The University Of Sheffield.

The Physiological and Psychological Effects of Electrical Vagus Nerve Stimulation in Patients with Refractory Epilepsy

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“Wouldn’t it be great if we could treat diseases not with drugs, but with devices.”

Harvey Leung 2016

I. Abstract

The vagus nerve is the longest cranial nerve in the body and has innervations and influence on many organs, yet the mechanisms that mediate these effects are still to be fully understood. Electrical stimulation of the vagus nerve has been used to treat refractory epilepsy for over 30 years despite an incomplete understanding of how it produces anti-epileptic effects. More recently, vagus nerve stimulation (VNS) has gained huge attention after the discovery that it may also have therapeutic benefit in mood and inflammatory disorders. This thesis explored the possible mechanisms that mediate the beneficial effects of VNS with a particular focus on the immune system in patients with refractory epilepsy. We also explored other potential therapeutic effects of VNS on anxiety, fatigue and perceived stress as well as heart rate variation As a non-invasive biomarker for the associated beneficial effects.

By 6 months post-VNS implantation, a trend in reduced perceived stress was observed and was independent of changes in seizure frequency, seizure severity, mood, age, and gender.

In heart rate variation studies, a significant decrease in high frequency relative power was seen at 6 months after VNS implantation, suggesting a reduction in sympathetic tone. This however did not correlate with other measures tested in the thesis.

Flow cytometry analysis did not identify a trend in increasing Treg frequency following VNS treatment. However, functional analysis suggests a reduction in Treg function in response to anti-CD3 antibody which mimics antigen-presenting cell mediated expansion of T cells. Finally, cytokine array assays identified a

significant reduction in the expression of BDNF, FGF-7, FGF-9, IL-1α, IL-1β, and MDC from plasma 3 months following VNS implantation.

In summary, this thesis has identified novel findings which open additional avenues for exploring the therapeutic potential of VNS to treat stress disorders and its effects on Tregs for anti-inflammatory effects.

II. Acknowledgements

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VI. List of Abbreviations

Abbreviation	Full name
3M	3 month
6M	6 month
α7nAChR	Alpha 7 subunit of nicotinic acetylcholine receptors
AC6	Adenylate Cyclase 6
ACh	Acetylcholine
AED	Anti-epileptic drugs
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
BDI	Beck Depression Inventory
BDNF	Brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CCR4	C-C chemokine receptor type 4
CD	Cluster of Differentiation
CG	Celiac Ganglion
ChAT	Cholinergic T cell
CIA	Collagen induced arthritis
CLP	Caecal ligation and puncture
CNS	Central Nervous System
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CSI	Cardiac sympathetic index
CVI	Cardiac vagal index
DMX	Dorsal motor nucleus of the vagus
ECG	Electrocardiogram
EEG	Electroencephalogram
FAS	Fatigue Assessment Scale
FDA	Food and Drug Administration
FGF-7	Fibroblast growth factor 7
FGF-9	Fibroblast growth factor 9
fMRI	Functional Magnetic Resonance Imaging
Foxp3	Forkhead box protein 3
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
GAD-7	General Anxiety Disorder assessment
GI	Gastrointestinal

Abbreviation	Full name
Ham-A	Hamilton Rating Scale for Anxiety
Ham-D	Hamilton Rating Scale for Depression
HF	High frequency
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish Peroxidase
HRV	Heart rate variation
ICAM1	Intercellular Adhesion Molecule 1
ICC	Immunocytochemistry
IFN-γ	Interferon gamma
IKK	IκB kinase
IL	Interleukin
ISR	Independent Scientific Review
IκBα	Inhibitor of κB
LF	Low frequency
LPS	Lipopolysaccharides
LSM	Lymphocyte separation medium
LSSS	Liverpool Seizure Severity Scale
MDC	Macrophage derived chemokine
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NDDI-E	Neurological Disorders Depression Inventory for Epilepsy
NF-κB	Transcription of nuclear factor kappa-light-chain-enhancer of activated B cells
NHS3	National Hospital Seizure Severity Scale
NK	Natural killer
NRES	National Research Ethics Service
NTS	Nucleus tractus solitarius
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pCREB	phosphorylated CREB
PS	Pre-stimulation
PSI	Parental Stress Index
PSNS	Parasympathetic nervous system
PSS	Perceived Stress Scale
PTSD	Post-traumatic stress disorder
RA	Rheumatoid Arthritis
RHH	Royal Hallamshire Hospital
RMS	Root mean squared
RMSSD	Root mean square of successive differences
SDNN	Standard deviation of RR intervals
SEB	Staphylococcal enterotoxin B
SNS	Sympathetic nervous system

Abbreviation	Full name
SSC-A	Side Scatter Area
SSQ	seizure severity questionnaire
STH	Sheffield Teaching Hospitals
TGF-β	Transforming growth factor beta
TLR	Toll-like receptors
tMCAO	Transient middle cerebral artery occlusion
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF-α	Tumour necrosis factor alpha
t-PA	Tissue plasminogen activator
tVNS	Transcutaneous VNS
UV	Ultraviolet
VLF	Very low frequency
VNS	Vagus nerve stimulation

Chapter 1. Introduction

1.1. The vagus nerve

1.1.1. Anatomy

The human body regulates many physiological processes through a plethora of different mechanisms to achieve homeostasis. This regulation is predominantly coordinated by the autonomic nervous system (ANS). Sub-divisions of the ANS include the sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS) which often act with opposing effects to regulate many physiological functions (Mathias and Low, 2010). Traditionally, sympathetic output is associated with the “fight or flight” response whereas parasympathetic output is associated with the “rest and digest” response (Cannon, 1929, 1932). Both the SNS and PSNS comprise of pre-and post-ganglionic fibres but differ anatomically (**Figure 1-1**). Sympathetic ganglia are located peripherally at the sympathetic trunk, whereas parasympathetic ganglia are located centrally at the midbrain and sacral regions of the spinal cord (Mathias and Low, 2010). The effects of ANS are mediated by the release of noradrenaline (NA) from the SNS and acetylcholine (ACh) from the PSNS at postganglionic synapses.

The vagus nerve is the major nerve of the PSNS and is the longest of the 12 cranial nerves. It is composed of 80% afferent fibres and 20% efferent fibres (Foley and DuBois, 1937; George *et al.*, 2000) with innervations to many organs within the body and extensive polysynaptic projections to various regions of the brain. The left and right vagus nerves emerge from the medulla in the midbrain and extend down the neck to the trunk of the body. The right vagus nerve contains efferents that partially supply the oesophagus and the heart whilst the left vagus

nerve contains efferents which partially supply the oesophagus and lungs. Both vagus nerves supply the stomach, duodenum, liver, pancreas, and small intestines (Berthoud and Neuhuber, 2000). Most vagal afferents project bilaterally to the nucleus tractus solitarius (NTS) which connects to many other areas of the brain including the thalamus, hypothalamus, the limbic system, and cerebral cortex (**Figure 1-2**) (Henry, 2002; Cheyuo *et al.*, 2011). Translating the complex anatomy of the vagus nerve to determine its function is challenging. Despite these challenges, some functions of the vagus nerve have been uncovered but many remain to be determined.

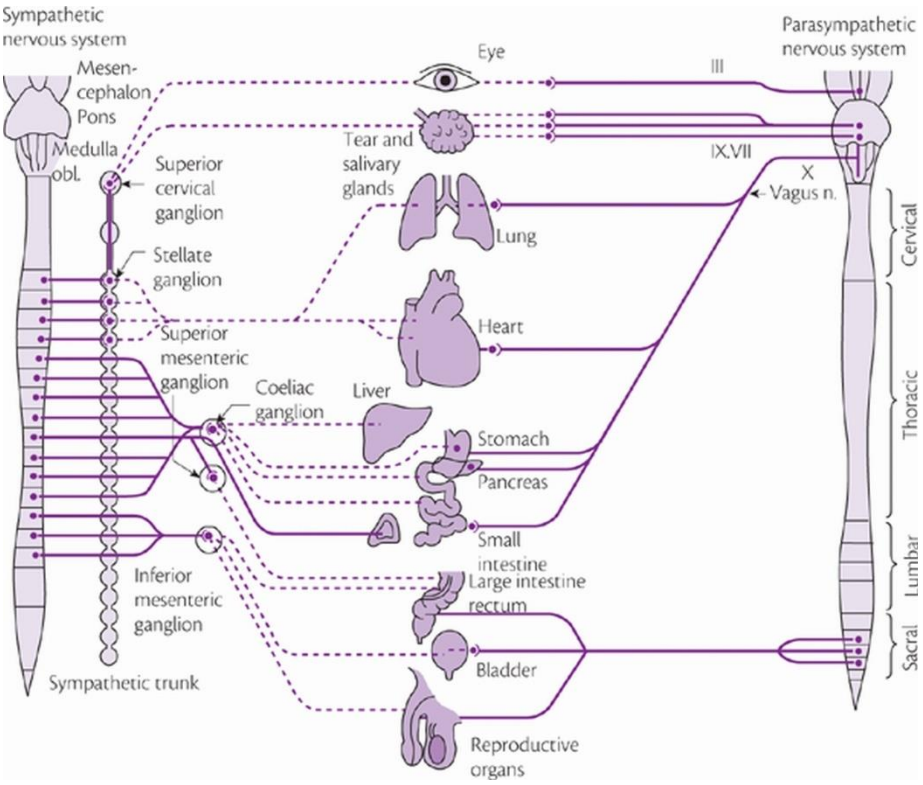

Figure 1-1 Sympathetic and parasympathetic input to organs
The ANS innervates many organs within the body, often with the SNS and PSNS acting on the same organ with opposing effects. mage taken from (Mathias and Low, 2010).

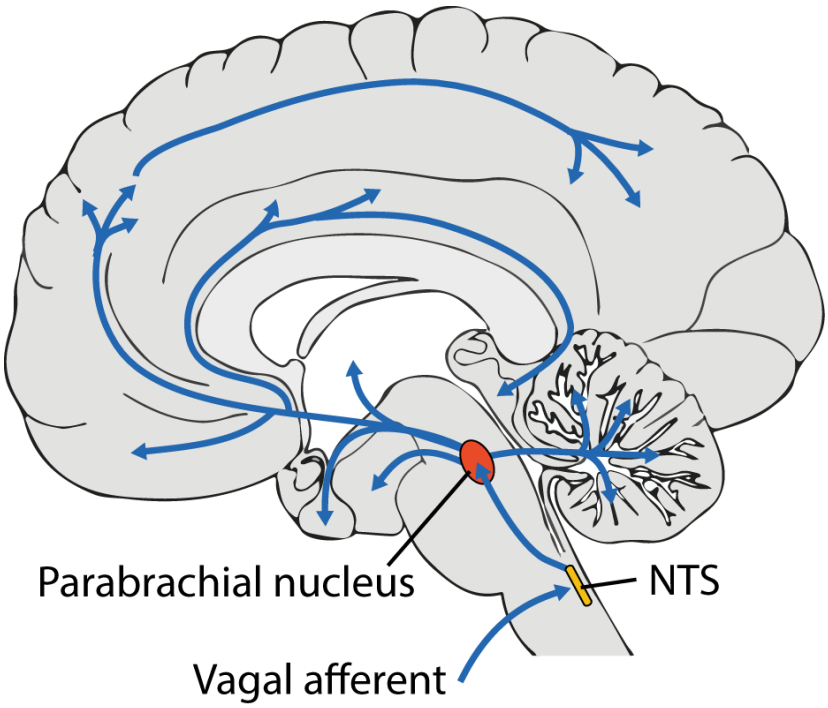

Figure 1-2 Polysynaptic central projection of vagal afferents via the NTS
Vagal afferents can activate many areas throughout the brain via the NTS and parabrachial nucleus. These areas include the hypothalamus, cerebellum, cerebral cortex, and the limbic system. For a full review on areas activated by vagal afferents, see review by Henry 2002. Vagal afferent pathway (blue arrow).

1.1.2. Function

The vagus nerve innervates many different organs and isolating the many functions of this nerve is experimentally difficult since it contains both afferent and efferent fibres.. Various methods such as vagotomy and stimulation of the vagus nerve through electrical or pharmacological means have been developed to investigate the function of the vagus nerve in animals. Some of these functions have been reviewed and are summarised in **Table 1-1** below.

Table 1-1. Functions of the vagus nerve

Reference/Review	Organ	Function
(Olshansky *et al.*, 2008)	Heart	Slows heart rate
(Canning, Mori and Mazzone, 2006)	Lungs	Cough reflex
(Mittal, 2016)	Oesophagus	Modulation of peristalsis
(Travagli *et al.*, 2006)	Stomach	Regulation of gastric motility
(Berthoud, 2008)	Liver	Sense glucose levels Suppress hepatic glucose production
(Babic *et al.*, 2012)	Pancreas	Regulatory (excitatory and inhibitory) roles in the secretion of insulin and pancreatic exocrine
(Matteoli and Boeckxstaens, 2013)	Small intestines	Modulating intestinal microenvironment for intestinal immune homeostasis

Heart

One of the first identified functions of the vagus nerve is its ability to slow heart rate. ACh released by vagal efferents activate muscarinic type 2 receptors in the heart and slows heart rate (Lund *et al.*, 1992; Olshansky *et al.*, 2008).

Lungs

The cough reflex can be evoked through many mechanisms including the mechanical and chemical activation of vagal afferent subtypes. Each subtype of vagal afferent innervating the lungs have differing anatomy, physiological properties, and responses to various pharmacological ligands such as bradykinin, adenosine triphosphate (ATP), serotonin, and capsaicin (Canning, Mori and Mazzone, 2006). Despite the identification of vagal afferent subtypes which mediate the cough response, its role and mechanisms in the central nervous system (CNS) is complex and less defined (Canning, Mori and Mazzone, 2006).

Oesophagus

Most of the gastrointestinal tract is innervated by the vagus nerve. In the oesophagus, vagal efferents exert control over different patterns of muscle contraction required for peristalsis (Mittal, 2016).

Stomach

Vagal afferents innervating the stomach provide sensory input to the NTS and modulate gastric function in a reflexive manner via vagal efferents from the dorsal motor nucleus of the vagus (DMX). Activation of different regions of DMX can produce either gastric contraction (rostral/medial) or relaxation (caudalmedial/rostrolateral) by modulating both inhibitory and excitatory pathways in gastric motility (Travagli *et al.*, 2006).

Liver

Vagal afferents innervating the liver contain glucose sensors and protect against the detrimental effects induced by excessive energy storage in the liver (Berthoud, 2008). Vagal efferents may also suppress hepatic glucose production and may modulate the development of obesity (Berthoud, 2008).

Pancreas

The vagus nerve modulates both the exocrine and endocrine functions of the pancreas through the activation of metabotropic glutamate receptors in the brainstem vagal neurocircuits. This decreases both excitatory and inhibitory synaptic transmission to DMX neurones projecting to the pancreas and reduces plasma insulin secretion while increasing pancreas exocrine secretion (Babic *et al.*, 2012).

Small intestines

Vagal innervation to the small intestines provide a vital role in modulating immune homeostasis in a reflexive manner (**Section 1.2.4.2**). Stimulation of the vagus nerve activates neurones of the myenteric plexus and release of ACh to deactivate resident macrophages in the intestines (Matteoli and Boeckxstaens, 2013).

In summary, it is evident that the vagus nerve exerts control over many different functions within the body. Despite having identified these functions, ongoing studies are exploring its other functions and how manipulation of the vagus nerve could be used therapeutically. Over the past 30 years, electrically stimulating the vagus nerve has shown anti-epileptic and anti-depressive effects, with growing interest in exploring its therapeutic potential for other conditions.

1.2. Epilepsy and the history of vagus nerve stimulation

1.2.1. Epilepsy

Epilepsy is a common neurological disorder affecting over 70 million people worldwide, encompassing a very diverse range of causes (Ngugi *et al.*, 2010) and manifestations (**Figure 1-3**) (Jette *et al.*, 2015). In epilepsy, uncontrolled electrical activity of the brain causes dysfunction of various regions of the cortex, resulting in a seizure. Depending on the location and characteristic of the abnormal electrical activity, different symptoms may arise. For example, focal/partial seizures may affect a localised part of the brain in a single hemisphere whereas generalised seizures often spread within and even between hemispheres of the brain, leading to complex responses. In many cases, seizures can be treated with anti-epileptic drugs (AEDs) or surgery. However, for patients who do not respond to AEDs or where surgical intervention is not possible, other treatments such as vagus nerve stimulation (VNS) may be offered.

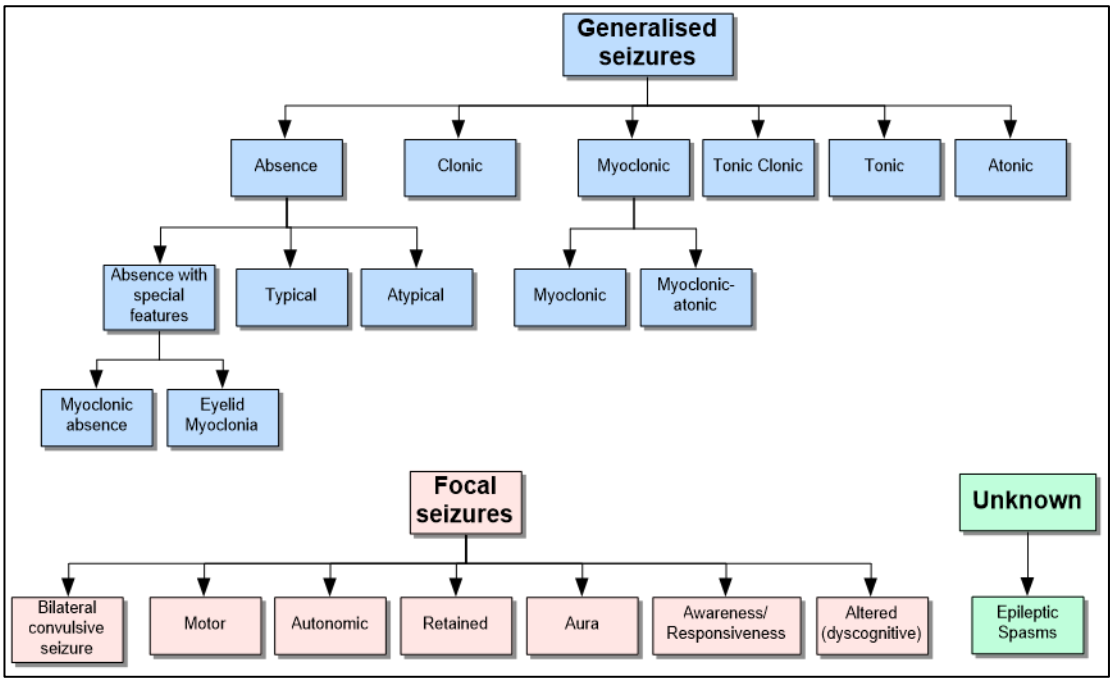

Figure 1-3 International League Against Epilepsy (ILAE) classification of seizures and epilepsies
Figure adapted from *Jette et al.* 2015.

