.

In hindsight there are inevitable changes that I would make to this project, some have already been discussed previously. The recruitment and retention for this project was good and the numbers were sufficient. I would like to recruit more participants to increase the number of participants classified as pre-diabetic and to investigate further this subgroup. There are some interesting questions that could be answered with a larger sample size, such as whether HRV and biochemical indicators that suggest autonomic tone is compromised earlier than first thought in this group. I would also like to look at a newly diagnosed diabetic group and consider the effect of weight loss and exercise on HRV and biochemical measures. I would also be very interested to see if we can detect early heart rate variability changes in early diabetes or pre-diabetes using our current assessment technique.

Chapter 14: Conclusions

The effects of inflammation on the autonomic nervous system have been well documented, (Vinik et al 2012 and 2013, Tracey et al 2007, Sajadieh et al 2004, Lombardi et al 2004, Haensel et al 2008). The research incorporated in this thesis is collectively linked by the investigation into the association between low grade inflammation and changes to the autonomic nervous system in three different clinical settings; where a low grade inflammatory provocation was either introduced to bring about temporary changes in heart rate variability (as in the case of the influenza vaccine, see chapter 3), or was already identified (as in the case of GORD and erosive oesophagitis, see chapter 6), or present in clinically obese patients (see chapter 10). Inflammation was then reduced through either pharmacological means (PPI) or through other measures to reduce body weight to bring about an improvement in autonomic tone. The subject matter of "inflammation" has recently been addressed in a 2012 and 2013 review-paper by leading authority in the field Professor Aaron Vinik which has made this research both relevant and timely.

A host of research referenced throughout this document has reported a link between inflammatory disease and autonomic impairment. In clinical conditions characterised by a known increase in inflammatory markers, (for example in diabetes, rheumatoid arthritis, lupus, sepsis and acute coronary syndromes), a reduction in heart rate variability is consistently observed in high risk patients, strengthening the connection between inflammation and autonomic dysfunction (Lombardi 2004), and suggests that potentially many of these diseases may be diseases of autonomic dysfunction (Czura and Tracey 2005). Other inflammatory disease (such as asthma, reflux oesophagitis, arthritis, and inflammatory bowel disease) may all lead to a temporary reduction in autonomic tone. We questioned what physiological mechanisms are involved in this process. Vinik et al (2013) states, *"there is increasing awareness that inflammation is central to the pathogenesis of diabetes and its complications*." The exact relationship between cardiac autonomic dysfunction and inflammation has yet to be fully elucidated. The traditional view of cardiac autonomic neuropathy is that there is

attenuation in parasympathetic function, however Vinik et al 2013 suggest it may be early augmentation of sympathetic tone with later sympathetic denervation Recent evidence suggests that the inflammatory response is in part controlled by the neural circuitry of ANS (Vinik et al 2013). The afferent arm senses endogenous or exogenous molecules from injury or infection and stimulate the vagal nerve. The increase in vagal nerve activity decreases the release of pro-inflammatory cytokines and suppresses inflammation (de Jonge and Ulloa 2007). In contrast the efferent arc of the inflammatory response is termed the cholinergic anti-inflammatory pathway, where the vagal nerve is implicated in a "hard wired" connection between nervous and immune systems and is considered the primary component of the immune-reflex (Czura and Tracey 2005), with consequent association between reduced heart rate variability and inflammation (Thayer 2009). Decreased vagal nerve activity and loss of inhibitory influence over the cholinergic anti-inflammatory reflex on immune responses and cytokine release may allow exaggerated cytokine responses to stimuli that would otherwise be harmless in good health.

In disease where there is a constant rise in low-grade inflammation such as in diabetes there is an early reduction in parasympathetic activity and corresponding increase in sympathetic tone that initiates a cascade of inflammatory responses culminating in significant morbidity and mortality. Recent research by Vinik et al (2013) suggests that dysautonomia "*impairs the ability of the ANS to regulate the cardiovascular system and may be a key component in the aetiology and clinical course of cardiovascular disease."* Vinik (2013) suggests "*there is strong evidence of inflammation with activation of inflammatory cytokines in diabetes, which correlate with abnormalities in the sympatho-vagal balance*." In sepsis and multi-organ disorders, research has demonstrated an inverse correlation between HRV and the immune response (Papaioannou et al 2013). Lanza et al (2007) demonstrated a significant increase in heart rate variability in a group of patients with dysautonomia after treatment with atenolol, which correlated with a reduction in C-reactive protein. In contrast the healthy control group were not treated with atenolol and as expected, no changes in HRV or CRP over time were identified. In this paper, Lanza (2007) suggests "*a causal link may exist between modulation of autonomic activity and inflammation*." Balance of opinion

in recent publications also suggests this is the case. We have not explicitly established such a causal link in our research. However we have shown a clear association between autonomic tone as measured by heart rate variability and 3 separate forms of mild inflammatory insults.

Reproducibility of the autonomic function testing technique used in this research was assessed using heart rate data from healthy volunteers, who did not have the influenza vaccination. We are continuing to recruit to increase the normal range further. There was no significant difference between repeated visits separated by 2-5 days in healthy volunteers, (see Table 15). Statistical analysis suggests the technique is reproducible with no significant difference between the initial visit and a second visit. We also established that there is no significant difference between our previous healthy normal range quoted in a previous study by Perring and Jones (2003) and the new data collected from healthy volunteers during the influenza study, see section 4.9.2B and Table 16).

The Influenza project extended over two vaccination periods over two years with successful recruitment in both years. Annually the composition of the trivalent vaccine reflects the strains of influenza that are predicted to cause disease for that year. Therefore the vaccine used was slightly different for each vaccination period. Analysis of the data collected showed no discernible statistical differences between the results for both years, (see Table 17).

We established that the 6 breaths per minute breathing rate was a more significant heart rate provocation than a more natural breathing rhythm of 10 breaths per minute and resulted in more significant heart rate variability pre and post vaccination (see section 4.5). We established that the inflammatory response to the influenza vaccination was large enough to have a significant effect on short measures of heart rate variability using frequency analysis during metronome guided breathing in subgroup 2 (most symptomatic post vaccination). Pupillometry was also performed and we found in a symptomatic subgroup there was a significant difference demonstrated in the standard

deviation of iris/ pupil ratio compared to an age matched normal range (see section 4.9 and Table 14). These findings have been peer reviewed and published in Clinical Physiology and Functional Imaging (2012), 32: 437-444.

The second tranche of research investigated the effects of gastro-oesophageal reflux disease on autonomic tone. We identified a non-erosive reflux disease (NERD) group of patients and an erosive oesophagitis reflux disease group of patients (ERD). Recruitment and retention was more difficult than expected and we encountered a noticeable dropout rate as a percentage of the total recruited due to the follow-up visit eight weeks later (see section 12.1). In keeping with our previous Influenza based research, we established that the presence of inflammation resulting from erosive oesophagitis had a significant effect on initial short measures of heart rate variability using frequency analysis during 2 minutes of metronome guided breathing. Analysis of the heart rate data during Ewing assessment showed significant differences between the two visits for the ERD group, in particular the result of forced breathing and lying/standing parameters (see section 8.5). Analysis of heart rate data collected during 2 minutes of metronome guided breathing also suggested there was a statistically significant difference in autonomic tone (DEV parameter) between the baseline and follow up visit for the ERD group, (see Table 22). Although we acknowledge we did not sample inflammatory markers, other research suggests that pro inflammatory cytokines such as IL6 are frequently associated with oesophagitis (Rieder et al 2007). We can infer that the difference in HRV between the two groups may be associated with the oesophageal inflammation noted during endoscopy in the (ERD) group, which resolved after eight weeks of PPI therapy. The NERD group did not show the same statistically significant changes in autonomic tone pre and post PPI therapy, (see Table 22). We could argue this is due to the absence of any inflammation. There was however, a trend in the NERD data towards a generalised improvement in HRV which supports other research by Dobrek 2004 and we posit that it may be possible that micro-inflammation related to GORD which is not identifiable during endoscopy may be present in the NERD group and therefore although not visible endoscopically,

inflammation may be present microscopically. We do not have evidence to support this at this time.

Statistical analysis of pupillometry demonstrated there was a statistically significant difference in the number of standard deviations below age matched normal values for iris/pupil ratio, before and after eight weeks of PPI therapy in the ERD group, (see Table 24). This difference was not evident in the NERD group.

This GORD based research demonstrated a link between autonomic dysfunction and gastro-oesophageal reflux disease. We established that the measurement technique implemented during this research was sufficiently sensitive to measure small but statistically significant changes in autonomic tone related to inflammation in the ERD group, (see Table 22). The results are in keeping with our earlier research relating to the Influenza vaccination whereby there was deterioration in autonomic tone following vaccination. We also saw a significant difference between GERD impact scale scores for both groups before and after eight weeks of PPI therapy (as shown in Table 20). We are confident in the use of this measurement technique and have validated the use of extended forced breathing in conjunction with spectral analysis as a sensitive method of assessing changes on autonomic tone.

Autonomic function is already a key component and indicator for cardiovascular health. The autonomic nervous system, specifically the parasympathetic branch is known to play an inhibitory role in a wide range of disease states; in particular it is associated with the regulation of allostatic systems associated with glucose regulation, hypothalamic-pituitary-adrenal axis function and inflammatory processes (Thayer and Sternberg 2006). There is an inverse association between reduced HRV and increased fasting glucose, urinary cortisol, HbA1c, pro-inflammatory cytokines and acute phase proteins and poor health.

Results of the third research project are in keeping with work by Thayer and Sternberg (2006). We saw an increase in serum adiponectin, and a decrease in leptin and biochemical measures correlating with concurrent and significant weight loss and

improved heart rate variability over an eight month period in subgroup A (participants with a BMI ≥36) (see section 12.9.1). Inflammation is known to play a key role in the development of heart disease and diabetes. Obesity is also a significant risk factor for both of these diseases and is a known inflammatory condition itself. The adipose tissue produces a variety of pro-inflammatory cytokines involved in insulin resistance and atherosclerosis (Bray et al 2009). In this third research project we performed measurement of autonomic function in essentially healthy, yet clinically obese individuals over an eight month period to assess subtle changes in autonomic tone relating to a significant change in weight and blood markers and associated adipose tissue related inflammation.

During analysis of heart rate data, significant differences were seen between the Ewing data and metronome guided breathing data for the full group and for weight related subgroups over the eight month period. We suggest this may be due to the significant weight loss observed and increased levels of activity. This also highlights the importance of maintaining the full Ewing protocol in addition to other assessments as an integrated assessment of cardiac autonomic function.

There were a small number of participants identified on the oral glucose tolerance blood test for impaired glucose tolerance as being pre-diabetic (N=5) although still falling within the normal range, (see section 12.16). Heart rate data for this group was compared with the rest of the group. There were no statistical differences in HRV between the groups.

The complete group was assessed for weight loss and other weight related parameters over the eight-month period and lost a significant amount of weight through lifestyle changes, diet and exercise. The cohort was subdivided using a threshold of percentage weight loss for each individual between baseline and eight months. The percentage thresholds were <12% and >12% weight loss. Analysis was performed on heart rate variability data. Analysis showed that for the <12% group four of the Ewing parameters were statistically different (see Table 36). For the group with >12% weight loss, three

of the metronome breathing parameters were significantly different (see Table 37). The difference in the results may be due to the sample size for the groups and the weight of the participants in each group at the start.

The full group was also subdivided according to their BMI. For subgroup A (those participants who had a BMI equal to or greater than 36 (N=14) at the baseline visit) (see section 12.9.1), data was analysed using analysis of variance (ANOVA) for heart rate data over the eight month period there were significant statistical differences between the data collected for four of the Ewing parameters (see Table 41). The heart rate data during two minutes of forced breathing at 6 breaths per minute was also analysed using ANOVA for paired data. No significant differences were seen in the HR parameters measured over eight months, (see Table 41). HR data for subgroup A was re-analysed using a paired t-test to look specifically for statistical differences between data from the start and the end of the study. Results show there was a significant difference in all parameters apart from (LPP and FCORR) (see Table 42); we suggest this is related to the increased BMI seen in this group.

The heart rate data for the full lifestyle group was compared with the heart rate data from the full influenza pre vaccination group; results show no real differences between the full lifestyle group and the influenza group, (see Table 34). However, for subgroup A the same comparison was made and statistical analysis was much more discerning with significant differences in metronome guided breathing parameters, (SD, A, LPP and DEV), see Table 43. This suggests that the varying levels of obesity in the full group may not be high enough to induce HRV changes related to adipose related inflammation. However, in the group with the highest BMI (subgroup A), the differences in HRV were significantly attenuated compared with a group of healthy individuals and we would suggest this is related to increased adiposity and the related inflammation associated with it.

A host of blood samples were taken during the study. Serum adiponectin and leptin for subgroup A (n=14) were sampled at baseline, four months and eight months. We saw a

significant statistical difference using analysis of variance between the samples over eight months. Correlational analysis also was performed and confirmed a relationship between serum adiponectin, leptin and concurrent weight loss; the results are shown in Table 40 and Table 45. The results of the present study confirm this relationship between plasma adiponectin and leptin levels and markers of central obesity. Vinik (2012) states "*there is strong evidence of inflammation with activation of inflammatory cytokines and leptin in newly diagnosed type two diabetes."*

Although inflammatory markers such as CRP and IL-6 were not measured directly in this study it is possible to infer from the adiponectin and leptin samples that the inflammatory process associated with obesity was active and had a subtle but negative effect on heart rate variability which improved in response to weight loss and lifestyle modification, (see section 12.9.2). We saw an increase in adiponectin, a decrease in weight and an improvement in heart rate variability, (see Table 40 and section 12.9.2). The results of this research show that mild adipose related inflammation is enough to negatively affect the performance of the autonomic nervous system with respect to HRV in this group of obese individuals with a familial history of diabetes.

In other recent and unpublished research performed by the medical physics department (part of a national study) (The Hypocompass Trial) we found that heart rate variability data was 0.5 standard deviations below age matched normal data in type one diabetics who were asymptomatic for postural symptoms. This is in keeping with previously as yet unpublished research by Kerr 2009 (personal communication), which prompted our involvement in the current lifestyle project.

Measures of extended resting heart rate variability were made for subgroup A (BMI ≥36) from the lifestyle study, along with measures of Fractal dimension, SDNN, PNN50, and LF/HF ratio. There were no statistically significant differences between any of the extended resting heart rate measures for subgroup A over eight months (see Table 44); it is noted that this was also true for the GORD study (pre and post PPI therapy), (see Table 23) and for the Influenza study (pre and post vaccination), (see

Table 11). Measures of extended resting heart rate is part of current clinical protocol and is useful in patients with chronic and long-term disease but has not proven to be a particularly sensitive measure of short term change in autonomic tone.

Unlike measures of extended resting heart rate, measures of heart rate variability during two minutes of metronome guided breathing at six breaths per minute remain a sensitive measure of changes in autonomic tone. We have shown a drop in autonomic tone after influenza vaccination, a reversal (improvement) in autonomic tone after eight weeks of PPI therapy in the GORD study and an improvement in autonomic tone in association with lifestyle changes and weight loss in the lifestyle study. For the full lifestyle group and the full influenza group (post vaccination) HRV measures were reduced compared with normal age matched values but were not statistically significant in sub groups of the most symptomatic and the most overweight the reduction in HRV measures was significant. Results from this work suggest that mild inflammation and the resultant changes in autonomic performance are temporary and reversible.
Our primary measure of HRV at six breaths per minute reflects respiratory variability which is associated with high frequency variation and is generally considered to be under parasympathetic control, however six cycles per minute falls just within the low frequency range, normally attributed to sympathetic activity. It is our suspicion that the changes we have observed during this research represent a generalised deterioration affecting the sympatho-vagal balance.

14.1 Clinical Implications of the Research

"Given that there are therapies that can reorient the functional abnormalities of the autonomic nervous system toward improved function, the importance of determining the presence of cardiovascular autonomic dysfunction early cannot be overemphasized" Vinik et al (2007 page 8).

The applications of this research are varied and may impact on clinical practice within the immediate department, and it is hoped in a wider clinical setting as well. We have ascertained that our measurement technique is sufficiently sensitive to be able to measure subtle and temporary changes in autonomic tone resulting from changes related to inflammation. The typical patients referred for autonomic assessment are usually symptomatic and on assessment are experiencing significant attenuation in heart rate variability compared to an age matched normal range. We can be confident that our assessment of autonomic function will be able to detect more subtle changes in HRV in patients with less marked symptoms or have been referred earlier in their management. With this in mind we are potentially in a position to offer routine annual screening for type two diabetics to detect subtle changes in HRV and direct patient management and focus input from the diabetes team where needed, as it is known that if caught early enough HRV deterioration can be reversed. Our assessment incorporates provocations that are directed at stressing different branches of the ANS or activating the resonant balance between the two branches, the majority of our testing provokes the vagal or parasympathetic branch, which is associated with respiration.

The three research projects have all incorporated pupillometry as a measure of autonomic function with varying results. It could be argued that this measurement has not been shown to be adequately sensitive enough to be used in a cohort of essentially healthy individuals, although in the influenza study, subgroup 2 did appear to show a statistically significant difference (P=0.027) (see Table 14). (In the reflux study statistical differences were demonstrated in the ERD group (p=0.037) (see section 8.7). In the lifestyle study, (see section 12.7), no statistical differences were seen in our full cohort over the study period (see Table 35). Our experience clinically is quite different and for symptomatic patients with long-term disease it is a useful marker of gross changes in autonomic control.

It could be suggested that for hospitalised patients where an acute or chronic inflammatory condition is diagnosed, autonomic assessment may be used to inform treatment in coronary care setting or other health management to assess improvements or deterioration in HRV alongside concomitant disease.

In order to restore or reverse the impact of dysautonomia on physiological function it is important to implement a range of therapeutic options, which target: identification, prevention and symptom relief. Motooka et al (2006) suggested dog walking in the elderly is enough to increase parasympathetic function. Vinik et al (2007) state that endurance training has been shown to improve vagal activity in diabetes. We have seen through a simple lifestyle and physical activity regimen, participants in subgroup A (BMI≥36) were able to improve HRV and serum markers.