1.2.2. Vagus nerve stimulation

Corning first described mechanical carotid artery compression for limiting blood flow to the brain in the 19th century and was shown to be an effective abortive treatment for epilepsy (Corning, 1882). This was later adapted and used in conjunction with transcutaneous electrical nerve stimulation at the neck to activate vagal cardiac afferents, hypothesising that this would reduce cardiac output and further limit blood flow to the brain to reduce seizure frequency (Corning, 1884). An implantable form of VNS was later developed in animal models of seizures in dogs (Zabara, 1992) and monkeys (Lockard, Congdon and DuCharme, 1990) which were found to suppress the onset of induced seizures.

These findings led to a clinical trial where 5 patients with refractory epilepsy were implanted with the VNS device and monitored for 24 weeks. Stimulation (intensity:1 mA, frequency: 50 Hz, pulse width: 250 μs, continuous stimulation pattern of on-time: 60 sec and off-time: 60 min) was applied only to the left vagus nerve to avoid activation of cardiac vagal fibres to induce bradycardia. This intervention was found to be well tolerated with only mild side effects; such as a tingling sensation or hoarseness whilst speaking. No noticeable changes in heart rate or electrocardiogram (ECG) morphology were reported (Uthman *et al.*, 1990). Subsequent functional magnetic resonance imaging (fMRI) studies in patients revealed that VNS alters blood flow to various areas of the brain and found that increased activity to the thalamus correlated with decreased seizures (Henry *et al.*, 1999; Narayanan *et al.*, 2002; Liu *et al.*, 2003). Since obtaining Food and Drug Administration (FDA) approval in 1997, VNS has been used to treat refractory epilepsy in over 100,000 patients worldwide (Terry, 2009).

Currently, VNS devices are manufactured by Livanova (formerly Cyberonics) and requires surgery to implant electrode cuffs around the left cervical vagus nerve which is then connected to a generator placed in a subcutaneous pocket (**Figure 1-4**). The patient will need to undergo surgery again to replace the device battery approximately every 10 years. Once implanted, the device can be remotely configured using a "magic wand" placed over the generator via short-ranged radio frequencies.

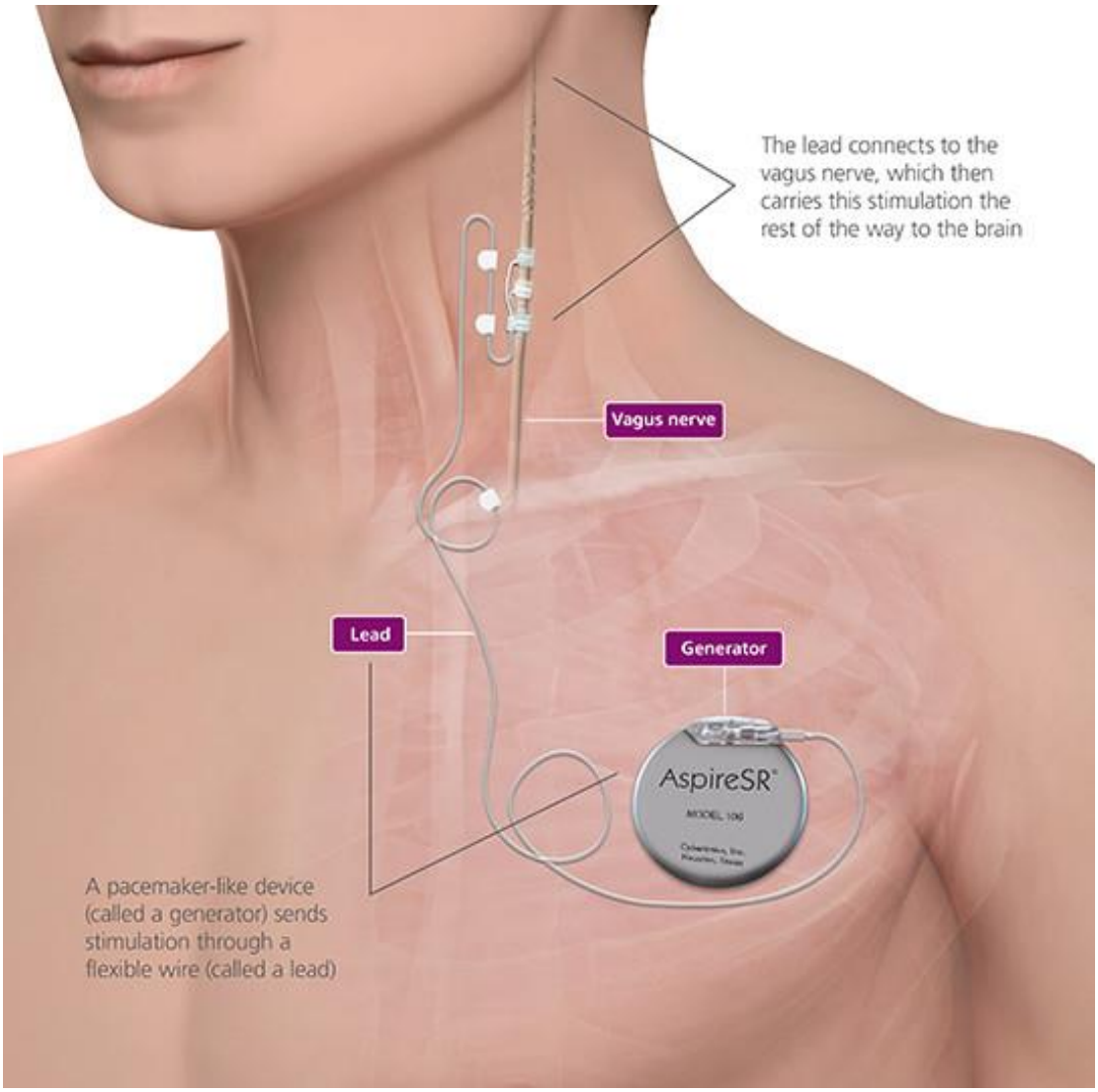

Figure 1-4 Placement of VNS
The current device is manufactured by Livanova. Figure taken from http://en.eu.livanova.cyberonics.com/vns-therapy/how-vns-therapy-works

Table 1-2 Livanova VNS device parameter settings available by manufacturer

Stimulation Parameters	Available Parameter Settings
Output current	0-2.0 mA in 0.125 mA steps 2-3.5 mA in 0.25 mA steps
Signal frequency	1, 2, 5, 10, 15, 20, 25, 30 Hz
Pulse width	130, 250, 500, 750, 1000 μsec
Signal ON time	7, 14, 21, 30, 60 sec
Signal OFF time	0.2, 0.3, 0.5, 0.8, 1.1, 1.8, 3 min and 5 to 180 min (5 to 60 in 5 min steps; 60 to 180 min in 30 min steps)
Example of settings for a VNS device. Table 14 in 5.3.1 (pg 105) of VNS Therapy® System Physician's Manual (US) for Model 106	

1.2.3. Stimulation settings and efficacy

In a meta-analysis of VNS studies comparing different doses of VNS stimulation, stimulation with intensities between 1.5-3 mA and frequencies between 20-30 Hz resulted in 31-39% of patients achieving a >50% reduction in seizure frequency. Stimulation with intensities and frequencies lower than this resulted in only 13-19% patients achieving a >50% reduction in seizure frequency (Cukiert, 2015). The studies reviewed by Cukiert compared 12 weeks baseline measurements against measurements up to 3 months following VNS treatment in children and adults (Holder, Wernicke and Tarver, 1992; Ben-Menachem *et al.*, 1994; Ramsay *et al.*, 1994; Handforth *et al.*, 1998) or adults only (The Vagus Nerve Stimulation Study Group, 1995). However, some studies investigated the efficacy of VNS for up to 9 months in children (Klinkenberg *et al.*, 2012, 2013) and up to 18 months in children and adults (Handforth *et al.*, 1998) following VNS treatment.

Side effects were usually mild with patients experiencing hoarseness or voice alteration. The occurrence of these were dose dependent with up to 67% of patients receiving higher stimulation intensities (>1.5 mA and 20-30 Hz) compared up to 30% of patients receiving lower stimulation intensities (<1.5 mA

and 1-2 Hz) (Cukiert, 2015). Cukiert concluded that patients with refractory epilepsy above the age of 12 may benefit from VNS treatment, and although higher doses of stimulation results in increased efficacy, so does the likelihood of experiencing side effects (Cukiert, 2015).

Due to the heterogeneity in the causes of seizures, there are currently no published studies with recommendations for specific stimulation parameters to use (Morris *et al.*, 2013). Therefore, settings are often adjusted according to individual tolerance (**Table 1-2**) and time taken to achieve ramp up stimulation settings may be limited to frequency of hospital visits to adjust settings.

Although the mechanisms for anti-epileptic effects by VNS are yet to be fully understood, fMRI findings studies suggests this may involve increased thalamic activity, as previously mentioned (Henry *et al.*, 1999; Narayanan *et al.*, 2002; Liu *et al.*, 2003). Furthermore, electroencephalogram (EEG) recordings in patients with refractory epilepsy have shown that responders to VNS (n=10) who achieved >50% seizure reduction generally have a significantly lower level of synchronisation in alpha and delta bands compared to non-responders (n=9) ($p<0.0001$) (Bodin *et al.*, 2015). This supports the hypothesis that VNS decreases synchronisation of EEG band waves to produce an anti-epileptic effect (Jaseja, 2010).

Despite an incomplete understanding of the anti-epileptic effects of VNS, other beneficial effects have been reported for diseases such as mood disorders (**Section 1.3**) and inflammatory disorders (**Section 1.5.3**). Furthermore, there is evidence that VNS produces cardiac effects via the hypothalamic-pituitary-adrenal (HPA) axis, presenting as changes in heart rate variation (HRV) (**Section 1.4**).

1.3. Vagus Nerve Stimulation in Mood Disorders

1.3.1. Depression

Depression is a major problem in the world affecting over 300 million people and is a leading cause of disability worldwide (World Health Organization, 2017). Despite many psychotherapeutic and pharmacological forms of treatment available, up to 20% of patients do not respond or become resistant to treatment (Burrows, Norman and Judd, 1994). Brain stimulation therapies such as electroconvulsive therapy and magnetic seizure induction have been used to treat depression with high efficacy. However, these treatments are often associated with adverse cognitive effects (Akhtar *et al.*, 2016) and therefore other forms of neurostimulation therapies for depression have been explored, including VNS.

Soon after VNS was granted FDA approval for treating refractory epilepsy, anecdotal clinical observations of improvements in mood emerged (Rawlins, 1997). This led to a pilot prospective trial which monitored changes in depression and anxiety in adult refractory epilepsy patients following VNS (Harden *et al.*, 2000). Following 3 months of VNS treatment, improvements in depression were found when compared to baseline ($P<0.05$) whilst no changes in anxiety were observed (Harden *et al.*, 2000). Due to the possible anti-depressive effects of VNS, FDA approval was granted for VNS to treat depression in 2005. Subsequently, this decision was met with criticism due to an inadequate number and poor quality of trials that supported the FDA decision to approve, as well as an “insufficient level of efficacy” to treat depression (O’Reardon, Cristancho and Peshek, 2006).

Many trials assessing the effects of VNS on mood have since taken place, and a systematic review on 18 studies observed the safety and efficacy of VNS on mood disorders deemed it safe (Daban *et al.*, 2008). However, they also noted that although anti-depressive effects were observed, additional blinded and randomised trials will be required to assess its efficacy for treating refractory depression (Daban *et al.*, 2008).

Dysfunction of serotonergic and noradrenergic systems are involved in the pathophysiology of depression and pharmacological treatments for depression aim to restore these systems (Millan, 2004). VNS may exert anti-depressive effects through similar pathways to increase NA and serotonin signalling. For example, studies on VNS in rats have shown elevated extracellular levels of NA in the cortex and hippocampus during the stimulatory phase (Roosevelt *et al.*, 2006), and increased activity in serotonergic and noradrenergic brain stem nuclei (Dorr and Debonnel, 2006).

Neuro-imaging studies have identified increased and decreased activities in various regions of the brain in treatment-resistant depression patients treated with VNS (Nahas *et al.*, 2007; Kosel *et al.*, 2011; Conway *et al.*, 2013). However, the mechanism underlying the anti-depressive effect of VNS remain unknown. Many factors including the complexity of the disease, heterogeneity of the patient population, influence from multiple medications, and conflicting study findings have posed difficulties in deciphering the anti-depressive mechanism of VNS.

1.3.2. Anxiety

Anxiety is a common feature in many mood disorders. Despite the wide range of pharmacological and psychological treatments available, many patients remain refractory to treatment and as such, other forms of treatment are needed (Kar

and Sarkar, 2016). Over the last century, various neuro-stimulation techniques have been developed for treating anxiety disorders (Kar and Sarkar, 2016). More recently however, there have been some indications from animal studies that VNS could treat anxiety.

Conditioned fear response experiments in rats have demonstrated that VNS treatment promotes plasticity between the infralimbic medial prefrontal cortex and the basolateral complex of the amygdala to enhance extinction of the conditioned fear response (Peña *et al.*, 2014). The authors of this study suggest its therapeutic potential for enhancing treatment of post-traumatic stress disorder (PTSD)-associated anxiety. However, there were no observed changes in anxiety in patients with major depression at 6 and 12 months post-VNS implantation according to the Beck Anxiety Inventory (Cristancho *et al.*, 2011), and 3-months post-VNS implantation in refractory epilepsy patients according to the Hamilton Rating Scale for Anxiety (Harden *et al.*, 2000). It remains unclear if VNS has a beneficial effect on anxiety in mood disorders due to the wide range of causes of anxiety.

1.3.3. Fatigue

Fatigue is the lack of physical and/or mental energy perceived by an individual which interferes with everyday activities (Staub and Bogousslavsky, 2001). In particular, post-stroke fatigue is a common symptom with over 50% of stroke survivors (Choi-Kwon *et al.*, 2005; Schepers *et al.*, 2006) and fatigue is 3 times more common in stroke survivors than in age-matched controls (van der Werf *et al.*, 2001). There are no evidence-based and effective treatments currently available for post-stroke fatigue (Acciarresi, Bogousslavsky and Paciaroni, 2014). Although some pharmacological, physical, and psychological treatments are

available, these tend to only alleviate the symptoms (Mead, Bernhardt and Kwakkel, 2012), thus there is a major need for effective post-stroke fatigue treatments.

Although fatigue was reported as an adverse event in 3/14 fibromyalgia patients following VNS treatment (Lange *et al.*, 2011), meta-analysis of VNS studies concluded that fatigue was not associated with VNS treatment (Panebianco *et al.*, 2015). Much of the effects of VNS on fatigue is currently unknown, and to our knowledge, changes in fatigue following VNS have not been previously studied.

1.3.4. Stress

Perceived stress affects overall health (Keller *et al.*, 2012) and is a contributor to diseases such as depression (Kendler, Karkowski and Prescott, 1999; Hammen, 2005), stroke (Booth *et al.*, 2015), and inflammatory gastrointestinal diseases (Targownik *et al.*, 2015). Despite the vast knowledge of the effects and mechanisms of stress, the effects of VNS on perceived stress are currently unknown, and no studies to our knowledge have investigated this.

Since vagal afferents have polysynaptic projections to the hypothalamus via the NTS (Henry, 2002), VNS may affect the HPA axis through this mechanism to produce observable cardiac effects. The HPA axis is a neuroendocrine system known for its role in the stress response and regulation of circadian rhythm through the secretion of cortisol (Spencer and Deak, 2016). Interestingly, VNS reduces serum corticosterone levels in rats (V. De Herdt *et al.*, 2009; Thrivikraman *et al.*, 2013) and reduces elevated serum levels of cortisol in depressed patients to levels typically observed in healthy volunteers (O'Keane *et al.*, 2005). In mood disorders including depression, the HPA axis is dysregulated and increased levels of cortisol may be released (Spencer and Deak, 2016).

Therefore, one method for studying this interaction could be monitoring changes in HRV.

1.4. Effects of Vagus Nerve Stimulation on Heart Rate Variation

HRV is the variation in the time between heart beats and may be calculated using a variety of methods. The Task Force of the European Society of Cardiology have described these time-domain, frequency-domain, and non-linear measurements (**Appendix Table 1**) (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996).

Time-domain measurements generally refer to measurements relating to the time between heart beats and are the simplest to calculate with the ability to calculate HRV with geometric methods. Frequency-domain measurements of HRV use power spectral density analysis to separate a complex signal into very low (VLF), low (LF, 0.04-0.15 Hz), and high frequency (HF, 0.15-0.4 Hz) bands. While LF is an indicator of parasympathetic or vagal tone, HF is more complex and largely considered as an indicator for sympathetic activity. The LF/HF ratio is often taken as an indicator of autonomic function. Lastly, HRV may be affected by a combination of complex interactions and non-linear analysis have been suggested as a potential tool for assessing elements of this (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996).

Although measures of HRV may not be directly involved with certain diseases, it has the potential to be used as a non-invasive surrogate biomarker for diagnosing or predicting disease and therapeutic outcome (Taralov, Terziyski and Kostianev, 2015).