Development of autonomic dysfunction is a multifactorial process and needs a multifactorial strategy to intervene. Our autonomic function testing technique could be used alongside other methods as part of a strategy, using HRV results (I) as a prognostic marker of heart rate change; (II) as a method of determining patient susceptibility; or (III) for measuring patient health recovery. Gaede et al (2008) suggest tight glycaemic control and a strategy aimed at lifestyle change with pharmacological correction of hyperglycaemia, hypertension, dyslipidemia and microalbuminuria. Bourcier and Vinik (2010) suggest immune therapy for hypertension and Ziegler et al (2011) suggest antioxidant therapy. Pavlov et al (2007) have suppressed the innate immune response causing a decrease in pro-inflammatory cytokine production through the use of selective antimuscarinic acetylcholine receptor agonists. Vinik et al (2011) suggest that the use of anti-inflammatory drugs must have a part to play in the management of inflammatory disease and autonomic dysfunction along with glycation end-product inhibitors, statins, carnitine, peroxisome proliferator activated receptors (PPARS), protein kinase C-β inhibitors. The prospects for treatment and intervention in autonomic dysfunction are varied and numerous, but early detection remains the key to restoring or reversing autonomic tone. Vinik (2012) page 12 suggests "*It is not beyond the realms of reason that we could reverse the unfortunate evolutionary profile by targeting the hypothalamic set point of autonomic balance*."

14.2 Future Work

14.2.1 Future work related to the Influenza study

- I intend to perform a small-scale study to establish whether changing the shape of the respiratory curve makes any difference to heart rate variability. This is on-going.

- There is a need to further refine our age matched normal ranges of autonomic heart rate variation based on metronome guided breathing and simultaneous measurement of chest plethysmography.

- A longer term study following up the most symptomatic volunteers at set periods after the vaccination would have been useful to establish when the autonomic tone returns to its pre vaccination state. This was not in the ethics protocol for this study and was not performed. Any future work will address this.

- Additional analyses will involve re-analysing the pupillometry data with new dynamic software for automated pupil recognition and shape mapping.

- If this study were repeated, collection and analysis of inflammatory blood markers would be an amendment to the protocol.

- Vassallo and Allen (1997) identified that patients often remain non-specifically ill for a significant period following pneumonia and as a result of the inflammatory process recovery takes much longer than would be expected. We would like to be involved in the clinical assessment of HRV in patients with significant inflammatory disease to chart their recovery time, in association with improved HRV. The ANS does appear to recover after an eight month

lifestyle programme, we posit after true inflammation resulting from disease or infection this would be longer.

14.2.2 Future work related to the reflux study

- If this research is repeated in the future, a significant change in the protocol will be made to allow us to measure inflammatory markers alongside the other biochemical measures.
- We are considering extending recruitment to the reflux study to give more power to the statistics.
- We hope to publish this work in due course.

14.2.3 Future work related to the obesity and lifestyle study:

- If this research is repeated in the future, a significant change in the protocol will be made to allow us to measure inflammatory markers alongside the other biochemical measures.

- Increasing recruitment in future projects may potentially increase the number of pre-diabetics in the study cohort.

- For future projects of a similar nature we may be able to acquire another Portapres or equivalent device for measurement of beat to beat blood pressure thus allowing us to comment on blood pressure changes and the baroreflex.

- Increasing recruitment in future projects may potentially increase the number of participants with a BMI≥36, which would improve statistical analysis of this subgroup, or changing the inclusion criteria to a higher BMI threshold.

- With obesity at epidemic levels worldwide one area for future work will involve assessment of how gastric bypass surgery will alter heart rate variability and change management of diabetes. We propose to work with the bariatric surgical team to put together a research project assessing autonomic function in patient's pre and post-surgery. We anticipate that many of the patients referred for surgery will be diabetic. We will aim to look at whether the surgery and resultant weight loss will improve HRV alongside eradicating diabetes in these patients.
- We are currently involved in a double blind randomised research project with the oncology department looking at the effect of two different chemotherapy options or stand-alone surgery for bowel cancer on the autonomic nervous system. Recruitment has started for this project.

- Anecdotal evidence from our colleagues around the hospital suggests that patients with autonomic dysfunction take slightly longer to recovery from surgery and other health problems such as influenza. We propose to work with orthopaedic consultants to assess heart rate variability pre and post-surgery to identify those who are more likely to make an unremarkable recovery from surgery and focus efforts on patients with reduced autonomic tone prior to surgery that may need additional support in their post-op recovery.

- We aim to work alongside our colleagues working in diabetes to implement the HRV assessment programme suggested by Boulton et al (2005) that all type 1 diabetics should be assessed for autonomic dysfunction every 5 years and Type 2 diabetics should be assessed annually after diagnosis to catch early attenuation in HRV.

- Vinik et al (2011) suggest the use of anti-inflammatory medication in the management of inflammatory disease and autonomic dysfunction. It would be interesting to see whether a research project using anti-inflammatory drugs in

specific key groups of patients would improve HRV, namely in elderly medicine. More research is needed in this area.

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Appendices

16.1 Clinical Physiology and Functional Imaging Publication

Clin Physiol Funct Imaging (2012) 32, pp437–444 doi: 10.1111/j.1475-097X.2012.01147.x

Assessment of changes in cardiac autonomic tone resulting from inflammatory response to the influenza vaccination

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Summary

A total of 71 healthy volunteers opting to have a routine influenza vaccination were investigated for potential changes in cardiovascular autonomic tone resulting from the temporary inflammatory effects of an influenza vaccination. A number of temporal and frequency domain parameters of heart rate and breathing were assessed 2–5 days prior to vaccination and 1–4 days postvaccination. Three lead electrocardiograph (ECG), beat-to-beat finger blood pressure and chest plethysmography signals were measured. After an extended resting period, patients performed metronome-guided breathing at six breaths per min for a period of 2 min. Standard Ewing tests of autonomic function were also performed. All volunteers completed a vaccine symptom questionnaire. A subgroup of 15 volunteers who reported significant symptomatic reaction to the vaccination for at least 24 h following vaccination were identified based on the results of the questionnaire. A significant reduction in measures of heart rate variability (HRV) obtained during metronome-guided breathing was noted following vaccination in the subgroup of 15 symptomatic volunteers. No significant changes were observed in standard Ewing assessment, fractal dimension analysis, baroreflex sensitivity assessment or resting HRV. There was no evidence of significant reduction in autonomic tone following vaccination in the full sample of 71 volunteers. Results suggest a significant change in HRV response to a small inflammatory provocation and suggest further investigation of the inflammatory causes of dysautonomia is of value.

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Introduction

Heart rate variability (HRV) describes the beat-to-beat variation in heart rate arising from the efferent activity of the sympathetic and parasympathetic branches of the autonomic nervous system (ANS). Reduced HRV as a measure of autonomic function is a powerful predictor of adverse diagnosis in patients and is associated with increased mortality (O'Brien et al., 1991). Diabetes mellitus and multisystem atrophy (Shields, 2009) have been shown to result in progressive and permanent loss of autonomic tone (Vinik & Erbas, 2001). However, there is increasing evidence that acute inflammatory disease leads to similar but temporary loss of autonomic tone, for example in pneumonia (Vassallo & Allen, 1997) and Respiratory syncytial virus in neonates (Stock et al., 2010). The implication is that autonomic failure contributes significantly to the constellation of symptoms and consequent morbidity associated with these conditions. Potentially chronic low-grade infection may also have an associated deterioration in ANS function accompanied by associated symptoms.

Inactive influenza vaccine has been available for inoculation since 1945 for vulnerable groups, such as pregnant women, the elderly, immunocompromised patients and infants under 2 years old (Whitley & Monto, 2006). More recently, vaccination has been offered to individuals whose occupation puts them at higher risk of contagion, such as front line hospital workers.

Vaccination with an influenza vaccine, such as Influvac, stimulates B lymphocytes to produce antibodies without causing influenza. When the body is exposed to the antigens in the vaccine, the immune response is the same as occurs in an influenza infection. These antibodies are specific to the antigens in the vaccine and protective immunity occurs within 2–3 weeks postvaccination and lasts up to 12 months. The influenza vaccine is effective in protecting against disease in approximately 70–90% of people vaccinated (Stohr, 2003). It is well known that inflammatory markers such as the liver-derived plasma proteins increase in concentration in response to an event such as infection (Steele & Whitehead, 1994). Acute phase reactants C-reactive protein (CRP) and serum amyloid A protein are elevated in the common cold and

influenza (Whicher et al., 1985). However, little information is available on the response of these inflammatory markers to influenza vaccination. Tsai et al. (2005) showed transient changes in inflammatory blood markers specifically CRP and plasma lipid concentrations at day one and day three after vaccination. These findings suggest that an inflammatory response is occurring at a chemical level, which is most significant for interleukin 6 (IL-6) and CRP on days one to three. Posthouwer et al. (2004) also found a similar response after vaccination with values peaking at 2 days postvaccination. Tsai et al. (2005) quote in their conclusion that 'Our findings indicate that influenza vaccination, like yellow fever vaccination; can be used as a model to study the response of mild stimulation of the inflammatory system.'

The aim of this project was to establish if it is possible to detect a small, temporary change in ANS function in response to the inflammatory provocation resulting from the influenza vaccination.