Patients with epilepsy, mood disorders, and inflammatory gastrointestinal diseases display a reduced HRV (Pellissier *et al.*, 2010; Ponnusamy, Marques

and Reuber, 2012; Sgoifo *et al.*, 2015). Although VNS electrodes are implanted onto the left vagus nerve to avoid bradycardia as mentioned in **Section 1.2.1**, patients with major depression display a significantly increased root mean square of successive differences (RMSSD, time-domain measure of HRV) during VNS stimulation when compared to baseline and stimulation-free intervals (Sperling *et al.*, 2010). Also, patients with refractory epilepsy display trends in decreasing in HF power (frequency-domain measure of HRV) following VNS treatment (Setty *et al.*, 1998; Galli *et al.*, 2003).

Interestingly, a clinical study by Liu *et al.* identified pre-operative cut-off values for HF power to predict the outcome of VNS treatment. They found that responders with a >50% reduction in seizure frequency following VNS have similar HF power prior to VNS implantation compared to healthy volunteers. However, non-responders to VNS treatment have a 2-fold reduction in HF power prior to VNS implantation which suggests ANS dysfunction is present in these patients (Liu *et al.*, 2017).

Furthermore, ANS dysfunction was identified in patients with inflammatory gastrointestinal diseases as indicated by blunted vagal tone (Pellissier *et al.*, 2010). A 6-month pilot study by Bonaz *et al.* tested safety and feasibility of VNS in Crohn's disease patients and found that VNS restored ANS balance (according to LF/HF ratio, frequency-domain measure of HRV) in patients which responded to treatment and achieved clinical and endoscopic remission (Bonaz *et al.*, 2016).

While HRV has been investigated as a predictive indicator of response to VNS treatment in refractory epilepsy patients, HRV biomarkers of treatment efficacy with VNS have not been identified for other diseases.

1.5. Vagus Nerve Stimulation and Immune System

1.5.1. Background to the Immune System

The immune system is a complex system which orchestrates the activity of many different immune cell types to protect the host against pathogens and facilitate tissue repair following an injury (Chaplin, 2010). This response requires the ability for the immune system to differentiate between foreign and self-tissue in a mechanism known as self-tolerance (Sakaguchi, 2004). This is necessary for avoiding damage to the host whilst combating pathogens, and failure of this may lead to inflammatory or autoimmune conditions.

The immune system is formed by two different systems: the innate immune system and the adaptive immune system. The innate immune system is considered the first line of defence against pathogens. This includes: physical barriers (e.g. skin and mucous), and chemical barriers (e.g. soluble substances with acidic pH and antimicrobial properties on physical barrier surfaces). Should pathogens penetrate these barriers, cellular innate immune responses are triggered within minutes (Turvey and Broide, 2010).

Toll-like receptors (TLR) are receptors expressed by cells of the immune system (such as natural killer (NK) cells, macrophages and dendritic cells) bind to a variety of pathogenic molecular patterns to activate the innate immune response (Medzhitov and Janeway, 2002). Circulating NK cells detect pathogens and abnormal cells via cell-cell contact and releases cytolytic granules to destroy them (Della Chiesa *et al.*, 2014). Circulating monocytes mature and differentiate into resident macrophages or dendritic cells (Geissmann *et al.*, 2010). Upon recognition of foreign antigens, resident macrophages and dendritic cells at the site of infection will phagocytose and degrade pathogens, decorating their cell

surface with foreign antigen fragments. Such antigen-presenting cells (APCs) activate cells of the adaptive immune system to mount a stronger and targeted immune response (Banchereau and Steinman, 1998; Owen, Punt and Stranford, 2013).

While the innate immune response eliminates common pathogens within hours, it relies on the detection of common foreign antigens by toll-like receptors and may not recognise subtly different foreign antigens (Owen, Punt and Stranford, 2013). The adaptive immune response on the other hand can detect such subtle differences and mount a highly specific targeted response through clonal selection (Burnet, 1959). During the maturation of B cells in bone marrow and T cells in the thymus, each cell expresses a randomly generated receptor against an antigen (Burnet, 1959). B cells express the receptor on their cell surface and produces antibodies against antigens to aid the destruction of pathogens by other immune cells (LeBien and Tedder, 2008). T cells express receptors against the antigen on their cell surface only and are classed into two major T cell types – T helper cells (usually expressing cluster of differentiation 4,CD4) which help regulate the immune response and T cytotoxic cells (usually expressing CD8) which eliminate foreign, infected or abnormal cells (Bevan, 2004; Laidlaw, Craft and Kaech, 2016). Upon encountering an antigen, specific clones of B cells or T cells expressing the complementary receptor will proliferate. This creates a greater population of cells able to recognise the antigen to destroy pathogens, infected, and abnormal cells which may take up to 8 days to eliminate the pathogen (Burnet, 1959; Owen, Punt and Stranford, 2013).

1.5.2. Inflammation

Inflammation is a response to infection and tissue injury characterised by heat, pain, redness, swelling, and loss of function. Medzhitov has previously reviewed its origin and physiological roles (Medzhitov, 2008). Briefly, endogenous or exogenous triggers, chemokines and cytokines are released and selectively recruit leukocytes and plasma proteins into the site of infection or injury to destroy pathogens and infected or damaged cells. Once these have been eliminated through a controlled immune response, apoptotic and necrotic cells are cleared and the site undergoes a resolution and repair phase (Medzhitov, 2008).

Resolution of inflammation is traditionally mediated by neutrophil and macrophage associated mechanisms including: 1. chemokine depletion through chemokine truncation and sequestration, 2. neutrophil apoptosis, 3. apoptotic neutrophil clearance, 4. switching macrophage phenotype from pro-inflammatory to resolution phase macrophage (Ortega-Gómez, Perretti and Soehnlein, 2013). Additionally, regulatory T cells (Tregs) emerged as one of the key mediators in the resolution of inflammation.

Firstly, Tregs are a specialised subset of immunosuppressive CD4+, CD25+, and forkhead box protein 3 (foxp3)-positive T cells which mediate the resolution of the immune response (Tao *et al.*, 2017). This is achieved by the secretion of anti-inflammatory molecules including interleukin (IL)-10, transforming growth factor-beta (TGF-β), granzyme B, and perforin. Tregs may also resolve inflammation through cell to cell mediated mechanisms resulting in the apoptosis of myeloid antigen-presenting cells (such as macrophages and dendritic cells) and inhibit the function of T cells (Ortega-Gómez, Perretti and Soehnlein, 2013; Zeng *et al.*, 2015).

However, when the inflammatory response cannot be controlled, such is the case in systemic inflammation or autoimmune disorders, excessive or chronic inflammation leads to damage and deterioration of host tissues (Medzhitov, 2008). Pharmacological treatments are traditionally used to suppress the immune response for such conditions, but emerging evidence has shown the vagus nerve can modulate inflammation.

1.5.3. Anti-Inflammatory Response

In addition to the potential interaction between the vagus nerve and HPA axis (**Section1.4**), both may also interact with the immune system. In the past 30 years, common signalling molecules, receptors, and pathways have been identified in the complex interaction between the nervous, endocrine, and immune systems (Taub, 2008). In particular, stress causes the release of hormones via the activation of the HPA axis and reduces lymphocyte populations and function, thus leaving an impaired immune system (Webster Marketon and Glaser, 2008). Stress may also produce similar immunosuppressive effects to the immune system changes in sympathetic tone and producing changes at spleen and lymph nodes (Bellinger *et al.*, 2008). Interestingly, pharmacological activation of the CNS via the vagus nerve produces anti-inflammatory effects (Borovikova, Ivanova, Nardi, *et al.*, 2000).

CNI-1493 is a known potent anti-inflammatory agent, and Borovikova *et al.* hypothesised its anti-inflammatory effect is exerted through CNS mechanisms. They investigated this through intracerebroventricular (i.c.v.) injection of CNI-1493 and found significantly reduced swelling in a carrageenan-induced paw oedema model of inflammation (Borovikova, Ivanova, Nardi, *et al.*, 2000). Furthermore, vagotomy studies revealed that intact vagus nerves are required for the anti-inflammatory effects of CNI-1493 (Borovikova, Ivanova, Nardi, *et al.*, 2000). Lastly, electrical stimulation of efferent vagus nerves in vagotomised rats for 10 minutes before and after carrageenan-induced paw oedema were also able to significantly reduce inflammation (Borovikova, Ivanova, Nardi, *et al.*, 2000).

Although these studies showed that the vagus nerve was required to modulate inflammation, its underlying mechanisms remained unclear. Subsequent *in vitro*

studies by the same group revealed that activation of cholinergic receptors on macrophages were able to reduce the inflammatory response (Borovikova, Ivanova, Zhang, *et al.*, 2000). Here, human macrophage cultures from peripheral blood mononuclear cells (PBMCs) and pre-conditioned with ACh. This inhibited the release of inflammatory cytokines (tumour necrosis factor alpha (TNF-α), IL-1β, IL-6, and IL-18) in a dose-dependent manner following exposure to lipopolysaccharides (LPS) (Borovikova, Ivanova, Zhang, *et al.*, 2000). To determine if this anti-inflammatory effect is influenced by the efferent vagus nerve, bilateral cervical vagotomy was performed in rats and the distal end of the vagus nerve was electrically stimulated following administration of a lethal dose of LPS. Rats with vagotomy alone expressed higher levels of TNF-α in serum at 19 ng/ml than sham vagotomy at 14 ng/ml where vagus nerves were exposed but not severed ($p<0.05$ vs sham). However, electrical stimulation of the vagus nerve greatly reduced serum TNF-α levels to 4 ng/ml ($p<0.005$ vs sham) (Borovikova, Ivanova, Zhang, *et al.*, 2000).

1.5.3.1. The Anti-Inflammatory Reflex

Tracey hypothesised that this anti-inflammatory effect was caused by the activation of vagal afferents by inflammatory mediators such as TNF-α. The vagal afferents would respond in a reflexive manner to activate the splanchnic nerve of the SNS via vagal efferents. This causes the release of ACh by cholinergic T-cells in the spleen which activate the alpha 7 subunit of nicotinic acetylcholine receptors (α7nAChR) on macrophages (**Figure 1-5**) (Tracey, 2002; Wang *et al.*, 2003). Downstream pathways inhibit the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inhibit the expression of TNF-α - a key mediator of inflammation (Tracey, 2002) (**Figure 1-6**). VNS has

been shown to be anti-inflammatory and protective on a cytokine, histological, functional, and survival level (**Table 1-3**).

Table 1-3 Effects of VNS in models of inflammation in rat

Reference	Model of inflammation	Inflammatory cytokine(s)	Histology	Function	Mortality
(Borovikova, Ivanova, Zhang, *et al.*, 2000)	LPS	⇩serum and liver TNF-α	NR	NR	NR
(Yamakawa *et al.*, 2013)	Heat stroke	⇩serum TNF-α	⇩tissue infiltration by inflammatory cells	NR	+35% survival rate
(Ay, Sorensen and Ay, 2011)	tMCAO	NR	⇩Infarct volume	⇧Neurological score	NR
(Boland *et al.*, 2011)	CLP	NS	NS	NR	Improved to 0% mortality
(Meregnani *et al.*, 2011)	TNBS-induced colitis	NS	⇩inflammatory lesions	NR	NR
(Khodaparast *et al.*, 2013)	Microinjection-induced focal ischaemia	NR	NS	Full forelimb recovery by 6 weeks	NR
(Levine *et al.*, 2014)	CIA	NS	⇩Joint damage scores	⇩Joint swelling	NR

NR = not reported, NS = no significant changes, collagen induced arthritis (CIA), caecal ligation and puncture (CLP), transient middle cerebral artery occlusion (tMCAO), 2,4,6-trinitrobenzenesulfonic acid (TNBS).

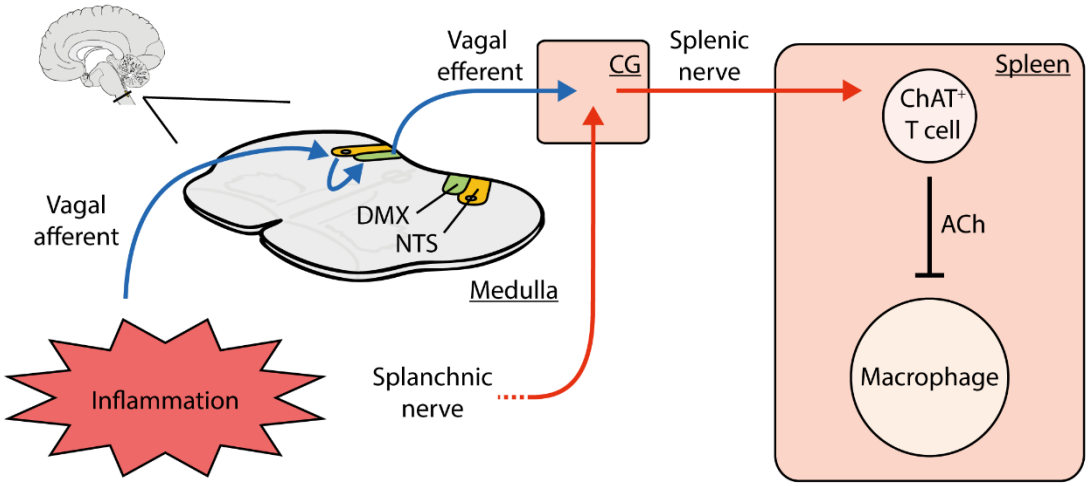

Figure 1-5 Anti-inflammatory reflex
Inflammation is detected by vagal afferents which activate neurones within the NTS. This activates neurons within the DMX and vagal afferents in a reflexive manner. Stimulation of the splenic nerve releases NA in the spleen, causing the release of ACh from cholinergic T cells (ChAT). ACh activates α7nAChR on the cell surface of macrophages in the spleen and inhibits TNF-α production, thereby dampening the inflammatory response. CG = Celiac ganglion. → = Vagal pathway, → = Sympathetic pathway.

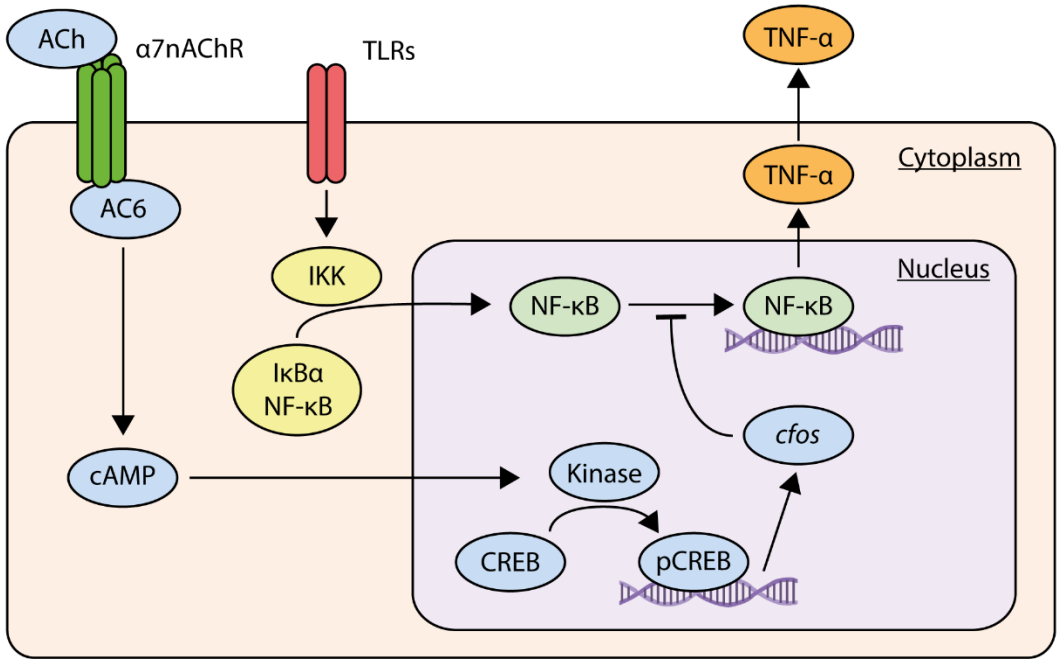

Figure 1-6 Activation of α7nAChR on macrophages inhibits secretion of TNF-α
Upon recognition of pathogens, activation of Toll-like receptors (TLRs) lead to the production and release of TNF-α via NF-κB activity. This response can be inhibited through activation of α7nAChR by ACh. Production of cyclic adenosine monophosphate (cAMP) in the cytoplasm are transported into the nucleus, mediating the transcription of *cfos*. AC6= Adenylate cyclase 6; IKK = IκB kinase; IκBα = inhibitor of κB; CREB = cAMP response element-binding protein; pCREB = phosphorylated CREB. Figure adapted from (Tracey, 2002).

1.5.3.2. Effects of VNS in Models of Inflammation

The anti-inflammatory effects of VNS have been studied in rat models of inflammation, a selection of which have been summarised in **Table 1-3**. As mentioned in the study by Borovikova *et al.* in **Section 1.5.3.1**, VNS administered before and after administration of LPS to induce sepsis in rats suppressed the expression of TNF-α in liver and serum below levels observed in sham-operated rats administered with LPS ($p<0.05$) (Borovikova, Ivanova, Zhang, *et al.*, 2000). A similar reduction in TNF-α levels in serum was shown in a study by Yamakawa *et al.* deploying VNS in rats following heat stroke-induced inflammation (Yamakawa *et al.*, 2013). Heat stroke is a life threatening condition whereby excessive heat may cause hyperthermia and result in systemic inflammation, multi-organ failure, and CNS dysfunction (Leon and Helwig, 2010). VNS administered in rats following heat stroke-induced inflammation displayed a >2-fold reduction in serum levels of TNF-α and IL-6 up to 6 hrs after heat stroke compared to rats with no VNS treatment ($p<0.05$) (Yamakawa *et al.*, 2013). Furthermore, a significantly reduced number of CD11b-positive cells (a marker of inflammatory cells) infiltrated into tissues under immunohistological examination of spleen (8-fold reduction) and lung (2-fold reduction) tissues in the VNS group compared to the no-VNS group ($p<0.05$) (Yamakawa *et al.*, 2013). They reported a significantly higher survival rate of 61% in mice treated with VNS compared to 26% in the no VNS stimulation group ($n=23$/group, $p=0.016$) (Yamakawa *et al.*, 2013).