Methods

A total of 71 healthy participants, aged 20–73 years were recruited (mean, 48·7 years; SD, 11·9 years; quartile range, 43–57 years). Of these 53 were women and 18 were men. All volunteers were workers or volunteers at Poole Hospital NHS Foundation Trust that had elected to have the routine yearly vaccination for influenza, which was offered and administered by the Hospital Occupational Health Department. Participants were selected only on the basis that they were having the vaccination as prophylaxis owing to the nature of their occupation, rather than for health reasons. Recruitment occurred during the 2008/2009 and 2009/2010 influenza vaccination cycles, that is, between October and November 2008 as well as between October and November 2009.

Volunteers were asked to complete a health screening questionnaire prior to inclusion into the project. Volunteers were excluded if they had any of the following pre-existing inflammatory conditions: Diabetes, rheumatoid conditions, coronary heart disease or hypertension, oesophagitis or gastritis, inflammatory bowel disease, asthma or other severe allergic reaction, pulmonary disease and multiple sclerosis. They were also excluded if they had received recent treatment for cancer, reported acute illness sufficiently severe to be absent from work in the previous week or had taken steroids or non-steroidal anti-inflammatory drugs in the previous week.

Baseline autonomic function assessment was performed on all volunteers between 1 and 5 days prior to the influenza vaccination. Autonomic reassessment was performed between 1 and 5 days after the vaccination (mean 2·6 days). 55 participants (77%) were assessed on day 2 or day 3 following vaccination. During the second visit, the volunteer's symptom response to the influenza vaccination was recorded using a questionnaire listing typical postvaccination symptoms.

To assess cardiac autonomic function, volunteers were connected to a multisignal bio-recorder ProComp Infiniti (Thought Technology, Montreal, Canada), which recorded the following parameters at the following sample rates:

1 ECG at 1024 Hz
2 Chest plethysmography at 256 Hz

Real-time systolic and diastolic blood pressure was also measured using a Portapres finger arterial blood pressure monitor (TNO-TPD Biomedical Instrumentation, Amsterdam, The Netherlands), with the blood pressure data transferred to the PC via the RS232 serial link.

Data were recorded in real time on a laptop using software developed in Visual Basic 6.0 (Microsoft Corp, Redmond, WA, USA).

Accurate measurement of heart rate was established by the method of rolling correlation coefficient introduced by Buttfield & Bolton (2005). The ECG data stream was compared with an archetype QRS complex from the data as a template. The template was moved through the data set. Precise timing of the onset of each QRS complex was identified as the point of maximum correlation. Instantaneous heart rate was calculated from the time between adjacent QRS complexes. Any data points lost because of excessive noise on the ECG signal or ectopic beats were manually identified, removed and replaced by cubic spline interpolation of the heart rates from adjacent valid beats. Finally, the heart rate data were regularized to a data set of heart rate values saved at an interval of eight samples per second, heart rate being interpolated from the irregular instantaneous heart rate data by cubic spline interpolation.

Volunteers were investigated in a quiet room and were familiarized with the equipment and the environment (Bloomfield et al., 2001). The room was maintained at an ambient temperature of approximately 23°C (Low & Pfeiffer, 1997). Volunteers were asked to refrain from caffeine and nicotine for 3 h (Low, 1993). Retesting occurred, where possible, at the same time of day as the initial assessment to avoid diurnal variation. Volunteers were positioned on the examination couch in a semi-recumbent position, connected to the equipment and allowed adequate time to relax.

The following sequence of provocations was used to assess autonomic cardiovascular reflexes:

1 An extended rest period (5 min).
2 A trial period of at least two cycles of deep breathing at six breaths per min.
3 Valsalva Manoeuvre (open glottis, pressure achieved >20 mmHg, maintained for 10 s).
4 Maximal hand grip (10 s squeeze without breath hold, repeated after 30 s).
5 Two minutes metronome-guided deep breathing at six breaths per min.

Between each provocation, a period of 2 min rest was maintained to allow the patient to relax.

Heart rate variations were analysed in the following manner for these provocation conditions:

1 Rest period: Average heart rate was measured during this period, as well as standard deviation of the RR interval (SDNN). Frequency analysis of HRV during the resting period was performed to establish the ratio of low-frequency (0·04–0·15 Hz) to high-frequency (0·15–0·4 Hz) range. Fractal dimension analysis was also performed for the resting period using Higuchi's algorithm (Addio *et al.*, 2007).

2 Valsalva manoeuvre, handgrip and slow breathing: Analysis was performed for these provocations as described by Ewing *et al.* (1985).

3 Two-minute metronome-guided deep breathing at six breaths per min. The following parameters of HRV were measured for this 2 min extended period of metronome-guided deep breathing:

- **a** Maximum correlation coefficient between HR and chest wall plethysmography signal, adjusting the timing of the heart rate curve by up to ±3 s until correlation between heart rate and breathing curves is at a maximum (CORR)
- **b** Average peak to trough HR amplitude (A) (bpm)
- **c** Standard deviation heart rate variation (SD) (bpm)
- **d** Log of the integral of peak of the frequency power curve following FFT at the breathing frequency of six breaths per min (LPP)
- **e** Correlation coefficient of the FFT graphs of breathing and HR (FCORR)

These parameters are explained in Fig. 1.

Although chest plethysmography was not calibrated, standard deviation chest wall movement was recorded during the 2-min metronome breathing period.

Baroreflex sensitivity was also assessed for the 2-min metronome-guided breathing period, comparing instantaneous heart rate with the beat-to-beat systolic blood pressure obtained from the Portapres finger blood pressure monitor. Baroreflex sensitivity was assessed in the frequency space by comparing the areas under the frequency peaks corresponding to six breaths per min in the heart rate and systolic blood pressure frequency spectra.

Heart rate variability data were reviewed for the sample group as a whole (71 volunteers). HRV was also reviewed for a subgroup of 15 volunteers (11 women, four men, mean age 45·7 years, standard deviation 12·9 years, quartile range 34–57 years) who reported a significant symptomatic response to the vaccination in their questionnaire on the following criteria:

1 Intramuscular discomfort or pain at the injection site and surrounding deltoid tissue
2 Cephalgia
3 Generalized malaise (including myalgia)
4 Pyrexia

All of the above lasting for at least 24 h following vaccination. Research by Tsai *et al.* (2005) and Posthouwer *et al.* (2004) suggest blood markers for inflammation peak at between 1 and 3 days. For the symptomatic subgroup ($N = 15$), the mean interval between vaccination and reassessment was 2·8 days. The remaining subgroup with no significant symptoms following vaccination was also reviewed ($N = 56$).

Approval was sought and granted by the Dorset Research Ethics Committee: Ref 08/H0201/73.

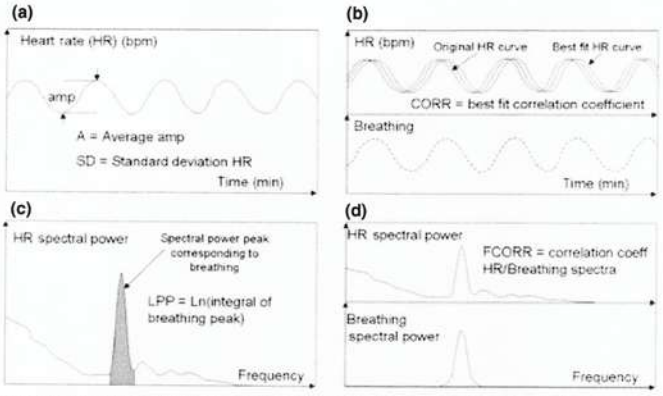

Figure 1 Illustration of the methods used to analyse heart rate variability and relationship to breathing in the temporal and frequency dimensions for a 2-min period of metronome-guided deep breathing at six breaths per min (a) Temporal dimension. A means average peak-to-trough heart rate variation, SD means standard deviation heart rate. (b) Temporal dimension. CORR means maximum correlation coefficient comparing heart rate and breathing curve, shifting heart rate curve in time to most closely match breathing curve. (c) Frequency dimension. LPP means log of the integral of the heart rate spectral power peak corresponding to six breaths per min. (d) Frequency dimension. FCORR means correlation coefficient comparing the heart rate and breathing frequency spectra.

Results

Ewing measures of cardiovascular autonomic tone

The heart rate responses to exercises recommended by Ewing prior to and following vaccination were assessed and are displayed in Table 1. Comparison of the measures pre- and postvaccination was made for the complete cohort and for the subgroups of 15 volunteers who reported significant reaction to the vaccination and the remaining 56 volunteers with no significant response to vaccination. No significant difference in heart rate responses was observed for the full group or either subgroups.

Measures of resting autonomic tone

Measures of resting cardiovascular autonomic tone were assessed for heart rate and blood pressure changes prior to and following the influenza vaccination. The parameters included and the results of paired *t*-test analysis are displayed in Table 2. There was no significant change in spontaneous breathing rate between the resting investigation period prior to and following vaccination (resting breathing rate (mean ± SD) 16·4 ± 3·9 cycles per min prior to and 16·08 ± 3·3 cycle per min following vaccination, $t = 0{\cdot}186$, $P = 0{\cdot}85$). The number of heart beat intervals obtained during the 5-min recording window for resting HRV study varied from 258 to 501 beats with a mean of 366 beats, SD 54·6 beats.

No significant difference was noted in any of these parameters for either the full group or the two subgroups.

Metronome-guided deep breathing parameters

The prevaccination metronome-guided breathing period was analysed for all volunteers and the responses compared with the volunteer's age. Correlation of the five parameters of metronome-guided breathing with age are displayed in Table 3. It was noted that, as previously reported (Perring & Jones, 2003), absolute measures of HRV deteriorated significantly with age. However, correlation of heart rate variation with chest plethysmography signal did not decay with age in normal volunteers, suggesting maintenance of the mechanism of the cardio-ventilatory coupling, even if the magnitude of the coupling effect decays with age. The relationship of the metronome-guided breathing parameters LPP and CORR to age are graphically represented in Fig. 2 to illustrate changes in heart rate amplitude and correlation with breathing associated with age.

Metronome-guided deep breathing parameters: effect of influenza vaccine

There was no significant difference in performance of the metronome breathing task between the two visits (breathing depth standard deviation (Mean ± SD, arbitrary units) prior to vaccination 111·3 ± 64·4, following vaccination 110·1 ± 69·5, $t = 0{\cdot}186$, $P = 0{\cdot}853$).

Analysis of the 2-min metronome-guided breathing periods was performed and results prior to the influenza vaccination were compared with those obtained following the vaccination. Table 4 displays the parameters of metronome breathing analysis before and after the vaccination for the full volunteer sample group of 71, the subgroup of 15 with symptoms following vaccination and the remaining 56. A small but significant reduction in three parameters of heart rate responsiveness (CORR, A, LPP) was noted in the symptomatic subgroup.

Further analysis of metronome-guided deep breathing

In a previous study by the authors, 44 normal volunteers were assessed in a similar fashion for metronome-guided deep breathing (Perring & Jones, 2003). Detailed description of this normal volunteer cohort has been described elsewhere. From this group of 44 healthy normal volunteers an age-related

Table 1 Summary of the differences of Ewing test parameters measured prior to and following influenza vaccination for the full volunteer group of 71 volunteers, the 15 volunteers who subsequently reported significant postvaccination symptoms and the remaining 56 volunteers with no symptoms.

Parameter	Prevaccination mean (SD)	Postvaccination mean (SD)	*t* Value (paired *t*-test)	*P* value
Full sample group (71 volunteers)				
Valsalva ratio	1·33 (0·18)	1·34 (0·18)	0·308	0·759
I/E difference (bpm)	14·18 (7·73)	14·06 (7·81)	0·209	0·835
Hand grip ratio	1·17 (0·09)	1·17 (0·08)	0·255	0·800
Sample with significant postvaccination symptoms (15 volunteers)				
Valsalva ratio	1·33 (0·25)	1·31 (0·19)	0·417	0·683
I/E difference (bpm)	14·76 (7·59)	13·35 (7·48)	1·541	0·146
Hand grip ratio	1·15 (0·07)	1·14 (0·05)	0·631	0·538
Sample with no significant postvaccination symptoms (56 volunteers)				
Valsalva ratio	1·33 (0·16)	1·34 (0·18)	0·486	0·629
I/E difference (bpm)	14·02 (7·82)	14·25 (7·95)	0·335	0·738
Hand grip ratio	1·17 (0·10)	1·17 (0·09)	0	1·0

Table 2 Summary of the differences between measures of resting heart rate variability and blood pressure prior to and following influenza vaccination for the full volunteer group of 71 volunteers, the 15 volunteers who subsequently reported significant postvaccination symptoms and the remaining 56 volunteers with no symptoms.

Parameter	Prevaccination mean (SD)	Postvaccination mean (SD)	*t* Value (paired *t*-test)	*P* value
Full sample group (71 volunteers)				
Resting heart rate (bpm)	73·45 (10·91)	74·34 (10·45)	1·079	0·285
Systolic blood pressure (mmHg)	124·1 (30·1)	117·8 (32·2)	1·653	0·103
Fractal dimension	1·394 (0·115)	1·358 (0·197)	1·401	0·166
SDNN (ms)	46·66 (25·84)	57·45 (59·46)	1·506	0·137
PNN50 (%)	8·80 (11·61)	8·87 (11·63)	0·051	0·960
LF/HF ratio	1·066 (0·083)	1·048 (0·137)	0·960	0·341
Sample with significant postvaccination symptoms (15 volunteers)				
Resting heart rate (bpm)	76·90 (13·65)	76·86 (14·56)	0·029	0·977
Systolic blood pressure (mmHg)	124·1 (26·8)	115·6 (24·6)	1·269	0·225
Fractal dimension	1·390 (0·092)	1·430 (0·121)	1·43	0·176
SDNN (ms)	42·60 (24·69)	46·61 (19·68)	0·611	0·551
PNN50 (%)	8·09 (9·09)	10·36 (13·88)	0·761	0·459
LF/HF ratio	1·374 (0·661)	1·356 (0·606)	0·389	0·703
Sample with no significant postvaccination symptoms (56 volunteers)				
Resting heart rate (bpm)	72·49 (9·96)	73·64 (9·04)	1·159	0·251
Systolic blood pressure (mmHg)	121·8 (35·1)	116·2 (37·5)	1·254	0·215
Fractal dimension	1·395 (0·121)	1·365 (0·100)	1·949	0·056
SDNN (ms)	46·92 (26·8)	59·41 (66·15)	1·414	0·163
PNN50 (%)	8·84 (12·22)	8·30 (10·99)	0·428	0·670
LF/HF ratio	1·065 (0·081)	1·063 (0·050)	0·292	0·771

Fractal dimension as defined by Higuchi's algorithm. SDNN means Standard deviation of RR interval. PNN50 means percentage of RR intervals where the gap between adjacent intervals is >50 ms. LF/HF ratio means ratio of integrated spectral power of the portions of the heart rate frequency plot in the low-frequency (0·04–0·15 Hz) and high-frequency (0·15–0·4 Hz) bands.

Table 3 Summary of the relationship between the parameters of heart rate variability during 1 min of metronome-guided breathing and age, parameters defined in Fig. 1.

Parameter	Pearsons correlation coefficient	*t* Value	*P* value
CORR	−0·077	0·63	0·529
A (bpm)	−0·521	5·03	<0·001
SD (bpm)	−0·502	4·79	<0·001
LPP	−0·489	4·63	<0·001
FCORR	−0·097	0·8	0·425

normal range was established for each of the parameters of metronome-guided breathing described above. The heart rate responses of volunteers in the current trial were then related to the previously established normal range by calculating the number of standard deviations from the lines of best fit as calculated below:

CORR = 0·765. Standard deviation from line of best fit = 0·104

A = 22·9–0·2297 * Age(years). SD from line of best fit = 5·467

SD = 9·04–0·094*Age(years). SD from line of best fit = 1·851

LPP = 7·715–0·0233*Age(years). SD from line of best fit = 0·4553

FCORR = 0·922. SD from line of best fit = 0·149

A combined parameter was then calculated by adding the deviations from the expected age-related norm for all five parameters. This combined parameter was then compared with data from the previous study to obtain an overall deviation from the expected age-related norm as calculated from the original study of 44 normal volunteers. This was calculated in number of standard deviations from the expected norm, was therefore a measure independent of the volunteer's age, and was labelled DEV.

Table 4 also displays the combined age-corrected parameter of HRV in response to metronome breathing (DEV) before and after the vaccination for the full volunteer sample group of 71, for the subgroup of 15 volunteers who reported significant reaction to the vaccination, and for the remaining 56. A highly significant reduction in the combined age-corrected parameter of HRV was seen in the symptomatic subgroup.

Discussion

When comparing heart rate data prior to and following influenza vaccination, there appeared to be a measureable reduction in autonomic HRV response to metronome-guided breathing following the influenza vaccination in those volunteers who reported a significant inflammatory response to the vaccination. There was no clear evidence to suggest the metronome breathing task had been performed in a different way

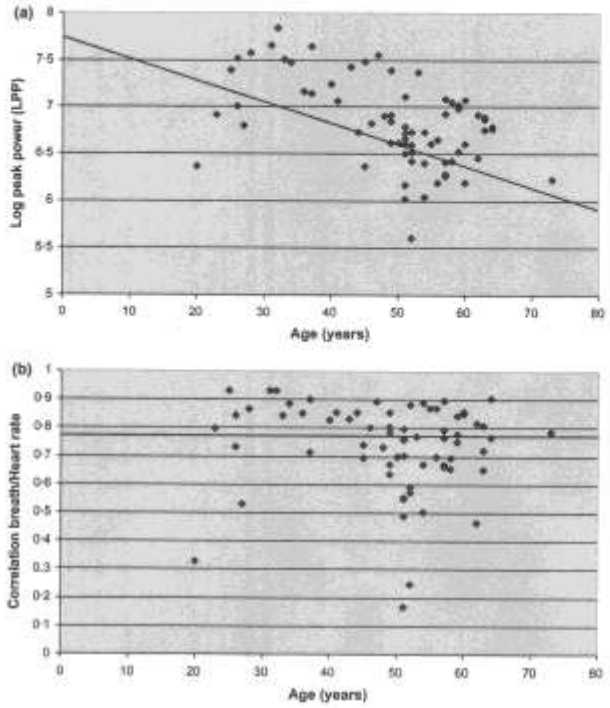

Figure 2 Graphs displaying the variation of (a) log peak power (LPP) and (b) heart rate/breathing correlation (CORR) with age for the 75 normal volunteers studied. Lines of best fit from the previous study of 44 normal volunteers reported in Perring & Jones (2003).

pre- and postvaccination. There was no difference in the spontaneous breathing rate or in blood pressure (systolic and diastolic) between visits. These observations suggest that the change in cardiovascular autonomic response did not result from altered emotional state or change in performance resulting from prior experience of the investigation.