In another model of inflammation, Boland *et al.* used caecal ligation and puncture (CLP) to induce peritonitis, VNS was administered 4 hrs later for 20 mins (15 V, 5 Hz, 2 ms). All rats in the VNS group had survived whilst the group which did not receive VNS treatment had a survival rate of 71% (Boland *et al.*, 2011).

The effects of VNS were studied in a rat model of inflammatory bowel disease by Meregnani *et al*. whereby colitis was induced through insertion of a cannula into the colon and administering 2,4,6-trinitrobenzenesulfonic acid (TNBS). VNS treatment started 1 hour prior to induction of colitis with a total of 3 hours stimulation a day for 5 days (1 mA, 5 Hz, 0.5 ms, on time 10 s, off time 90 s, continuous cycle). Histological examination of colon tissue at the end of the study revealed that VNS had visibly reduced inflammation. No significant changes in the expression of TNF-α, IL-1β, Intercellular Adhesion Molecule 1 (ICAM1), and IL-6 messenger ribonucleic acid (mRNA) from colonic tissue samples were associated with VNS treatment (Meregnani *et al.*, 2011).

It is evident that VNS administration is capable of attenuating inflammation at these levels and reducing mortality under various models of inflammation in rats. Translating these findings into a clinical setting is a potentially invaluable benefit which may reduce inflammation and improve quality of life.

1.5.3.3. Therapeutic potential of VNS in Inflammatory Diseases

Clinical studies using VNS have displayed similar anti-inflammatory effects seen in studies deploying VNS in animal models of inflammation (**Table 1-3**) and show promise for its use to treat a variety of inflammatory diseases in humans. Here, we focus on the therapeutic applications of VNS in rheumatoid arthritis (RA), stroke, and inflammatory gastrointestinal diseases.

Rheumatoid arthritis

RA is an autoimmune disease characterised by pain, chronic synovial inflammation (synovitis), excessive production of inflammatory cytokines, and hyperplasia at joints leading to, deterioration of cartilage and bone, and ultimately immobility (McInnes and Schett, 2011). The majority of therapies for treating RA have primarily focused on inhibiting TNF-α function (McInnes and Schett, 2011). Interestingly, VNS administered in rats with collagen induced arthritis (CIA) showed attenuated TNF-α release and reduced swelling (Levine *et al.*, 2014) (**Table 1-3**). Furthermore, functional assays assessing cytokine production from 7 epilepsy patients with VNS in response to endotoxin revealed a >2-fold reduction in TNF-α, IL-6 and IL-1β levels compared to pre-surgery levels ($p<0.05$) (Koopman *et al.*, 2016). Following these findings, the therapeutic efficacy of VNS was assessed in a pilot study with RA patients (Andersson and Tracey, 2012).

In one of the first RA patients to be implanted with the device, joint pain was alleviated and serum levels of C-reactive protein (CRP) had reduced to levels seen in healthy volunteers after 8 weeks of VNS treatment (Andersson and Tracey, 2012). Following this, a multi-centre clinical trial implanted 18 RA patients and which provided a single 60 s stimulation (0.25-2.0 mA, 10 Hz, 250 μs) 1-4

times a day. This led to significantly reduced levels of TNF-α, IL-6, and CRP in serum compared pre-implantation levels ($p<0.05$). However, when the stimulator was switched off, the levels of these molecules would increase again, thereby demonstrating treatment of RA with VNS requires a daily stimulation (Koopman *et al.*, 2016).

Stroke

Stroke is a leading cause of disability worldwide, and the initial cell death and is followed by a secondary inflammatory phase which causes further cell death to areas surrounding the site of ischaemia (Wang, Tang and Yenari, 2007). All clinical trials of pharmacological treatments for stroke arising from rodent studies have failed, and the only approved treatment of stroke is tissue plasminogen activator (t-PA) which has a narrow window for therapeutic benefit. Since VNS attenuates inflammation (Borovikova, Ivanova, Zhang, *et al.*, 2000; Yamakawa *et al.*, 2013) it is predicted that VNS may limit inflammation following stroke. In preclinical studies (**Table 1-3**), VNS administered 30 mins after transient middle cerebral artery occlusion (tMCAO) in rats had significantly reduced infarct volume by approximately 50% compared to the no VNS group ($p<0.05$) (Ay, Sorensen and Ay, 2011). In addition to this, neurological scores assessing motor ability had significantly improved 1 to 3 days following tMCAO in the VNS group compared to a control group receiving no VNS treatment following tMCAO ($p<0.05$) (Ay, Sorensen and Ay, 2011).

Limb impairment following stroke is common outcome found in >80% of stroke survivors (Nakayama *et al.*, 1994) and treatments for recovering arm function is one of the key research priorities relating to life after stroke (Pollock *et al.*, 2012). Interestingly, VNS paired with forelimb rehabilitation after microinjection-induced focal ischaemia in rats displayed an improved rate of rehabilitation (Khodaparast

et al., 2013) (**Table 1-3**). Rats were first trained to perform a pulling task upon which triggers an automated pellet dispenser to deliver a sucrose pellet when the pulling force exceeded 120 g. Focal ischaemia was induced by microinjecting endothelin-1 at the motor cortex and VNS was delivered within 30 ms of exceeding 120 g pulling threshold. Paired stimulation of VNS to a successful pull showed significantly improved rate of forelimb recovery to pre-ischaemic levels 6 weeks following focal ischaemia (VNS group n=6, no VNS group n=9; successful pull rate in VNS group = 88% vs no VNS = 63%, $p<0.05$; average peak pull force in VNS group = 105% of pre-ischaemic force vs no VNS = 57% of pre-ischaemic force, $p<0.05$) (Khodaparast *et al.*, 2013).

These findings in animal studies suggest that VNS paired to forelimb movement may enhance forelimb rehabilitation following a stroke, and indeed VNS-paired rehabilitation significantly improved motor function in a 6-week pilot study with stroke patients (Fugl-Meyer assessment score difference from baseline: VNS= +9.6 (n=9) vs rehabilitation only = +3.0 (n=10), p=0.038) (Dawson *et al.*, 2016).

Inflammatory gastrointestinal diseases

The vagus nerve innervates the majority of the gastrointestinal (GI) tract from the oesophagus to the small intestines (Berthoud and Neuhuber, 2000) (**Section 1.1.1**) and forms part of the brain-gut axis. Interactions in the brain-gut axis involves bi-directional communication between: the enteric nervous system, peripheral and central nervous systems, HPA axis, and gut walls (Collins and Bercik, 2009) to modulate aspects of behaviour and gut function in response to external and bodily demands (Mayer, Tillisch and Gupta, 2015).

Autonomic dysfunction, indicated by a significantly blunted vagal tone, has been observed in patients with inflammatory bowel disease (Pellissier *et al.*, 2010). Furthermore, TNF-α is highly expressed in patients with Crohn's disease (Pellissier *et al.*, 2014). VNS has been suggested as a treatment for inflammatory gastrointestinal diseases to restore the vagal tone and reduce inflammation. Indeed, a rat study discussed above (**Section 1.5.3.2**) found administration of VNS had attenuated inflammation following induction of colitis (Meregnani *et al.*, 2011).

Furthermore, a 6-month pilot study performed by Bonaz *et al.* studied the therapeutic effects of VNS in 7 patients with Crohn's disease (Bonaz *et al.*, 2016). 2 patients withdrew from the study 3 months following VNS treatment due to worsening of disease, however, the remaining 5 patients displayed improvements in Crohn's disease activity indices (4 patients in clinical remission for disease activity at 6 months) and Crohn's disease endoscopic index of severity (5 patients in endoscopic remission at 6 months) (Bonaz *et al.*, 2016).

In summary, despite an incomplete understanding of the mechanisms in VNS, it is evident that VNS produces an anti-inflammatory effect which can benefit conditions such as rheumatoid arthritis, stroke, and inflammatory GI diseases. Findings in animals have been directly translatable to clinical trials, and advances in understanding the pathways responsible for the anti-inflammatory effect by VNS will allow identification of biomarkers such as HRV which will aid the monitoring and refinement of treatment efficacy in the future.

1.6. Aims

Although much has been deciphered about the physiological and pathological roles of the vagus nerve, much remains unknown. There is a major need for a better understanding of the mechanistic pathways which mediate the effects of VNS and whether VNS could also be beneficial in other disabling conditions like fatigue, stress, and anxiety. A better understanding of how VNS influences HRV is also needed and whether this indicates improvement in health and treatment efficacy. Moreover, the effect of VNS on anti-inflammatory cells such as Tregs and pro- (e.g. TNF-α, IL-6, IL-1) and anti-inflammatory cytokines (e.g. TGF-β, IL-10) also need to be determined.

Determining the changes in immune cells, cytokines, and HRV after VNS could lead to the identification of key biomarkers that could aid the optimisation of VNS therapy such as optimum stimulation settings to attain a therapeutic response in individual patients. Moreover, biomarkers could also serve as surrogate endpoints in clinical trials, thus potentially reducing the number of patients needed.

In the current study, we recruited patients with refractory epilepsy who were referred to Royal Hallamshire Hospital (RHH) for VNS implantation. While this is not an ideal patient group for our study since many patients have structural brain abnormalities, learning disabilities, co-morbidities, and are taking many medications, insertion of a VNS device which requires invasive surgery could not be ethically justified in normal individuals which arguably would be the ‘ideal’ group to explore mechanistic pathways. We collected samples before and up to 6 months after VNS implantation.

The primary aims of this study were:

Aim 1: To determine the effect of VNS on other unexplored mood disorders such as anxiety, fatigue and stress

Hypothesis: VNS will have beneficial effects on anxiety, fatigue, and stress.

Aim 2: To determine the effects of VNS on HRV

Hypothesis: VNS will enhance HRV and this will correlate with the beneficial effects of VNS.

Aim 3: To determine changes in anti-inflammatory cell frequency and function and in pro and anti-inflammatory cytokines after VNS

Hypothesis: VNS will alter cell frequencies and expression of cytokines in blood to promote immune tolerance. Furthermore, VNS will enhance the functional capacity of Tregs to promote immune tolerance.

In summary, this is the first study to observe the psychological, cardiac, and neuroimmune effects of VNS simultaneously. Findings from this study will aid the understanding of the complex function of the vagus nerve and the mechanisms that mediate VNS. Furthermore, these findings may allow the identification of biomarkers to help track or determine effectiveness of VNS therapy, to aid optimisation of VNS settings/regime, and suggest other therapeutic uses of VNS.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Reagents

Table 2-1 Table of materials

Description	Manufacturer	Catalogue number
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Bioscience	552843
Anti-Rat Ig, κ/Negative Control Compensation Particles Set	BD Bioscience	552844
Cell Proliferation Dye eFluor® 450	eBioscience	65-0842-85
Foxp3 Transcription factor staining buffer set	eBioscience	00-5523-00
Glass Sodium Heparin Plasma tube	BD Bioscience	VS368480
Human Cytokine Array C5	RayBiotech	AAH-CYT-5-4
Lymphocyte separation medium 1.077	Lonza	LZ17-829F
RPMI 1640 + L-glutamine cell culture media	Lonza	LZBE12-702F
Staphylococcal enterotoxin B (SEB) from Staphylococcus aureus	Sigma	S4881-1MG
UV cell viability dye	Life technologies	L-23105

Table 2-2 Table of antibodies

Antibody/Isotype control	Manufacturer	Catalogue number
Mouse anti-human CD4:FITC	eBioscience	11-0048-42
Mouse anti-human CD25:PE	eBioscience	12-0259-42
Mouse anti-human CD8a APC-eFluor780	eBioscience	47-0088-41
Mouse anti-human CD14:PerCP-Cy5.5	eBioscience	45-0149-41
Mouse anti-human CD19:PE-Cy7	eBioscience	25-0199-41
Rat anti-human Foxp3:APC	eBioscience	17-4776-42
Mouse IgG2b K Isotype Control FITC	eBioscience	11-4732-42
Mouse IgG1 K Isotype Control PE	eBioscience	12-4714-42
Mouse IgG1 K Isotype Control APC-eFluor780	eBioscience	47-4714-80
Mouse IgG1 K Isotype Control PerCP-Cy5.5	eBioscience	45-4714-80
Mouse IgG1 K Isotype Control PE-Cy7	eBioscience	25-4714-80
Rat IgG2a K Isotype Control APC	eBioscience	17-4321-81
Anti-human CD3 Functional Grade Purified (aCD3)	eBioscience	16-0039-81

2.1.2. Software

Microsoft excel, SPSS (version 22), and Graphpad Prism 7 were used for statistical analysis and creating graphical outputs. ECG data were uploaded using the FirstBeat uploader and analysed using Kubios HRV analyser. Flow cytometry data were analysed using FlowJo (version 7.6.5). Figures were created in Adobe Illustrator.

2.1.3. Questionnaires

Table 2-3. List of questionnaires and purpose

Questionnaire	Purpose	Reference
Liverpool Seizure Severity Scale (LSSS)	Seizure number and severity for the past 4 weeks.	(Scott-Lennox *et al.*, 2001)
Neurological Disorders Depression Inventory for Epilepsy (NDDI-E)	Rapid screening of major depression in patients with epilepsy over the past 2 weeks. Patients with a score of >15 are likely to suffer from major depression.	(Gilliam *et al.*, 2006)
General Anxiety Disorder assessment (GAD-7)	Screening tool and severity measure for generalised anxiety disorder over the past 2 weeks.	(Spitzer *et al.*, 2006)
Fatigue Assessment Scale (FAS)	Measure of chronic fatigue.	(Michielsen, De Vries and Van Heck, 2003)
Perceived Stress Scale (PSS)	Measure of perceived stress in the past month.	(Cohen, Kamarck and Mermelstein, 1983)
Note: For full copies of questionnaires, see **Appendix Section 8.4**		

2.2. Methods

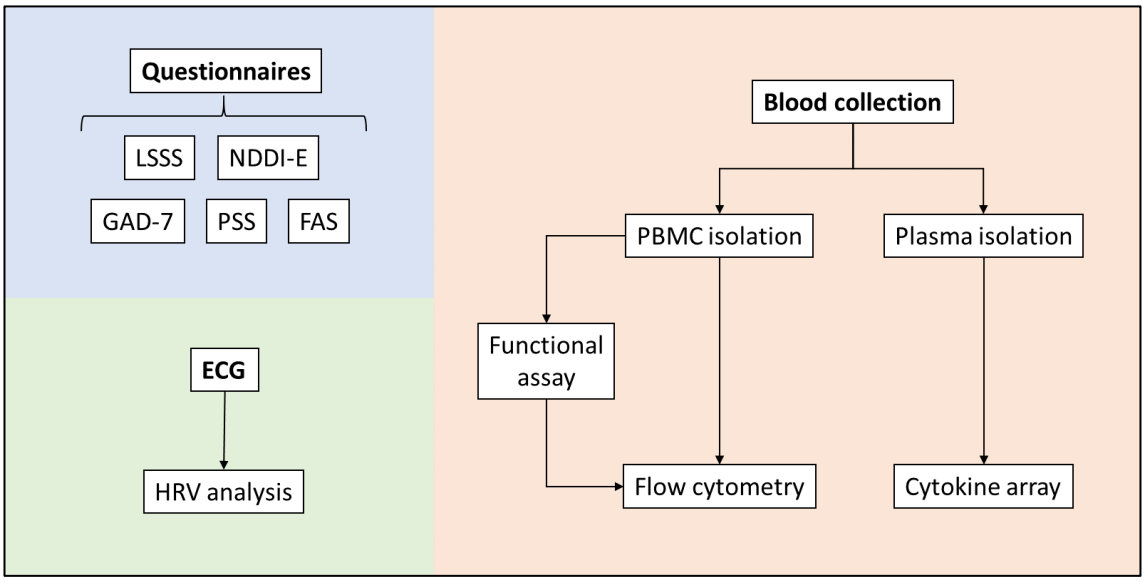

Figure 2-1 Summary of method workflow
At each visit, patients will complete a series of questionnaires, undergo a 5-minute ECG, and provide a blood sample.

2.2.1. Ethics

An Independent Scientific Review (ISR) was performed prior to the submission of ethics. Ethical approval was granted by the ethics committee at Sheffield Teaching Hospitals (STH) Research department for RHH, Sheffield, England and the National Research Ethics Service (NRES) committee North West – Haydock (14/NW/1221). See **Appendix Section 8.1** for approvals and **Appendix Section 8.2** for application and supporting documents for ethics approval including protocol, information sheets, and consent form templates. All samples were assigned a unique identifier number and anonymised at the point of collection.

2.2.2. Recruitment

Healthy volunteers were approached at the University of Sheffield, and refractory epilepsy patients were identified and approached at RHH in Sheffield. Both healthy volunteers and patients were screened according to the inclusion and exclusion criteria below:

The inclusion criteria are as follows:

- Participants must be above 18 years or older
- Participants without a history of autoimmune disorders
- Participants without a history of long term immunomodulatory treatment

The exclusion criteria are as follows:

- Participants under the age of 18 years
- Participants with a history of autoimmune disorders
- Participants who have previously received immunomodulatory treatment
- Who already have an electrical implant such as a pacemaker
- Already taking part in another intervention study
- Patients that have experienced seizure(s) 48 hours prior or presenting with signs of clinical infection on the day of blood sampling may be asked to have the routine consultation and sampling postponed as a measure of control for the study where possible.

Healthy volunteers and patients fulfilling the criteria were given information sheets regarding the study and a formal written consent was obtained for subjects recruited into the study.

2.2.3. Surgery and Follow-up

A detailed protocol describing the implantation procedure can be found in section 12 of the VNS Therapy System Physician's Manual (version 5.17, https://us.livanova.cyberonics.com/healthcare-professionals/resources/product-training accessed 03/07/2017). Briefly, a small incision is made along the left side of the neck to expose the left vagus nerve, and another incision is made below the collar bone in the chest or arm pit, forming a subcutaneous pocket to house the pulse generator. A lead is tunnelled from the incision at the neck to the pocket, and the electrodes are attached to the left vagus nerve. The lead is then connected to the pulse generator and placed inside the subcutaneous pocket. The generator is switched on immediately to test the device, and stimulation intensity is gradually increased according to patient's tolerance at each follow-up appointment.