This cardiovascular autonomic response only appeared to be significant in the subgroup of volunteers who reported a significant inflammatory symptomatic response to the influenza vaccination, although for the most part, this group had recovered from any symptoms of malaise that they had reported by the time of retesting. The mean time between vaccination and reassessment for the symptomatic subgroup was 2·8 days.

Other measures of cardiovascular autonomic tone used in this investigation did not show sufficient response to influenza vaccination to register a statistically significant change.

Resting heart rate and blood pressure measures were not significantly changed by the intervention of the influenza vaccination, suggesting that the reduction in autonomic tone is not biased towards either the sympathetic or parasympathetic branches of the ANS. Rather this suggests a reduction in the vegetative balance of the two branches, leading to a generalized reduction in responsiveness. The lack of any significant difference in heart rate, blood pressure or spontaneous breathing rate prior to and following the vaccination also suggests that the changes in HRV responses observed were not a direct result of the malaise following vaccination.

This project was restricted by practicalities of assessing pre- and postvaccination HRV in a sample group of busy health professionals and volunteers having prophylactic vaccination for reasons of their occupation and therefore minimizing the invasiveness and time taken by the intervention. We acknowledge the limitation of the study resulting from the lack of direct data on blood markers of inflammation and would have preferred that data to be available. However, we observed a measureable fall in autonomic response close to the time when the postvaccination inflammatory response was expected to be at its peak in the volunteers who reported significant inflammatory symptoms to the vaccination. Although it would have been useful to have performed long-term ambulatory studies of HRV using holter studies, other studies have indicated that short-term HRV studies have equivalent capability to identify reduction in autonomic tone as a result of

Table 4 Summary of the differences between HRV parameters and baroreflex sensitivity for metronome breathing measured prior to and following influenza vaccination for the full volunteer group of 71 volunteers, the 15 volunteers who subsequently reported significant postvaccination symptoms and the remaining 56 volunteers with no symptoms.

Parameter	Prevaccination mean (SD)	Postvaccination mean (SD)	*t* Value (paired *t*-test)	*P* value
Full sample group (71 volunteers)				
CORR	0·729 (0·189)	0·718 (0·216)	0·432	0·667
A (bpm)	13·38 (6·74)	13·33 (6·36)	0·100	0·921
SD (bpm)	5·497 (2·465)	5·359 (2·336)	0·754	0·453
LPP	6·825 (0·456)	6·786 (0·467)	0·889	0·377
FCORR	0·896 (0·181)	0·905 (0·196)	0·392	0·697
Baroreflex sensitivity (bpm $mmHg^{-1}$)	0·887 (1·021)	0·982 (1·390)	0·623	0·536
DEV	0·292 ± 1·188	0·252 ± 1·231	0·386	0·702
Sample with significant postvaccination symptoms (15 volunteers)				
CORR	0·748 (0·173)	0·673 (0·232)	2·740	0·016
A (bpm)	14·71 (7·64)	12·62 (6·54)	1·471	0·027
SD (bpm)	5·787 (2·546)	5·193 (2·305)	1·830	0·089
LPP	6·956 (0·386)	6·792 (0·397)	3·627	0·003
FCORR	0·913 (0·161)	0·867 (0·261)	0·964	0·351
Baroreflex sensitivity (bpm $mmHg^{-1}$)	0·826 (1·077)	1·205 (1·304)	1·257	0·229
DEV	0·284 ± 1·466	−0·287 ± 1·516	4·71	<0·001
Sample with no significant postvaccination symptoms (56 volunteers)				
CORR	0·710 (0·215)	0·717 (0·232)	0·204	0·839
A (bpm)	12·79 (6·67)	13·29 (6·55)	0·94	0·351
D (bpm)	5·321 (2·544)	5·318 (2·451)	0·064	0·949
LPP	6·668 (1·020)	6·663 (1·027)	0·091	0·927
FCORR	0·875 (0·220)	0·899 (0·212)	0·910	0·366
Baroreflex sensitivity (bpm $mmHg^{-1}$)	0·904 (1·014)	0·920 (1·419)	0·089	0·930
DEV	0·295 (1·118)	0·396 (1·115)	0·815	0·419

HRV parameters defined in Fig. 1. DEV means combined age-corrected measure of all five HRV parameters.

disease, for example, diabetes (Migliaro & Contreras, 2003). Simultaneous measurement of breathing does appear to confirm added diagnostic value to HRV studies performed during short-term metronome-guided breathing.

The inflammatory process is complex, and research by Lampert *et al.* (2008) suggests that inflammatory and autonomic processes may be linked and that the risk factors for coronary heart disease may be proinflammatory associated with autonomic dysregulation. Lombardi (2004) also found measures of inflammation, such as CRP or IL6, have been proven as risk factors for cardiovascular mortality in both healthy subjects and patients with different cardiovascular diseases. There is increasing evidence indicating that inflammation plays a determinant role in the pathogenesis and progression of atherosclerosis. This finding may well explain the strong epidemiological association between inflammation and cardiovascular morbidity and mortality (Libby *et al.*, 2002).

Research has been published reporting a link between inflammatory disease states such as rheumatoid arthritis, diabetes and other autoimmune disorders and autonomic impairment (Toussirot *et al.*, 1993). It is known that increasing age, gender, medications, infection and respiratory disease can all have a negative effect on autonomic tone (Vassallo & Allen, 1997; Sandroni, 1998; Vinik & Erbas, 2001). Vassallo & Allen (1997) discuss a link between infection and respiratory disorders and a temporary reduction in autonomic function. It is still unclear the exact mechanism between acute inflammation and temporary dysautonomia, however, is likely to be multifactorial. Recent evidence suggests the presence of the 'cholinergic anti-inflammatory pathway' where the vagal nerve is implicated in a 'hard wired' connection between nervous and immune systems (Czura & Tracey, 2005) with consequent association between HRV and inflammation (Thayer, 2009). Stimulation of the vagal nerve attenuates the production of proinflammatory cytokines and inhibits the inflammatory process (de Jonge & Ulloa, 2007).

In clinical conditions characterized by an increase in inflammatory markers, for example, diabetes, rheumatoid arthritis, sepsis and acute coronary syndromes, a reduction in HRV is consistently observed in high-risk patients, strengthening the connection between inflammation and autonomic dysfunction (Lombardi, 2004) and suggesting that potentially many of these diseases may actually be diseases of autonomic function (Czura & Tracey, 2005).

It may therefore be appropriate to follow-up patients with chronic inflammatory conditions, such as cardiovascular disease or diabetes, to assess their autonomic status and identify those at increased risk of sudden death associated with dysautonomia. Previous research has shown that the performance of the ANS is significantly attenuated in asymptomatic diabetics compared with a healthy age-matched cohort (Perring & Jones, 2003). Gerritsen *et al.* (2001) suggest the finding that dysautonomia is particularly significant in 'at risk' patient groups may validate the hypothesis that reduced autonomic

tone may complicate underlying cardiovascular disease rather than being a risk factor.

Conclusions

The results are suggestive that it is possible to measure changes in autonomic tone resulting from a low level of inflammation associated with routine influenza vaccination, although more work is needed to clarify this further. These results therefore suggest that the measurement of cardiovascular autonomic function using metronome-guided breathing may be a sensitive measure of autonomic response to even mild inflammatory conditions.

The technique for assessment of autonomic function used in this study is ideally suited to longitudinal studies of disease progression, as has been suggested already for monitoring of patients with diabetes by Vinik & Erbas (2001). It may be of value to routinely assess patients with a range of inflammatory conditions for reduced autonomic tone.

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16.2 Vaccine Evaluation Centre Questionnaire

Adverse events reported following administration of influenza vaccine	
Adverse event	
Allergic reaction	
Allergic reaction -unspecified	
Allergic reaction -respiratory	
Allergic reaction -skin	
Allergic reaction -skin & respiratory	
Fever	
Fever -temperature not recorded	
Fever -39.9^{o}-40.4^{o} C	
Fever >= 40.5^{o} C	
Severe vomiting and/or diarrhoea	
Rash	
Rashes -unspecified	
Rashes -generalized	
Rashes -localized	
Severe pain and/or swelling	
Severe pain and/or swelling -unspecified	
Severe pain and/or swelling -lasting >= 4 days	
Severe pain and/ or swelling -extending past nearest joint(s)	
Severe pain and/or swelling -lasting >= 4 days and extending past nearest joint(s)	
Arthralgia/arthritis	
Adenopathy enlargement of glands	
Anesthesia/paresthesia	
Anaesthesia/paraesthesia -specified	
Anaesthesia/paraesthesia -localized	
Anaphylaxis	

Abscess	
Sterile abscess/nodule/necrosis	
Infective abscess	
Infective abscess -virulent discharge	
Parotitis (Inflammation of one or both parotid glands)	
Paralysis limb/facial/cranial	
Guillain-Barré syndrome	
Convulsions/seizures –febrile (fever) **or afebrile** (no fever)	
Meningitis/encephalitis/encephalopathy/encephalomyelitis	
Orchitis Inflammation of one or both testes	
Hypotonic-hypo responsive episode	
Allergic reaction	
Allergic reaction -unspecified	
Allergic reaction -respiratory	
GI symptoms	
Abdominal pain	
Diarrhoea	
Nausea	
Vomiting	
Anorexia	
Other: dyspepsia, appetite increased, faeces discoloured, flatulence, halitosis, hiccups, malabsorption, melaena	
Skin and local reactions	
Rash	
Pruritis (Itching)	
Urticaria (hives)	
Injection site reaction	
Facial oedema swelling	
Cellulitis	