2.2.4. Sample Collection

Patients completed questionnaires, ECGs were recorded, and blood samples were taken prior to VNS implantation (at pre-operation assessment or on the day of the surgery) and at 3- and 6-month follow-up appointments.

Questionnaires

Validated questionnaires were self-administered or assisted by the next of kin at each visit. The questionnaires administered included are listed in **Table 2-3**. These were used to monitor seizure frequency and severity (LSSS), mood/depression (NDDI-E), general anxiety (GAD-7), fatigue (FAS), and perceived stress (PSS). Questionnaire scores were deduced according to scoring

methods described in their respective published studies (**Table 2-3** and **Appendix Section 8.4**)

ECG

A 5-minute single-lead ECG (FirstBeat Bodyguard 2) recording was performed at each visit. Briefly, patients were laid down in a supine position and asked to remain still for the duration of the recording. Electrodes were attached to the right collarbone and left side of the patient according to manufacturer's protocol (**Figure 2-2**). The device automatically detects electrical discharge and records heart beats.

Blood sampling

Approximately 20 ml of blood was drawn from healthy volunteers and patients from the cubital vein into vacutainers containing lithium heparin anticoagulant. Samples were mixed well by gently inverting the vacutainers several times to ensure thorough mixing of blood with heparin.

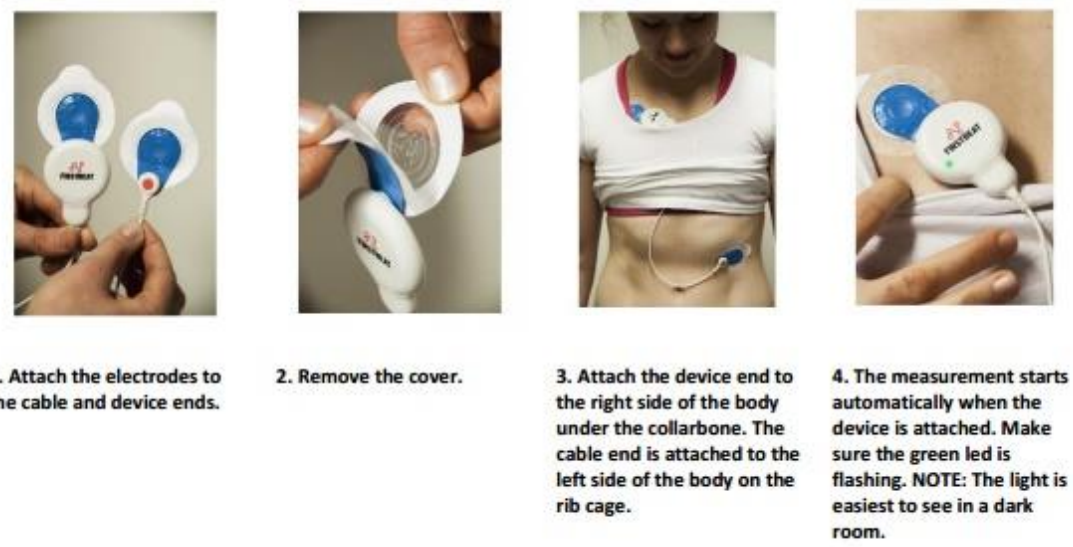

Figure 2-2 Firstbeat Bodyguard 2 manufacturer's protocol

2.2.5. Deducing Measures of HRV

5-minute single lead ECG recordings were imported into Kubios HRV software and a 3-minute artefact-free interval was selected for automated analysis (**Appendix Figure 1** for full output of analysis). The key measures of HRV selected for analysis of this study and their descriptions are outlined in **Table 2-4**. In addition to this, Toichia and colleagues described non-linear methods of calculating sympathetic and vagal activity using the formulae below (Toichi *et al.*, 1997):

(1) $\text{Cardiac sympathetic index (CSI)} = \text{SD1} \times \text{SD2}$

(2) $\text{Cardiac vagal index (CVI)} = \log_{10}[\text{SD1} \times \text{SD2}]$

These nonlinear measures of sympathetic and vagal indices were also included in the analysis of the effects of VNS on HRV.

The key measures of HRV observed in this study include:

Table 2-4 Key HRV measures observed

HRV parameter	Description
Mean HR (bpm)	Mean of heart rate in beats per minute
STD HR (bpm)	Standard deviation of heart rate in beats per minute
HRV Triangular Index	The integral of the RR interval (time between beats) histogram divided by the height of the histogram as a geometric measure of overall heart rate variation
LF Relative Power (%)	Relative power (signal energy) of the low-frequency band as a percentage of total HRV power.
HF Relative Power (%)	Relative power (signal energy) of the high-frequency band as a percentage of total HRV power.
LF/HF Ratio	Ratio between LF and HF, a commonly used measure of autonomic tone. A higher LF to HF ratio suggests greater X activity whereas a lower LF to HF ratio suggests greater X activity.
CSI	A non-linear measure of sympathetic activity
CVI	A non-linear measure of vagal activity
References: (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Toichi *et al.*, 1997)	

Outputted measures of HRV were transferred into SPSS and Graphpad for statistical analysis.

2.2.6. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Plasma

Heparinised blood was diluted with sterile phosphate buffered saline (PBS) in a 1:1 ratio. 20 ml of diluted whole blood was carefully layered on top of 10 ml lymphocyte separation medium (LSM) as per the manufacturer's protocol. Samples were centrifuged at 400 x *g* for 40 minutes with soft start and no brake to create a density gradient with distinct plasma, white buffer coat, LSM, and red blood cell layers (**Figure 2-3**).

Fresh diluted plasma was used fresh for cytokine array assays (**Section 2.2.10**). The white buffy coat layer containing PBMCs was washed with approximately 30 ml sterile PBS and centrifuged at 600 x *g* for 12 minutes. The supernatant was discarded, and the cell pellet was resuspended and washed with 15 ml sterile PBS for two further washes at 600 x *g* for 5 minutes. The remaining PBMCs pellet was resuspended in 1 ml RMPI-1640 media containing 20% FBS. A 5 µl sample of PBMCs was added to 95 µl of trypan blue to estimate the number of viable live cells in the sample. This was estimated using a haemocytometer according to the figure and equation below (**Figure 2-4**). PBMCs were diluted with media for a final working concentration of $1x10^7$ cells/ml for subsequent assays.

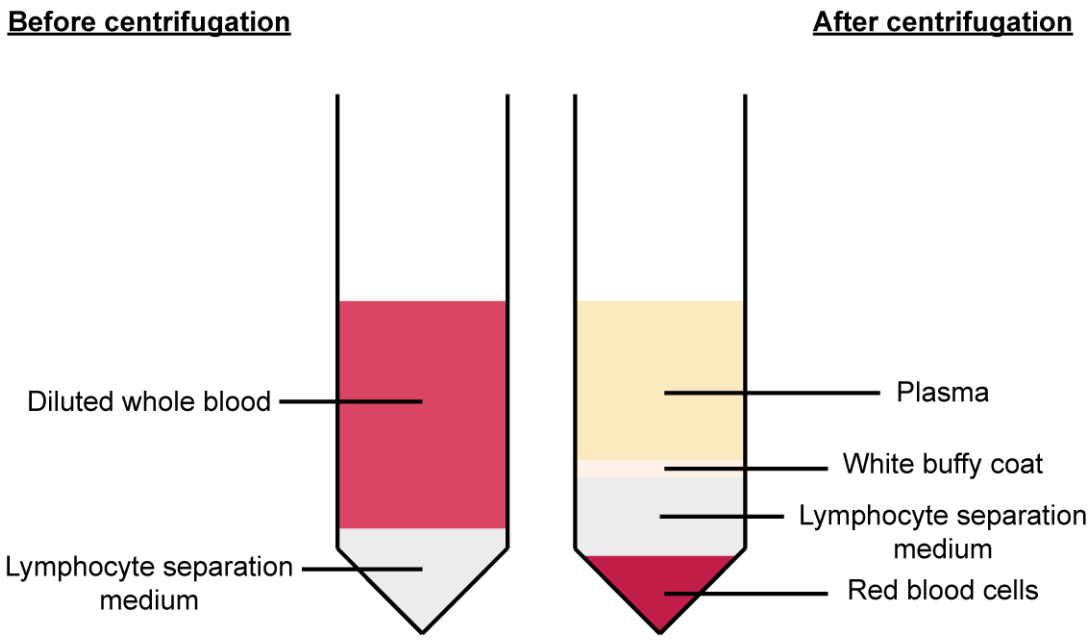

Figure 2-3 PBMC isolation
Centrifugation of diluted whole blood on LSM produces a density gradient which separates the components of blood into plasma, a white buffy coat (containing PBMCs) and red blood cells.

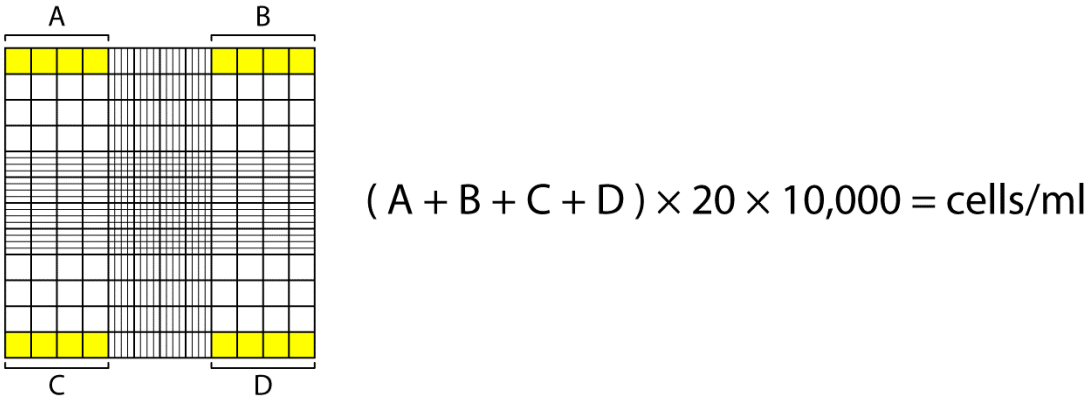

Figure 2-4 Method for estimating number of viable cells using a haemocytometer
Yellow highlighted squares indicate the squares where viable cells were counted. The sum of cells in A-D were taken as the average number of cells to estimate number of cells in the sample using the equation above.

2.2.7. Immunocytochemistry (ICC)

$1x10^6$ PBMCs were aliquoted into separate tubes and washed twice with 1 ml PBS supplemented with 0.1% Bovine Serum Albumin (BSA) (5 mins at 400 x *g* and 4°C). Samples were subsequently incubated with ultraviolet (UV) cell viability dye and flow cytometry antibodies, or isotypes controls (**Figure 2-5**). Flow cytometry beads were incubated with individual fluorescent antibodies for calculating the compensation matrix during analysis.

Briefly, PBMCs were incubated with fluorescent antibodies targeting extracellular CD4, CD25, CD8, CD14, and CD19 in addition to UV cell viability dye for 1 hour at 4°C in the dark. Since foxp3 is expressed intracellularly, PBMCs were subsequently fixed and permeabilised for 45 minutes at 4°C in the dark then washed 2 times. Lastly, cells were incubated with anti-foxp3 fluorescent antibody for 35 minutes at 4°C in the dark and washed a further 2 times before being analysed using the flow cytometer.

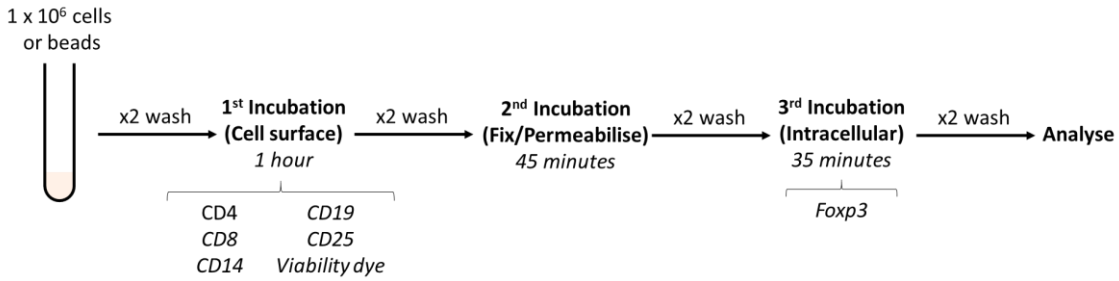

Figure 2-5 Immunocytochemistry protocol
Samples were washed with 1 ml PBS supplemented with 0.1% Bovine Serum Albumin (BSA) and centrifuged for 5 mins at 400 x *g* and 4°C. Incubations steps were performed at 4°C in the dark.

2.2.8. Functional Assay

Following PBMC isolation, cells were stained using cell proliferation dye eFluor 450 according to manufacturer's protocol. It is important to note that this dye is not harmful to the cell and does not affect cell processes according to the manufacturer. Briefly, 1 x 10^7 cells were washed with sterile 4 ml PBS and incubated with 1 ml of dye for 20 mins in the dark at room temperature. Samples were blocked with 4 ml media for 5 mins on ice, and subsequently washed 3 times with 5 ml media at room temperature. Supernatants were discarded and cell pellets containing PBMCs were resuspended with media to make a working concentration of 1 x 10^7 cells/ml.

1 x 10^6 PBMCs were aliquoted into wells on a 48-well plate and incubated at 37°C and 5% CO_2 for 96 hours under three different conditions: (1) unstimulated control with only media (2) wells pre-coated with 250 ng anti-human CD3 antibody (aCD3) to mimic activation by antigen presenting cell (APC) (3) media containing 400 ng Staphylococcal enterotoxin B (SEB) to activate TLR pathways. Unstained PBMCs and PBMCs stained with the proliferation dye were also incubated with only cell culture media as controls for flow cytometry analysis. Following incubation, cells were resuspended and centrifuged for 5 minutes at 400 x *g* and 4°C, following the ICC staining protocol outlined above (**Figure 2-5**).

2.2.9. FlowJo Analysis

Flow cytometry data was analysed using FlowJo version 7.6.5 Compensation beads and sample controls (unstained, isotype, UV-only, and proliferation dye-only) were used to calculate the compensation matrix required for compensation.

In both cases, monocytes (**Figure 2-6A**) and lymphocytes (**Figure 2-6B**) were gated according to size (forward scatter) and granularity (side scatter) from an unstained control sample and analysed separately to reduce background noise in data arising from the monocyte population. In **Figure 2-6A**, the monocyte population was interrogated according to size and area of cells and only single cells were gated. This reduces the likelihood of false positives arising from doublets passing through the flow cytometer. Within the single cell population, viable cells were gated according to UV fluorescence. Finally, viable cells expressing high expression of CD14 were recorded as monocytes. Likewise, in **Figure 2-6B**, lymphocytes were gated in a similar way to monocytes above but except the high expression of CD4, CD25, CD8a, CD19 were recorded instead. Additionally, CD4+ and CD25+ viable cells were gated, and foxp3+ cells within this population were gated as Tregs. Cell populations were represented as percentage of live (viable) cells from either the monocyte gate or lymphocyte gate.

Proliferation is measured using FlowJo's proliferation platform tool (**Figure 2-6C**). Briefly, a built-in algorithm creates and fits a number of peaks from a histogram of fluorescence intensity. The number of peaks were determined according to the lowest root mean squared (RMS) value outputted by the algorithm as lower values indicate a more accurate model (Roederer, 2011). Control samples without *in vitro* stimulation were used to calibrate the fluorescence intensity for

the undivided cells peak in all groups within an assay. The level of proliferation is quantified by the:

Division index – The average number of divisions a cell has undergone within a sample including non-dividing cells, and the;

Proliferation index – The average number of divisions a cell has undergone within a sample excluding non-dividing cells.

The division index is a measure of overall function of the sample, whereas the proliferation index is a measure of the function of responding cells only.