Other: bullous eruption (blisters), erythema multiforme, herpes simplex, herpes zoster, hot flushes, purpura (subcutaneous bleeding), rash, rosacea (flushed skin), skin disorder, skin necrosis, dry skin, skin exfoliation, acne, anaphylactoid reaction	
Oculo-respiratory syndrome: bilateral red eyes and/or at least one chest/respiratory symptom (cough, sore throat, difficulty breathing, chest tightness, wheezing) and/or facial oedema	
Bilateral red eyes	
Chest/respiratory symptoms	
Bronchospasm A spasm of the bronchi that makes exhalation difficult and noisy; associated with asthma and bronchitis	
Chest pain	
Dyspnoea laboured breathing	
Cough	
Sore throat	
Difficulty breathing	
Chest tightness	
Wheezing	
Other: hyperapnoea, hypoapnoea, pneumonia, respiratory disorder, haemoptysis, hyperventilation, asthma, pulmonary congestion	
Facial oedema	
Other eye symptoms	
Eye pain	
Photophobia	
Abnormal vision	
Other: blepharitis (Inflammation of the eyelids), blepharospasm, (spasm of the eyelid), eye abnormality, eye infection, eyelid retraction, haemorrhage, lacrimation abnormal, retinitis	
Ear/nose/throat symptoms	
Laryngitis	
Pharyngitis	
Rhinitis	
Throat tightness	

Oedema mouth	
Dysphonia speech disorder	
Dysphagia difficulty swallowing	
Other: dry mouth, oedema mouth, saliva increased, sinusitis, tongue disorder, tongue paralysis, tongue oedema, upper respiratory tract infection, angioedema swelling, epistaxis nose bleeds, ear ache, ear disorder, hearing decreased, tinnitus, aphasia	
Neurological symptoms	
Myelitis Inflammation of the spinal cord	
Paresthesia tingling itching or burning	
Paralysis	
Other: agitation, impaired concentration, confusion, abnormal coordination, abnormal crying, delirium, depression, diplopia (visual impairment), dyskinesia (abnormal voluntary muscle movements), euphoria, fall, gait abnormal, hallucination, insomnia, nervousness, somnolence, vertigo, tremors, stupor, hypertonia, hypoesthesia, hypokinesia, anxiety, ataxia	
Musculo-skeletal symptoms	
Skeletal pain	
Back pain	
Myalgia (muscle pain)	
Other cardiovascular symptoms: arrhythmia bradycardia (slow heart rate), cardiac arrest, cardiac failure, atrial fibrillation, hypotension, mitral insufficiency, palpitation, weak pulse, tachycardia, syncope, hypertension, hypertension aggravated	
Other metabolic disorders: glucocorticoid increase, hyperglycemia, metabolic disorder, thyroid stimulating hormone	
Others	
Dizziness	
Fatigue	
Asthenia	
Flushing	
Malaise	
Influenza-like illness	

Headache	
Oedema (including peripheral oedema)	
Rigors	
Increased sweating	
Other: leg pain, livido (skin disclouration) reticularis, fatty liver, ischemic necrosis, neoplasm, orchitis, pallor, parotitis, polymyalgia rheumatica, speech disorder, splenomegaly (enlarged spleen), sputum increased, trigeminal neuralgia, tooth ache, xerophthalmia (dry eyes), yawning, sweating increased, taste perversion, torticollis, urine abnormal, urinary tract infection, vasculitis, temperature change sensation, aura, apathy, cystitis, dehydration, infection, muscle weakness	

16.3 GORD Project Statistical Power Calculation

-----Original Message-----
From: Zoe Sheppard
Sent: 17 March 2010 15:08
To: 'Jones, Emma'; 'S.Perring@poole.nhs.uk'
Cc: Peter Thomas; Reuben Ogollah
Subject: Sample Size Recommendation for Pilot Study

Dear Emma Jones and Steve Perring

This e-mail is to confirm that you have consulted the Research and Development Support Unit with regards to the sample size for your pilot study investigating the influence of proton pump inhibitors on measures of autonomic function. Because of the pilot nature of the study, a sample size of thirty participants is recommended (Browne 1995 cited by Lancaster et al. 2004 p308). We will continue to be available for advice and support during the course of your study.

Many Thanks

Zoe Sheppard

References:
Browne, R.H. (1995). On the use of a pilot sample for sample size determination. *Statistics in Medicine*, 14: 1933-1940.
Lancaster, G.A., Dodd, S., and Williamson, P.R. (2004). Design and analysis of pilot studies: recommendations for good practice. *Journal of Evaluation in Clinical Practice*, 10 (2): 307-312.

Research Fellow in Research Methods
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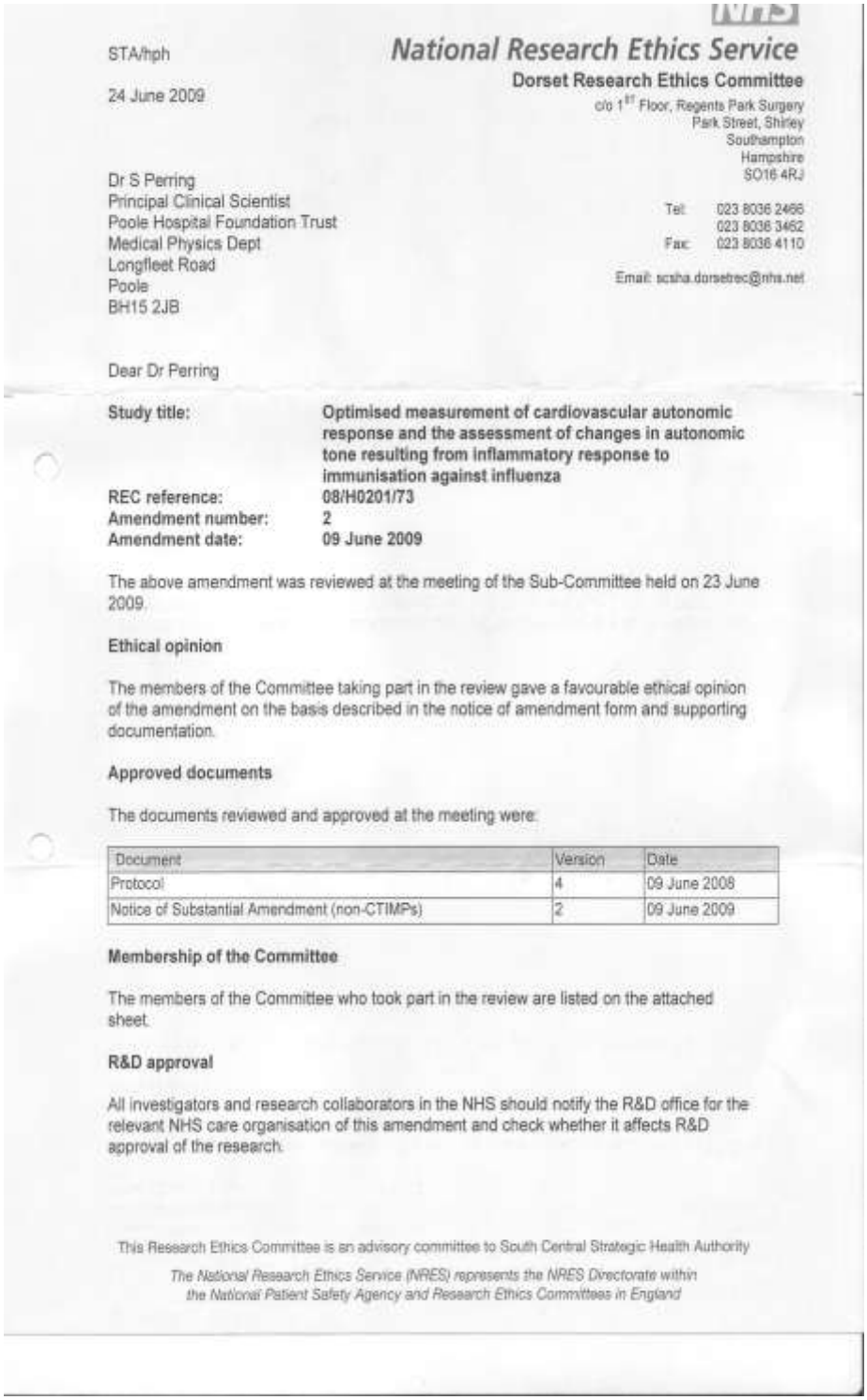
16.4 Ethics Documentation

16.4.1 Influenza Study Ethics Documentation



STA/hph

24 June 2009

National Research Ethics Service

Dorset Research Ethics Committee

c/o 1st Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
SO16 4RJ

Tel: 023 8036 2466
023 8036 3462
Fax: 023 8036 4110

Email: scsha.dorsetrec@nhs.net

Dr S Perring
Principal Clinical Scientist
Poole Hospital Foundation Trust
Medical Physics Dept
Longfleet Road
Poole
BH15 2JB

Dear Dr Perring

Study title: **Optimised measurement of cardiovascular autonomic response and the assessment of changes in autonomic tone resulting from inflammatory response to immunisation against influenza**

REC reference: **08/H0201/73**
Amendment number: **2**
Amendment date: **09 June 2009**

The above amendment was reviewed at the meeting of the Sub-Committee held on 23 June 2009.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	4	09 June 2008
Notice of Substantial Amendment (non-CTIMPs)	2	09 June 2009

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

08/H0201/73:	Please quote this number on all correspondence

Yours sincerely

Mrs Sharon Atwill
Acting Committee Co-ordinator

E-mail: scsha.dorsetrec@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Mrs Mary Burrows
Poole Hospital NHS Trust

16.4.2 Reflux Study Ethics Documentation

NHS

National Research Ethics Service
Southampton & South West Hampshire LREC (B)
1st Floor
Regents Park Surgery
Shirley
Southampton
Hampshire
SO16 4RJ

Telephone: 0118 918 0566
Facsimile: 0118 918 0559

14 June 2010

Dr Stephen Perring
Clinical Scientist
Poole Hospital
Longfleet Road
Medical Physics Dept
Poole
BH15 2JB

Dear Dr Perring

Study Title: **Proposal to evaluate the effects of Proton Pump Inhibitors on Measures of Autonomic Function in Reflux Patients with Either Non Erosive or Erosive Oesophagitis.**

REC reference number: **10/H0504/31**

Thank you for your letter of 10 May 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		15 March 2010
REC application		15 March 2010
Protocol	2	15 March 2010
Investigator CV	: Dr S Perring	
Participant Information Sheet	2	15 March 2010
Letter of invitation to participant	2	15 March 2010
Letter from Statistician		17 March 2010
Referees or other scientific critique report		18 September 2009
Questionnaire: GERD Impact Scale		
Unfavourable Opinion Letter		08 February 2010
Summary of ISRCTN		12 March 2010
Investigator CV: Mrs E Jones		
Participant Information Sheet	3	10 May 2010
Participant Consent Form	3	10 May 2010
Response to Request for Further Information		10 May 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0504/31	**Please quote this number on all correspondence**

Yours sincerely

Dr Helen McCarthy
Chair

Email: scsha.SWHRECB@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Emma Jones, Poole Hospital NHS Foundation Trust*

16.4.3 Lifestyle Study Ethics Documentation

NHS

National Research Ethics Service
Southampton & South West Hampshire REC (B)

Building L27
University of Reading
London Road
Reading
Berkshire
RG1 5AQ

Tel: 0118 9180566
Fax: 0118 9180559

06 December 2010

Dr Joe Begley
Consultant Medical Biochemist
Clinical Biochemistry Department
Royal Bournemouth Hospital
Castle Lane East
BH7 7DW

Dear Dr Begley

Study title:	**The Effect of Intensive 8-months Lifestyle Intervention on Hormonal Factors Regulating Food Intake in Obese Non-Diabetic Adult Population**
REC reference:	**09/H0504/61**
Amendment number:	**Amendment 3**
Amendment date:	**05 November 2010**

The above amendment was reviewed at the meeting of the Sub-Committee held on 24 November 2010.

Ethical opinion

The Sub-Committee request that the researchers change the last paragraph of the amendment description on the amendment form, from "These amendments don't alter the research design or methodology and doesn't affect the scientific value of the study." To read something like "The design has been changed to add scientific value to the study", as the research design has been altered.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
GP/Consultant Information Sheets	4	01 November 2010
Participant Consent Form	5	01 November 2010
Participant Information Sheet	5	01 November 2010
Protocol	5	01 November 2010
Notice of Substantial Amendment (non-CTIMPs)	Amendment	05 November 2010

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

	3	
Covering Letter		05 November 2010

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

09/H0504/61:	**Please quote this number on all correspondence**

Yours sincerely

Miss Kate Gardner
Committee Co-ordinator

E-mail: scsha.SWHRECB@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Robert Chapman, Royal Bournemouth Hospital NHS Trust*
Royal Bournemouth Hospital
Castle Lane East
Bournemouth
BH7 7DW

Southampton & South West Hampshire REC (B)

Attendance at Sub-Committee of the REC meeting on 24 November 2010

Name	*Profession*	*Capacity*
Professor Ron King	Retired Mathematician	None
Dr Helen McCarthy	Haematology Consultant	Expert

NHS
National Patient Safety Agency

National Research Ethics Service

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at { HYPERLINK http://eudract.emea.eu.int/document.html#guidance }.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.nres.npsa.nhs.uk/applicants/review/after/amendments.htm.

Details of Chief Investigator:

Name:	**Dr Joe Begley**
Address:	Bournemouth Diabetes and Endocrine Centre Royal Bournemouth Hospital Castle Lane East Bournemouth BH7 7DW
Telephone:	**01202704603**
Email:	**Joe.begley@rbch.nhs.uk**
Fax:	

Full title of study:	**THE EFFECT OF INTENSIVE 8-MONTHS LIFESTYLE INTERVENTION ON HORMONAL FACTORS REGULATING FOOD INTAKE IN OBESE NON- DIABETIC ADULT POPULATION WITH A FAMILY HISTORY OF DIABETES.**
Name of main REC:	Southampton & South West Hampshire Research Ethics Committee B
REC reference number:	**09/H0504/61**
Date study commenced:	**11/06/2010**
Protocol reference *(if applicable)*, current version and date:	Version 5 **dated 01/11/2010**
Amendment number and date:	Number 3, **dated 01/11/2010**

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the NRES Application Form

Yes *No*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes *No*

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

Please see Protocol Version 5 dated 01/11/2010 (attached).

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes *No*

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Please see Patient Information Sheet Version 5 dated 01/11/2010 and Consent Form Version 5 dated 01/11/2010, GP letter Version 4 dated 01/11/2010 (attached).

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes *No*

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study. In the case of a modified amendment, highlight the modifications that have been made.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

The changes proposed in this amendment are as follows:

As per the protocol (version 4, 30/03/2010), subjects will visit the Diabetes department for clinical assessment and fasting blood sampling at baseline, 4 months and 8 months. Each of these visits takes 30 min - 120 min (fasting blood test, 30min blood test after injecting 75 g glucose solution and additional 120 min blood test at the 1st visit only). In Patient Information Sheet, version 4, dated 30/03/2010, we have stated that blood test and assessment requirements per each session is 60 min. From our practice, we have learned that the time demands from patient are 120 min at each session. During each of the visits we would like to add an additional non invasive clinical investigation - Autonomic function

testing (AFT) to the protocol which will be performed during the 2 hour period in between blood sampling and assessment. We do not propose that this additional investigation will add any time to the overall length of time subjects are at the hospital.

The autonomic nervous system controls automated body functions including heart rate, blood pressure, digestion and metabolism. It is subdivided into the parasympathetic and the sympathetic pathways that work antagonistically to provide a fine degree of control over the target organs. A healthy heart does not work at a fixed rate but varies in milliseconds from moment to moment in response to physical, physiological, and environmental changes. Low heart rate variability reflects generally poor autonomic tone. Autonomic function testing (AFT) is a simple method of investigating heart rate variability. It is performed using a simple 3 lead ECG. We will also place a band around the chest to measure chest wall movement and place a sensor on a finger to measure pulse volume. We perform a series of simple measurements involving resting tidal breathing, blowing through a tube against resistance, 2 minute timed breathing at a set (6 breaths per minute) breathing rate, hand grip, lying-standing and we also photograph one eye while the patient is sat in the dark using infra-red goggles. None of the provocations will be painful and it should take no longer than 20 minutes.

We are proposing this additional assessment to give more information regarding the cardiovascular autonomic health of the subjects taking part in the study. For a long time it has been well established that the performance of the autonomic nervous system deteriorates with increasing age. We know that disease states such as diabetes have a significant impact on the autonomic nervous system. Previous research performed by Perring and Jones 2003 showed a difference in autonomic tone between age matched normal subjects and asymptomatic diabetics. We also know that research performed by Vassallo and Allen 1997 showed autonomic tone to be temporarily affected by acute pneumonia and with gradual improvement in the patient's health they documented a corresponding improvement in the autonomic tone. It was previously thought that loss of autonomic tone was irreversible. We now know that this is not always the case.

There is host of evidence linking autonomic function and weight loss, exercise and diabetes. In this project the additional autonomic function testing will allow us to establish whether we can detect changes in the autonomic tone between the baseline visit and the four and eight month follow up. In those subjects where significant weight loss has occurred and a sustained level of increased physical activity has been achieved we may see a change in autonomic tone which correlates well to the results of the blood samples. The autonomic nervous system is a physiologically interesting system to evaluate in the association between diabetes and physical activity. One motivation for such study is the responsiveness of the autonomic nervous system to lifestyle changes. The positive impact of physical activity and weight loss on autonomic function may prove to be a key benefit in the prevention of diabetes.

~~These amendments don't alter the research design or methodology and doesn't affect the scientific value of the study.~~ The design has been changed to add scientific value to the study. No addititional scientific critique for these amendments has been obtained.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

List of enclosed documents

Document	*Version*	*Date*
Protocol	5	01/11/2010
Patient Information Sheet	5	01/11/2010
Consent Form	5	01/11/2010
GP letter	4	01/11/2010
Covering letter		05/11/2010

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: .....

Date of submission: .....