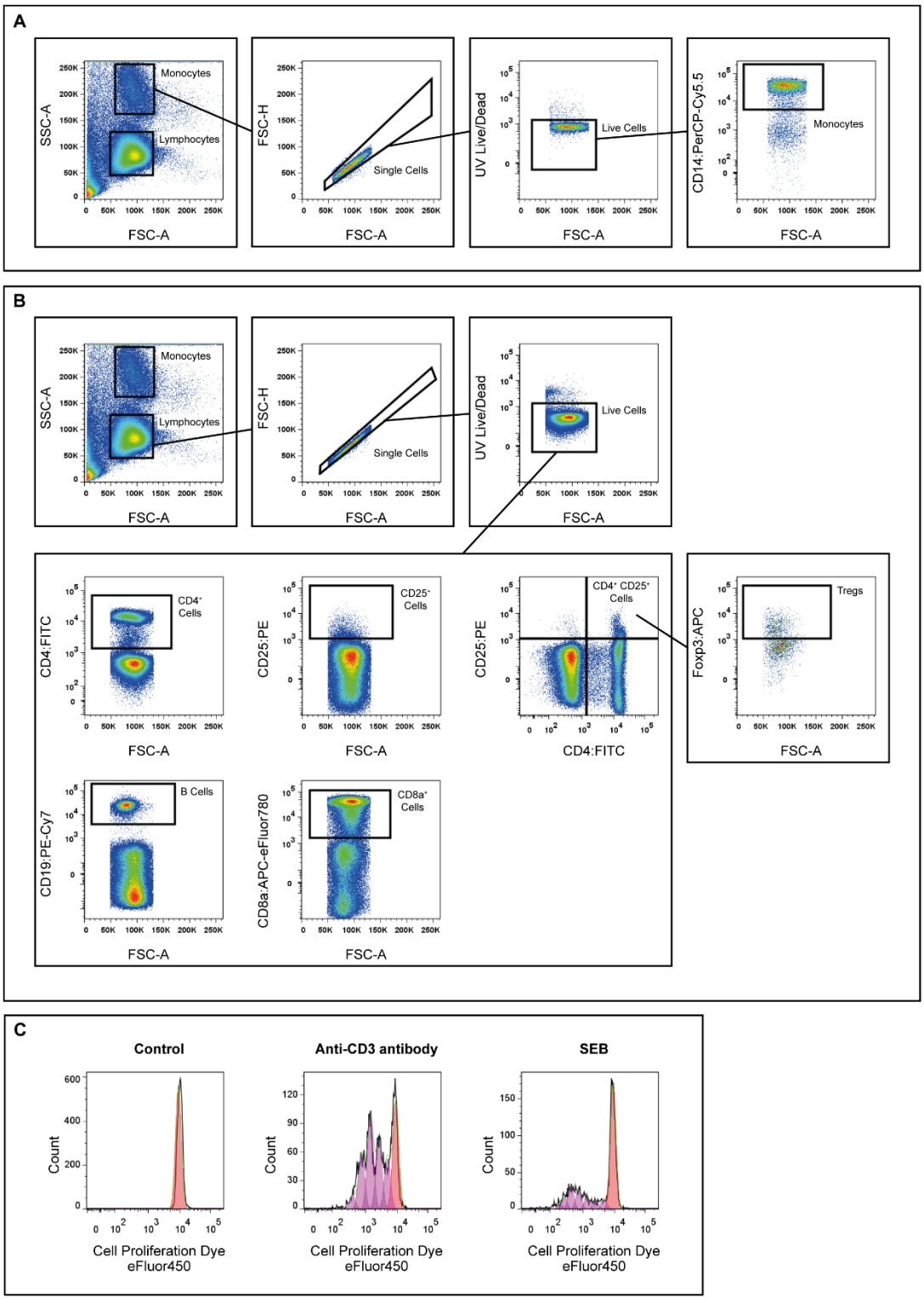

Figure 2-6 Flow cytometry gating strategy and analysis
Gating strategies for (A) monocytes and (B) lymphocytes. (C) Example of cell division histograms for analysing cell proliferation via FlowJo's proliferation platform. FSC = Forward scatter (FSC-A= by cell Area, FSC-H = by cell Height), SSC-A = Side scatter by cell area, Red peaks = undivided cells, purple peaks = divided cells.

2.2.10. Cytokine Array

Ray Biotech cytokine arrays were used according to manufacturer's protocol to observe semi-quantitative changes in the relative expression of 80 inflammatory cytokines. Briefly, membranes were blocked with the supplied blocking buffer for 30 mins and incubated with human plasma overnight at 4°C. Membranes were washed using supplied washing buffers and incubated with an antibody cocktail for 2 hours, these were washed and incubated with horseradish peroxidase (HRP)-Streptavidin for a further 2 hours. Following a final wash, enhanced chemiluminescence reagents were added onto the membranes and imaged using the intelli-chemi setting on the G-box to observe relative expression of cytokines in the sample. Signal intensity of cytokines were expressed as a percentage relative to the average of positive controls and the average of negative controls were used for background correction.

2.2.11. Statistics

In this exploratory study,one-way analysis of variance (ANOVA) with repeated measures and Tukey correction was used to detect differences in the observed parameters between timepoints. Paired T-tests were used to compare differences between two timepoints in patients with an incomplete set of data. Furthermore, paired T tests with Bonferroni correction was used for the analysis of cytokine arrays.

Power analysis was performed using G*power 3.1. The mean of difference and standard deviation of difference were used to calculate the effect size. An α error probability was set to 0.05, and power (1-β error probability) was set to 0.80. These parameters were used perform a two-tailed matched-pair T test to

calculate the sample size required to detect a significant change in variables between two timepoints.

Chapter 3. Mood

3.1. Introduction

Since VNS was granted FDA approval for use in refractory epilepsy, beneficial changes in mood have been observed (Rawlins, 1997; Harden *et al.*, 2000; Daban *et al.*, 2008). Although its anti-depressive efficacy remains to be fully evaluated (O'Reardon, Cristancho and Peshek, 2006), others have explored its mechanism and effects on mood in rats (Peña *et al.*, 2014). Peña and colleagues suggested VNS may also benefit other mood disorders such as anxiety, but the effects of VNS on anxiety were unclear when studied in humans (Harden *et al.*, 2000).

Fatigue has been reported as an adverse event following VNS treatment in one study (Lange *et al.*, 2011), however it was not significantly associated with the treatment (Panebianco *et al.*, 2015). The effects of VNS on fatigue are currently unknown, and no studies to our knowledge have studied the changes in fatigue before and after VNS treatment.

Perceived stress is a contributor to depression (Kendler, Karkowski and Prescott, 1999; Hammen, 2005) and activates the HPA-axis to release stress hormones to produce physiological stress responses (Herman *et al.*, 2012). Since vagal nerve afferents innervates the NTS which has connections to the hypothalamus (Henry, 2002), VNS may alter the stress response and perceived stress. To our knowledge, no studies have investigated the effects of VNS on perceived stress.

In this study, we explore other side effects of VNS on mood; focusing on its effects on anxiety, fatigue, and perceived stress. Validated questionnaires were administered to patients to assess changes in seizure severity and frequency

(Scott-Lennox *et al.*, 2001), depression (Gilliam *et al.*, 2006), anxiety (Spitzer *et al.*, 2006), fatigue (Michielsen, De Vries and Van Heck, 2003), and perceived stress (Cohen, Kamarck and Mermelstein, 1983). These short questionnaires were chosen to encourage patient adherence to the study and act as a screening tool to identify aspects of mood to investigate in future studies.

Aim 1: To determine the effect of VNS on other unexplored mood disorders such as anxiety, fatigue and stress

Hypothesis: VNS will have beneficial effects on anxiety, fatigue, and stress.

3.2. General Methodology

Patients with refractory epilepsy were asked to complete a series of short questionnaires prior to VNS implantations and at their 3- and 6-month follow up appointments. Questionnaires administered include: Liverpool seizure severity scale (LSSS, 100-point scale), Neurological disease depression inventory for epilepsy (NDDI-E, 24-point scale), General Anxiety Disorder Assessment (GAD-7, 21-point scale), Fatigue Assessment Scale (FAS, 50-point scale), and Perceived Stress Scale (PSS, 40-point scale) (**Appendix Section 8.1**).

One-way ANOVAs with repeated measures and Tukey correction were used to analyse full sets of data (excluded data from patients 007 and 010 where data for 6M was not available). Separate pair-wise T test comparisons with Bonferroni correction were used to test for differences in questionnaire scores between pre-stimulation (PS) and 3-months including data from patients 007 and 010.

Questionnaire scores were compared against each other to investigate potential trends in seizures and mood following VNS implantation. Additionally, changes in questionnaire scores were compared to differences in age and gender.

3.3. Results

3.3.1. Recruitment

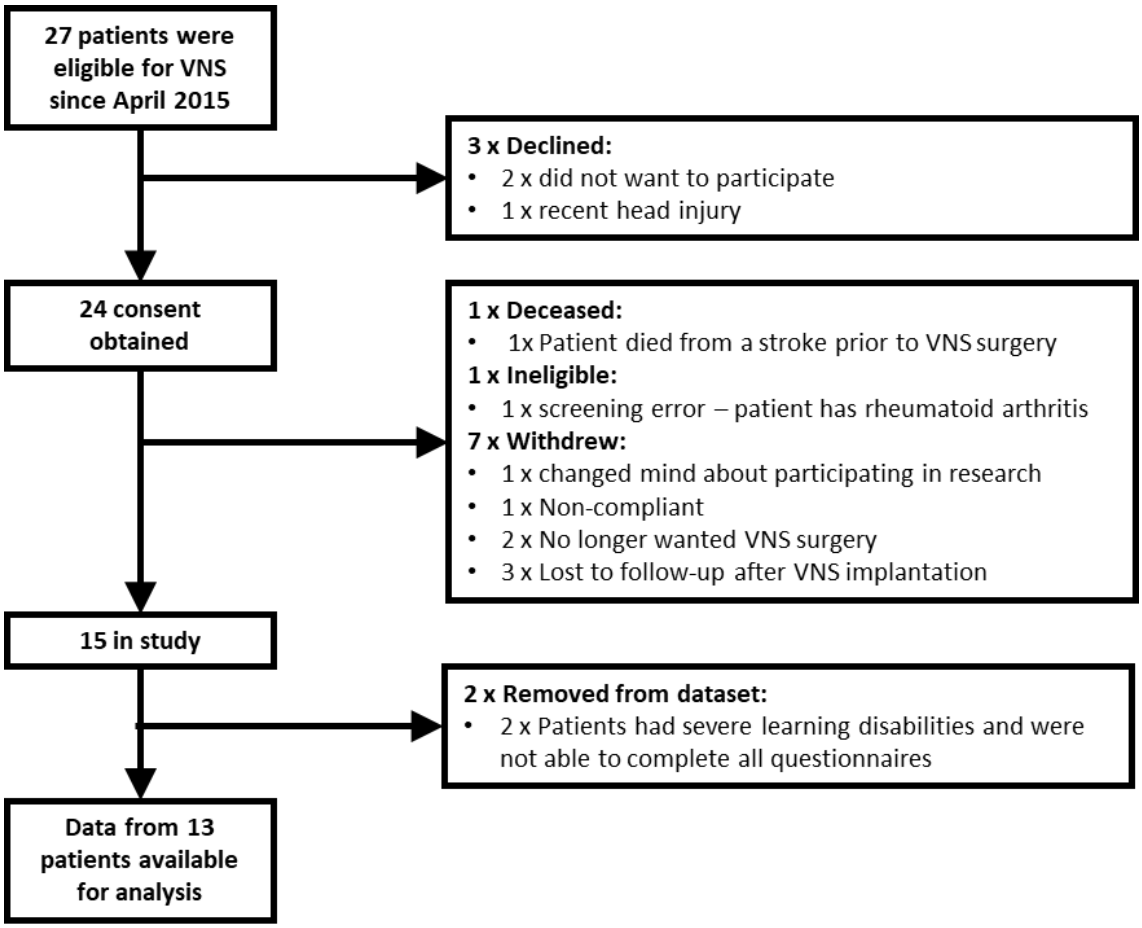

Figure 3-1 Summary of recruitment for assessing effects of VNS on mood

Table 3-1 Summary of recruited patients for assessing effects of VNS on mood

Patient	Age (years)	Gender	Questionnaire	Stimulator model	Intensity (mA)	Frequency (Hz)	Pulse width (μs)	On time (sec)	Off time (min)
001	39	F	✓*	105	1.25	20	250	30	5
004	72	F	✓	106	1.00	20	250	30	5
007	42	F	3M*	106	1.00	15	250	30	5
010	46	F	3M	106	1.00	25	250	30	5
011	22	M	✓	106	1.13	20	250	30	1.8
013	34	M	✓	105	1.25	20	250	30	3
014	46	F	✓	105	1.00	20	250	30	1.8
015	23	M	✓	105	1.75	25	250	30	1.8
016	25	M	✓	105	0.75	30	250	30	3
020	21	M	✓	105	1.75	25	250	30	1.8
022	42	F	✓	105	1.50	20	250	30	3
023	53	F	✓	105	1.25	20	250	30	1.8
024	22	M	✓	105	1.50	20	250	30	1.8

✓=full set of data available,
3M=pre-stimulation and 3-months only,
*No FAS questionnaire data available

Patients 003 and 021 were severely disabled, unresponsive and could not complete the questionnaires, therefore they were removed from data analysis. Patients 007 and 010 were lost to follow up after 3 months of VNS treatment but were included in data analysis. FAS scores were not available for patients 001 and 007 as this questionnaire was added to the study after these patients were implanted with the VNS device. A full set of data was available for the remaining 11 patients (**Table 3-1**).

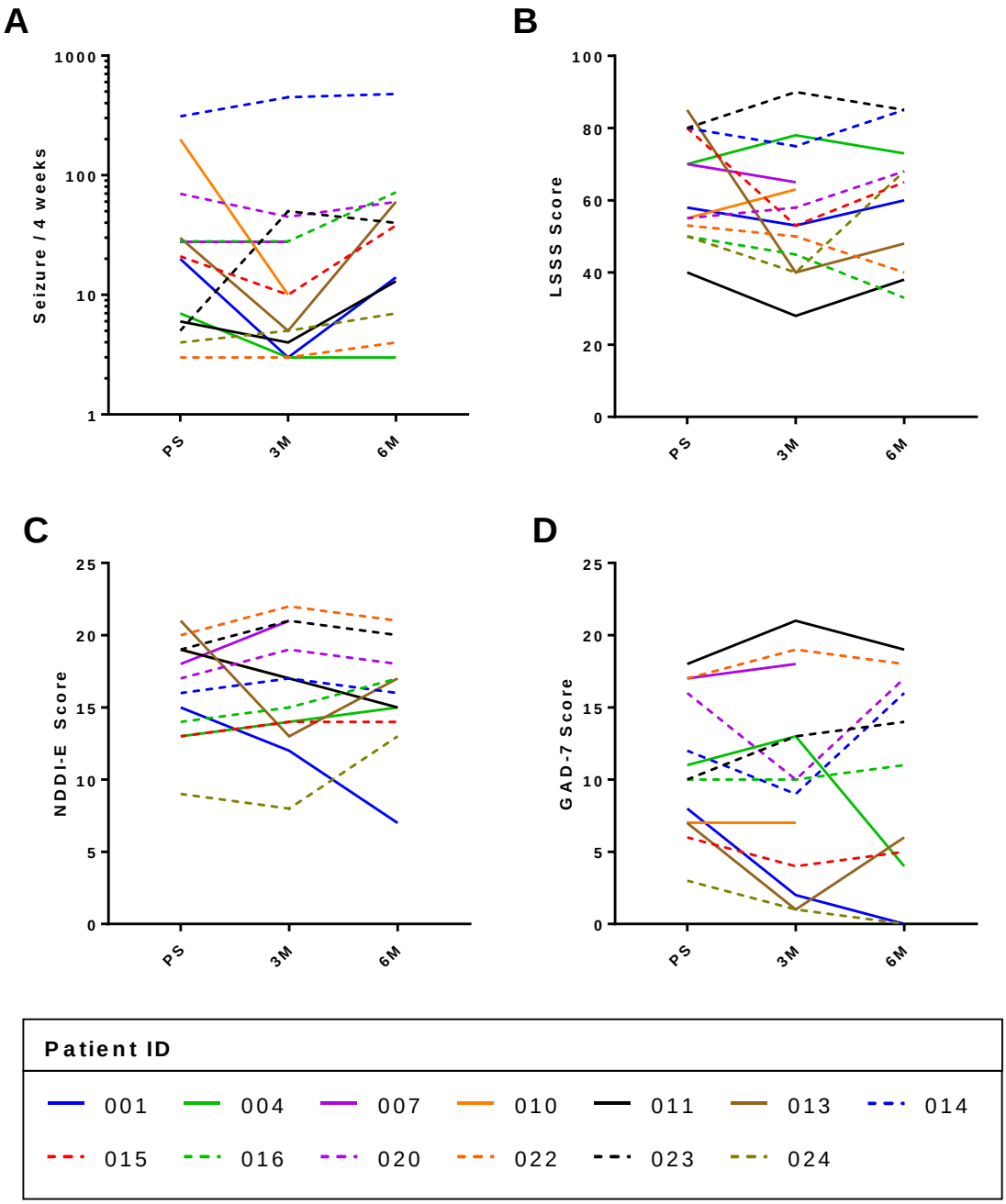

Figure 3-2 Individual change in seizure frequency and questionnaire scores in individual patients
Summary of individual changes in (A) seizure frequency over a 4-week period, (B) seizure severity, (C) depression in epilepsy, (D) anxiety, (E) fatigue, and (F) perceived stress. PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

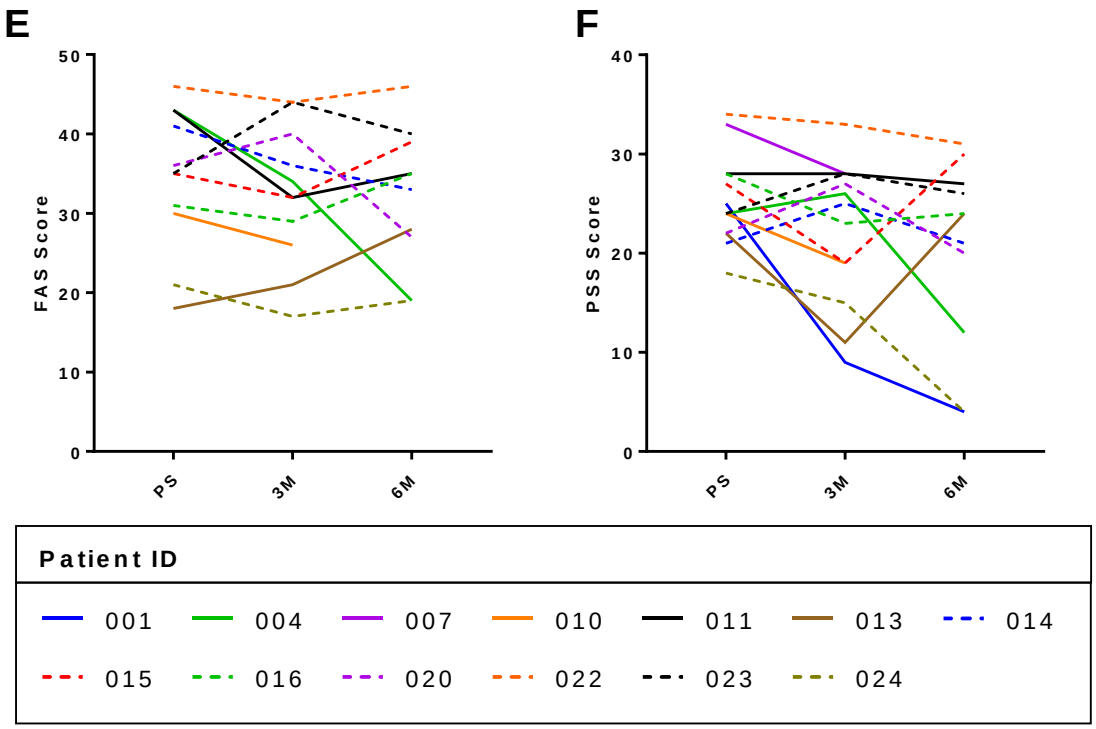

Figure 3-2 Individual change in seizure frequency and questionnaire scores in individual patients (cont'd)
Summary of individual changes in (A) seizure frequency over a 4-week period, (B) seizure severity, (C) depression in epilepsy, (D) anxiety, (E) fatigue, and (F) perceived stress. PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

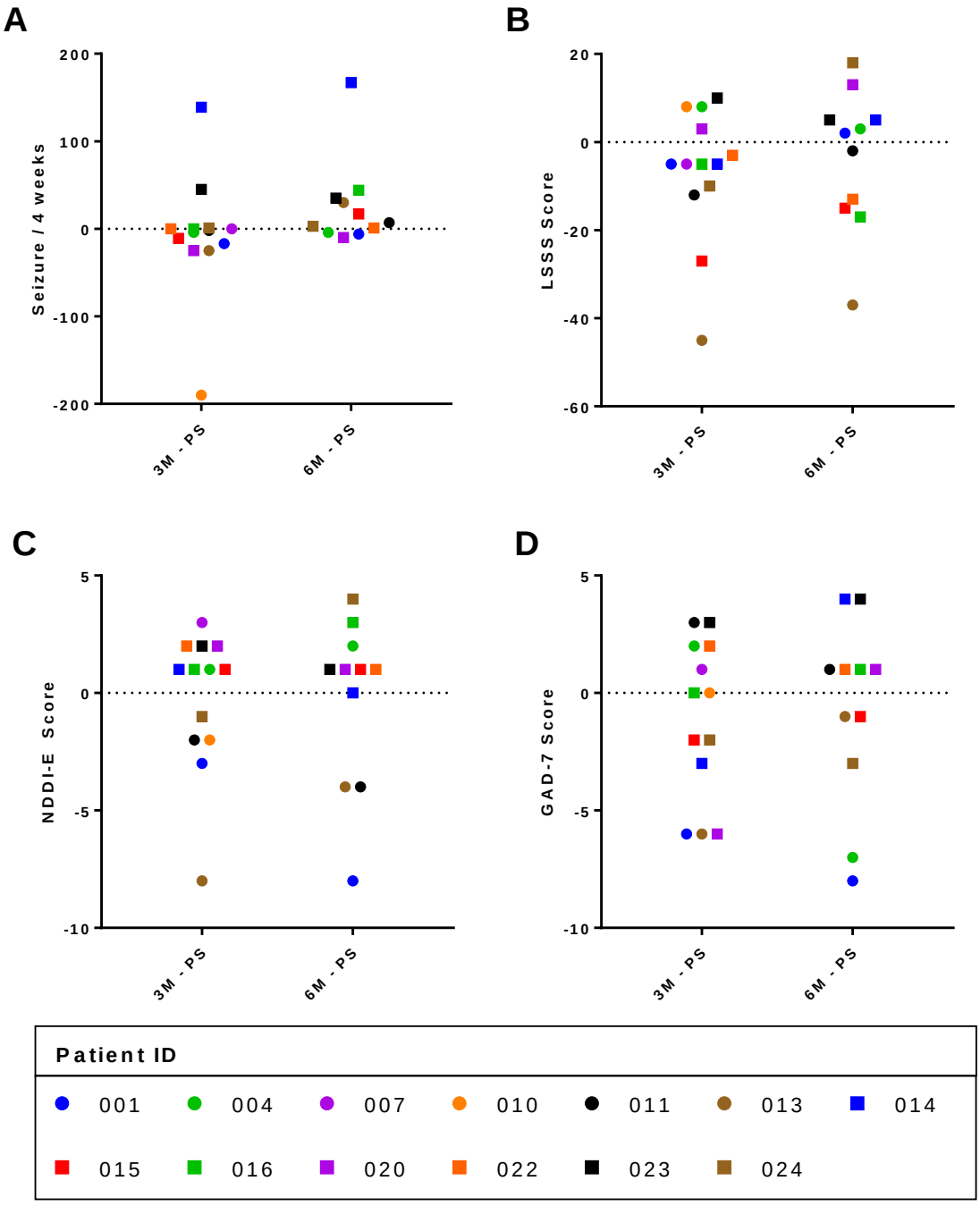

Figure 3-3 Change in seizure frequency and questionnaire scores in individual patients
Summary of differences for (A) seizure frequency over a 4-week period, (B) seizure severity, (C) depression in epilepsy, (D) anxiety, (E) fatigue, and (F) perceived stress. PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

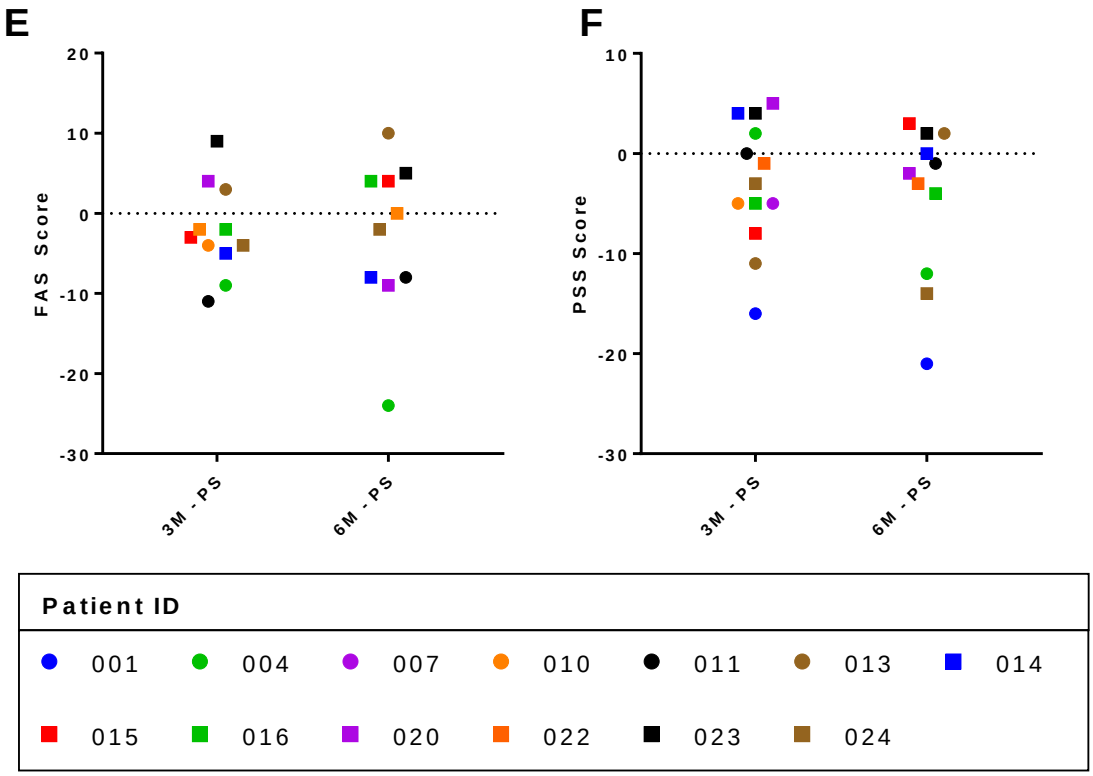

Figure 3-3 Change in seizure frequency and questionnaire scores in individual patients (cont'd)
Summary of differences for (A) seizure frequency over a 4-week period, (B) seizure severity, (C) depression in epilepsy, (D) anxiety, (E) fatigue, and (F) perceived stress. PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

Table 3-2 Statistical analysis of questionnaires

	One-way ANOVA p value (n=6)	3M – PS (n=13)			6M – PS (n=11)		
		Mean diff.	SD of diff.	T test p value	Mean diff.	SD of diff.	T test p value
LSSS	0.1929	-6.846	15.19	0.1301	-3.636	15.75	0.4615
NDDI-E	0.9114	-0.231	2.976	0.7846	-0.273	3.58	0.8057
GAD-7	0.537	-1.077	3.378	0.2727	-0.727	3.927	0.5527
FAS*	0.5045	-2.182	5.741	0.2362	-2.8	9.818	0.3906
PSS	0.1653	-3.000	6.258	0.1095	-4.545	7.751	0.0804
* for FAS, n=11 for 3M - PS, and n = 10 for 6M - PS							

3.3.2. Seizure Frequency and Severity

A total of 5/13 patients at 3 months and only 1/11 patient at 6 months achieved a >50% reduction in seizure frequency **(Figure 3-2A** and **Figure 3-3A**). An average change in seizure frequency of -6.85 ± 69.83 seizures was observed at 3 months and +24.08 ± 48.16 seizures at 6 months (**Table 3-2**), equating to a relative seizure frequency of +40.52% ± 261.86% observed at 3 months and +110.41% ± 206.05% at 6 months compared to pre-stimulation. No significant change in seizure frequency was detected by one-way ANOVA and T test analysis following VNS implantation (**Table 3-2**).

A decrease in seizure severity was observed in 9/13 patients at 3-months and 5/11 patients at 6-months (**Figure 3-2B** and **Figure 3-3B**). Seizure severity scores reduced on average by -6.846 ± 15.19 at 3 months and -3.636 ± 15.75 at 6 months compared to pre-stimulation (**Table 3-2**). No significant changes in LSSS scores were detected by one-way ANOVA and T test analysis following VNS implantation (**Table 3-2**), and changes in seizure severity were independent of changes in seizure frequency.

3.3.3. Depression in Epilepsy

A decrease in depression was observed in 5/13 patients at 3 months and 5/11 patients at 6 months (**Figure 3-2C** and **Figure 3-3C**). NDDI-E score reduced on average by -0.2308 ± 2.976 at 3 months and -0.2727 ± 3.58 at 6 months compared to pre-stimulation (**Table 3-2**). No significant changes in NDDI-E scores were detected by one-way ANOVA and T test analysis following VNS implantation (**Table 3-2**). Changes in NDDI-E scores were independent of age, gender, and changes in seizure frequency and severity. No trends were found

when comparing changes in NDDI-E scores to changes in PSS, GAD-7, or FAS scores.

3.3.4. Generalised Anxiety

A decrease in generalised anxiety was observed in 6/13 patients at 3 months and 5/11 patients at 6 months (**Figure 3-2D** and **Figure 3-3D**). GAD-7 scores reduced on average by -1.077 ± 3.378 at 3 months and -0.7273 ± 3.927 at 6 months compared to pre-stimulation (**Table 3-2**). No significant changes in GAD-7 scores were detected by one-way ANOVA and T test analysis following VNS implantation (**Table 3-2**). Changes in GAD-7 scores were independent of age, gender, and changes in seizure frequency and severity. Furthermore, no trends were found when comparing changes in GAD-7 scores to changes in NDDI-E, PSS, or FAS scores.

3.3.5. Fatigue

A decrease in fatigue was observed in 8/11 patients at 3 months and 5/10 patients at 6 months (**Figure 3-2E** and **Figure 3-3E**). FAS scores reduced on average by -2.182 ± 5.741 at 3 months and -2.8 ± 9.818 at 6 months compared to pre-stimulation (**Table 3-2**). No significant changes in FAS scores were detected by one-way ANOVA and T test analysis following VNS implantation (**Table 3-2**). No trends were found when comparing changes in FAS scores to changes in NDDI-E, PSS, or GAD-7 scores.

3.3.6. Perceived Stress

A decrease in perceived stress was observed in 8/13 patients at 3 months and 7/11 patients at 6 months (**Figure 3-2F** and **Figure 3-3F**). PSS scores reduced on average by -3.00 ± 6.258 at 3 months and -4.545 ± 7.751 at 6 months

compared to pre-stimulation (**Table 3-2**). A trend in decreasing PSS scores was observed and was approaching statistical significance at 6 months (T-test p=0.08, one-way ANOVA p=0.1653, **Table 3-2**). Changes in PSS scores were independent of age, gender, and changes in seizure frequency and severity.

3.4. Discussion

3.4.1. Seizure Frequency and Severity

VNS did not reduce seizure frequency in the majority of patients at 3- and 6-months post-VNS implantation (**Figure 3-2A**, **Figure 3-3A**) with only 1 patient achieving >50% seizure reduction by 6 months (**Figure 3-2A**, **Figure 3-3A**). Cukiert's study reports 31-39% of VNS patients achieving a >50% reduction in seizure frequency 2 years post-VNS implantation (Cukiert, 2015). Since stimulation intensities were still being adjusted by the end of the study, the individual optimal VNS stimulation paradigm is yet to be achieved in the majority of patients in the current study.

A small mean decrease in seizure severity was present (**Figure 3-2B**), and approximately half of all patients in the study achieved a reduction in seizure severity (**Figure 3-3B**). Our findings were in line with other studies which have used the seizure severity questionnaire (SSQ) (Fisher *et al.*, 2015) and the National Hospital Seizure Severity Scale (NHS3) (Boon *et al.*, 2015; Fisher *et al.*, 2015) to monitor changes in seizure severity. In both studies, they detected a small but significant reduction in seizure severity scores at 3-, 6-, and 12-months following VNS implantation. Furthermore, Shahwan *et al.* assessed seizure severity through monitoring the frequency and duration of seizures, need for emergency medications to stop seizures, and intensity of seizures and speed of recovery. They also report a small reduction in seizure severity following >18 months VNS implantation in children (Shahwan *et al.*, 2009).

3.4.2. Mood

We report a trend in decreasing perceived stress scores and minor reduction in depression scores over time. We did not find changes in anxiety or fatigue following VNS treatment.

Depression

We observed a minor decrease in NDDI-E scores for only 3 patients up to 6 months after VNS implantation. However, this was not found to be statistically significant. This was similar to the NDDI-E scores observed in a randomised long-term effectiveness trial for VNS patients which found a mean reduction in NDDI-E scores after 6 months (-1.1 ± 3.3) and 12 months (-1.0 ± 2.2) of VNS treatment (when compared to best medical practice control group, at 6 months p=0.02, at 12 months p=0.28) (Ryvlin *et al.*, 2014). Similar reductions in depression were found 3-months post-VNS implantation with the Beck Depression Inventory (BDI, mean difference vs baseline: -2.6, p=0.045) and Hamilton Rating Scale for Depression (Ham-D, mean difference vs baseline: -4.1, p=0.017) (Harden *et al.*, 2000).

Anxiety

No change in anxiety was observed up to 6 months following VNS implantation. This finding was similar to the pilot study by Harden *et al.* where anxiety (assessed by the Hamilton Rating Scale for Anxiety (Ham-A)) did not show any significant change 3-months post-VNS implantation (mean difference vs pre-stimulation: -1.0, p=0.277) (Harden *et al.*, 2000). Despite this, George *et al.* suggested deploying VNS in patients with anxiety disorders (obsessive compulsive disorder, post-traumatic stress disorder, and panic disorder) following 4/9 patients having received sustained improvements in anxiety scores (mean

change in HAM-A: -8.1, p>0.05) 4 years post-VNS implantation compared to pre-stimulation (George *et al.*, 2008).

Fatigue

Fatigue did not significantly change in the current study. Although one study assessing safety and efficacy of VNS in treating fibromyalgia reported increased fatigue in 3 of 14 patients (Lange *et al.*, 2011), a Cochrane review on VNS in partial seizures concluded that fatigue was not significantly associated with VNS treatment (Panebianco *et al.*, 2015). In the current study, 4/10 patients had increased fatigue according to the FAS but did not report fatigue as a side effect or adverse event as a result of VNS.

Perceived stress

We identified a trend in reducing PSS score 6 months after VNS implantation. Subsequent sample size calculations predict an additional 9 patients would be sufficient to detect this change after 6 months of VNS treatment. Although there are currently no studies reporting changes in perceived stress following VNS in adults, one study investigated changes in parenting stress in parents of children with refractory epilepsy before and after VNS implantation reports reduced parent-child interaction-associated stress following VNS implantation in children (Li *et al.*, 2017). This study used parental stress index (PSI) to assess various aspects of stress associated with parent-child interaction with separate domains assessing theses stresses in parents and children. Scores from the children domain of the questionnaire show no significant changes in stress 12 months following VNS (n=30) (Li *et al.*, 2017). The differences in changes in stress observed between the current study and Li *et al.* is possibly due to differences in

tools used. Whilst the PSS scale is targeted for general perceived stress in adult, the PSI focuses on stress arising from interaction between parent and child.

In conclusion, while efficacious VNS settings have not yet been achieved, we report a trend in reducing perceived stress independent of age, gender, and changes in seizure, depression, anxiety and fatigue at 6 months following VNS treatment.

Chapter 4. Heart Rate Variation

4.1. Introduction

A dysfunctional ANS or blunted vagal tone from measures of HRV can be found in patients with epilepsy, mood disorders, and inflammatory gastrointestinal diseases from measures of HRV (Pellissier *et al.*, 2010; Ponnusamy, Marques and Reuber, 2012; Sgoifo *et al.*, 2015). VNS treatment in these patients altered various measures of HRV which indicated a restored balance in autonomic tone (Setty *et al.*, 1998; Galli *et al.*, 2003; Sperling *et al.*, 2010; Bonaz *et al.*, 2016). HRV has also been identified as a potential indicator to predict responsiveness to VNS treatment in refractory epilepsy patients (Liu *et al.*, 2017). In this study, we explored other changes in HRV following VNS treatment and compared this to the other beneficial changes observed from questionnaires (**Chapter 3**), cell frequencies, functional capacities of cells, and cytokine expression (**Chapter 5**)

Aim 2: To determine the effects of VNS on HRV

Hypothesis: VNS will enhance HRV and this will correlate with the beneficial effects of VNS.

4.2. General Methodology

5-minute ECG recordings were obtained at each patient visit and a 3-min artefact-free recordings were used to calculate HRV parameters in Kubios. Additionally, non-linear measures of autonomic tone: CSI and CVI were calculated according to the equations in **Section 2.2.5**.

One-way ANOVAs with repeated measures and Tukey correction were used to analyse full sets of data excluding patients 007 and 010 which data for only pre-stimulation (PS) and 3 months were available. Separate pair-wise T test comparisons with Bonferroni correction was used to test for differences in measures of HRV between timepoints for all samples. Changes in measures of HRV were compared to differences in age and gender.

Values and differences between timepoints for measures of HRV in individual patients were plotted against questionnaire scores (**Chapter 3**), cell frequencies, measures of cell function, and expression of cytokines (**Chapter 5**). Due to the small sample size, resulting graphs were visually inspected for trends between variables to identify potential measures of HRV as indicators of other outcomes in the study.

4.3. Results

4.3.1. Recruitment

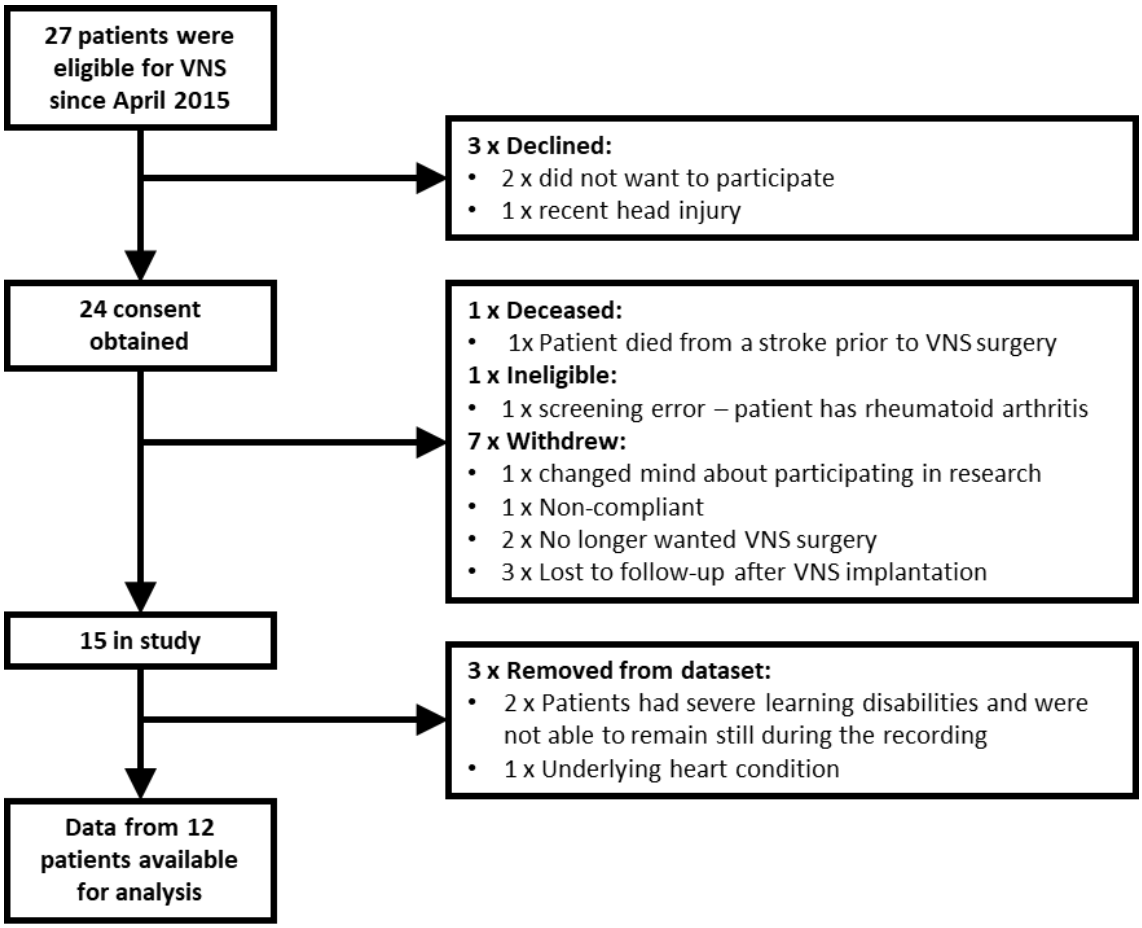

Figure 4-1 Summary of recruitment for HRV analysis

Table 4-1 Summary of recruited patients for HRV analysis

Patient	Age (years)	Gender	ECG	Stimulator model	Intensity (mA)	Frequency (Hz)	Pulse width (μs)	On time (sec)	Off time (min)
001	39	F	✓	105	1.25	20	250	30	5
007	42	F	3M	106	1.00	15	250	30	5
010	46	F	3M	106	1.00	25	250	30	5
011	22	M	3M	106	1.13	20	250	30	1.8
013	34	M	3M	105	1.25	20	250	30	3
014	46	F	✓	105	1.00	20	250	30	1.8
015	23	M	6M	105	1.75	25	250	30	1.8
016	25	M	✓	105	0.75	30	250	30	3
020	21	M	✓	105	1.75	25	250	30	1.8
022	42	F	✓	105	1.50	20	250	30	3
023	53	F	✓	105	1.25	20	250	30	1.8
024	22	M	✓	105	1.50	20	250	30	1.8

✓=full set of data available,
3M=pre-stimulation and 3-months only,
6M=pre-stimulation and 6-months only

A total of 15 patients were recruited into the study. Patients 003 and 021 were removed from the study due to severe learning disabilities and were not able to remain still during ECG recordings. Patient 004 was removed from the dataset due to an underlying heart condition. Patients 007 and 010 were lost to follow up after 3 months of VNS treatment but were included in data analysis. Data from patient 015 at 3 months and patients 011 and 013 at 6 months did not contain 3-minute artefact-free samples and were not included in data analysis.

4.3.2. HRV

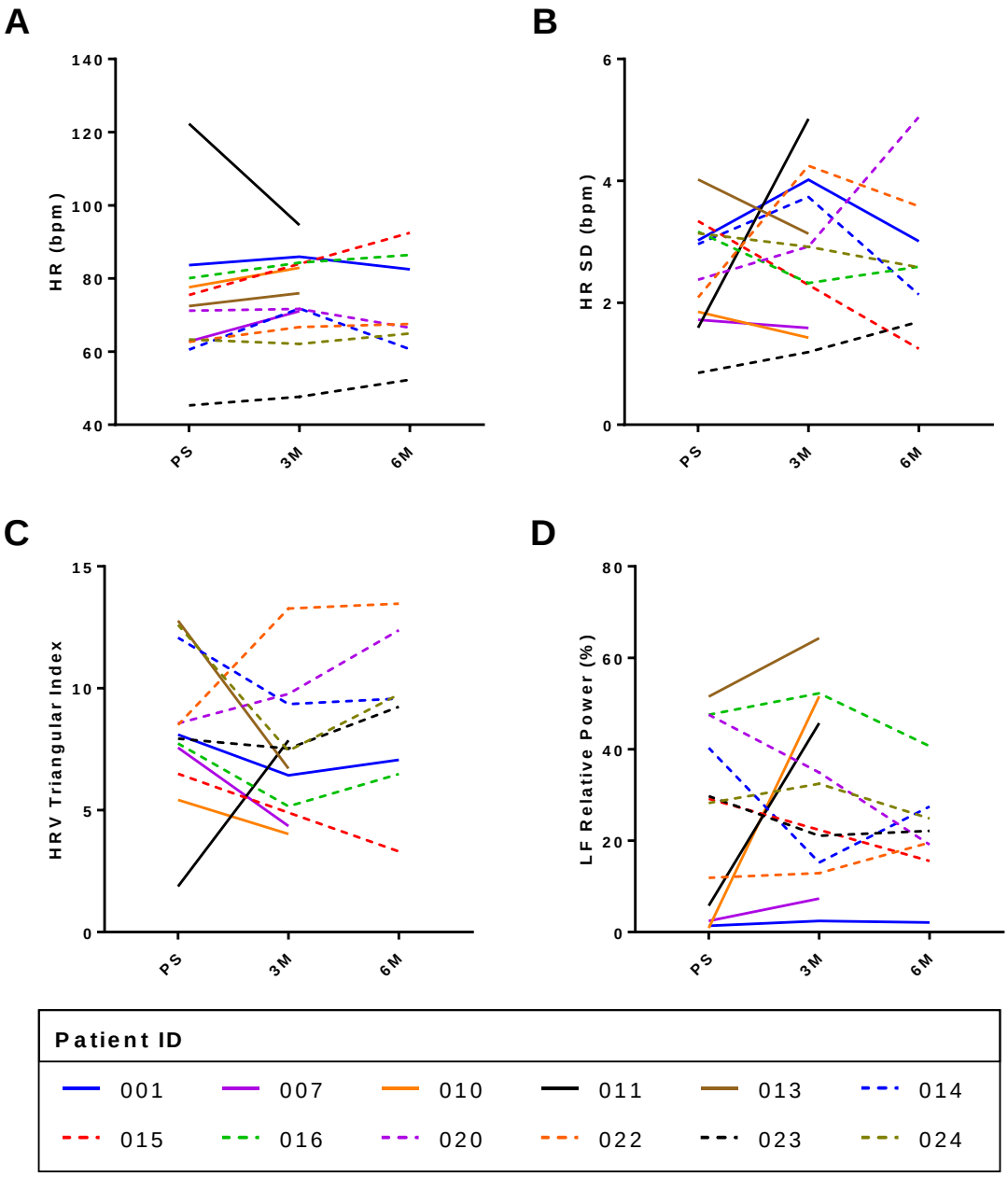

Figure 4-2 Cardiac effects in individuals following VNS in individual patients
Summary of individual changes in (A) heart rate, (B) standard deviation in heart rate, (C) HRV triangular index, (D) LF relative power, (E) HF relative power, (F) LF/HF ratio, (G) cardiac sympathetic index (CSI), and (H) cardiac vagal index (CVI). PS = Pre-stimulation, 3M = 3 months, 6M = 6 months. ** = p<0.01.

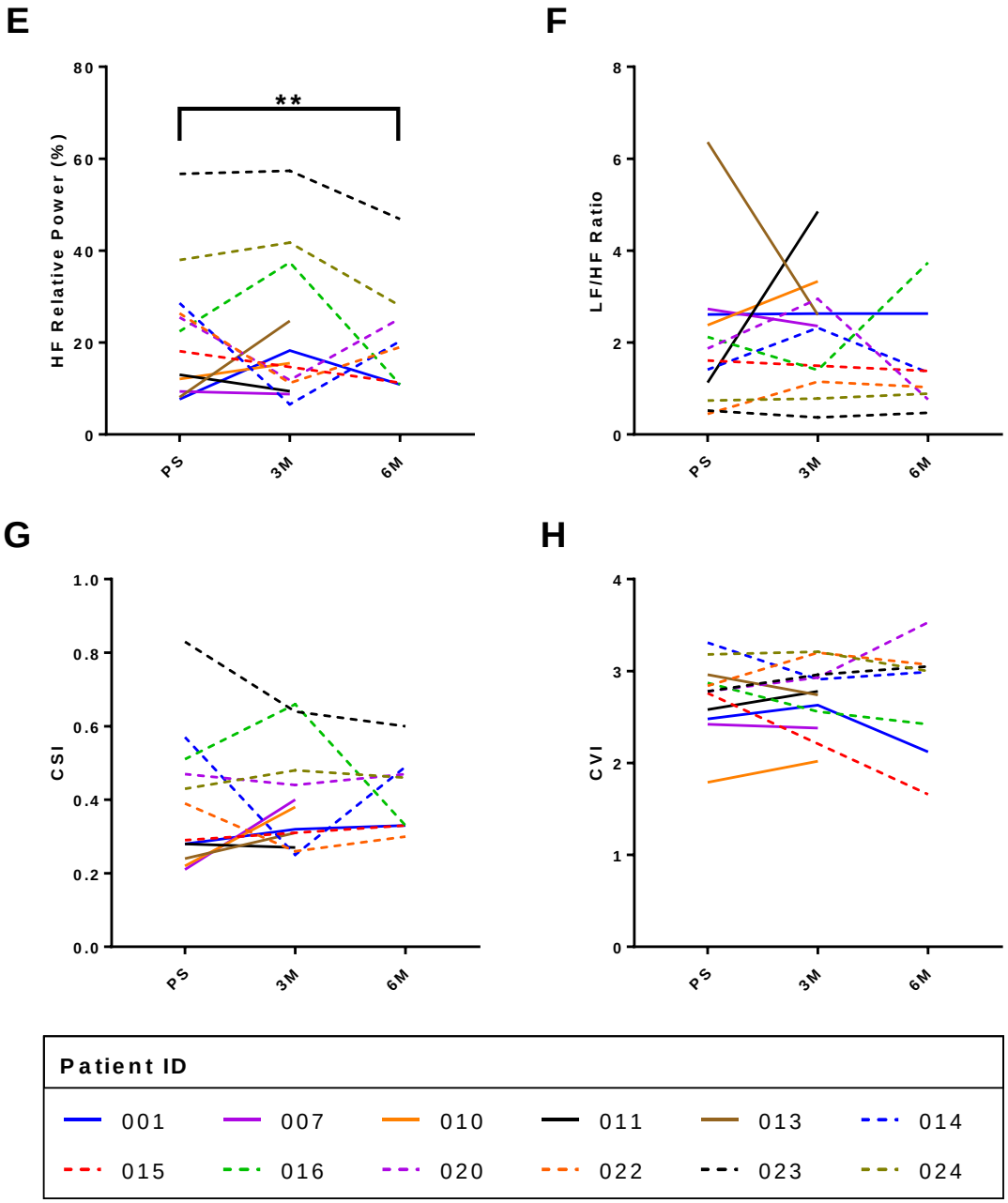

Figure 4-2 Cardiac effects in individuals following VNS in individual patients (cont'd)
Summary of individual changes in (A) heart rate, (B) standard deviation in heart rate, (C) HRV triangular index, (D) LF relative power, (E) HF relative power, (F) LF/HF ratio, (G) cardiac sympathetic index (CSI), and (H) cardiac vagal index (CVI). PS = Pre-stimulation, 3M = 3 months, 6M = 6 months. ** = $p<0.01$.

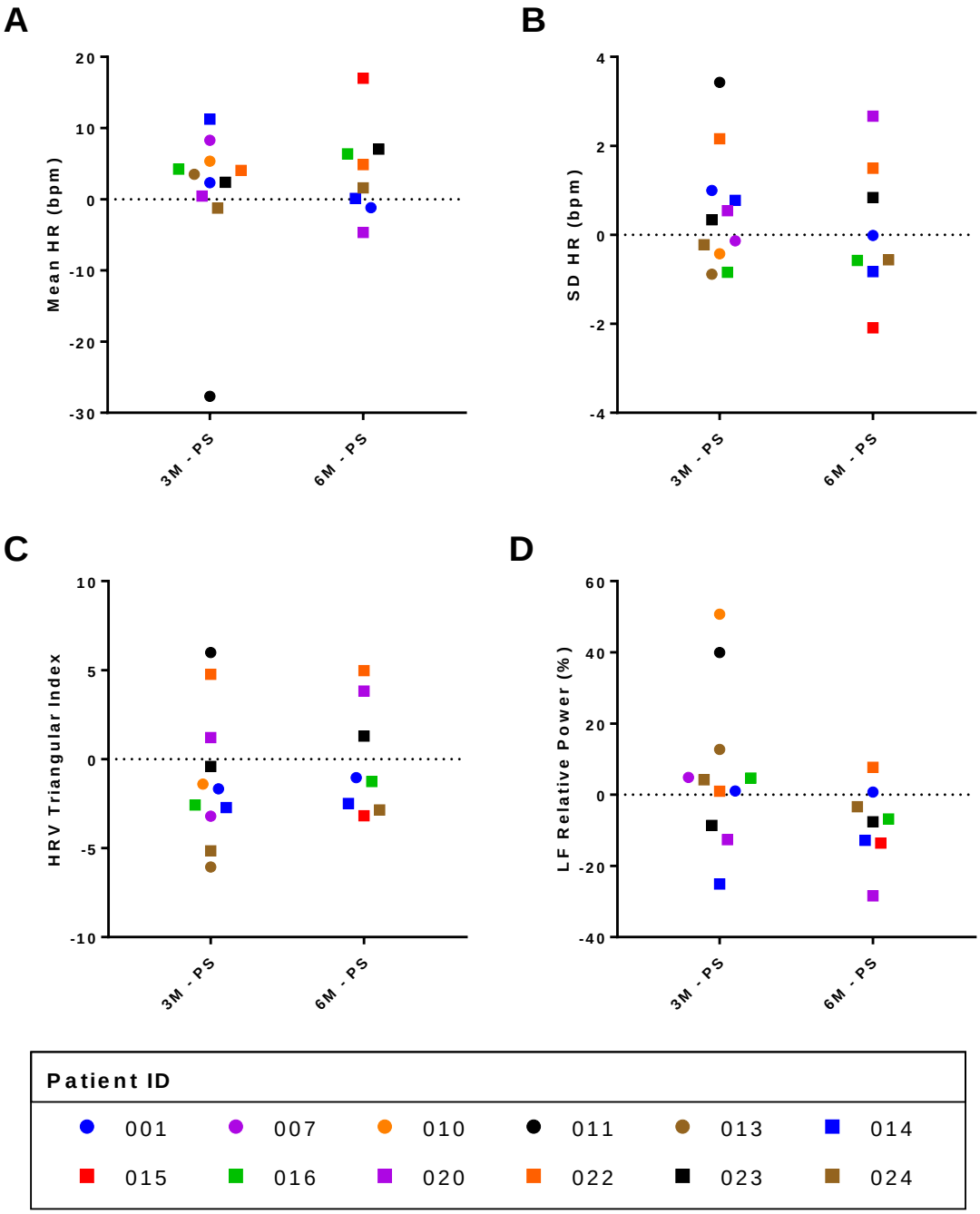

Figure 4-3 Changes in measures of HRV in individual patients
Summary of differences for (A) heart rate, (B) standard deviation in heart rate, (C) HRV triangular index, (D) LF relative power, (E) HF relative power, (F) LF/HF ratio, (G) cardiac sympathetic index(CSI), and (H) cardiac vagal index (CVI). PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

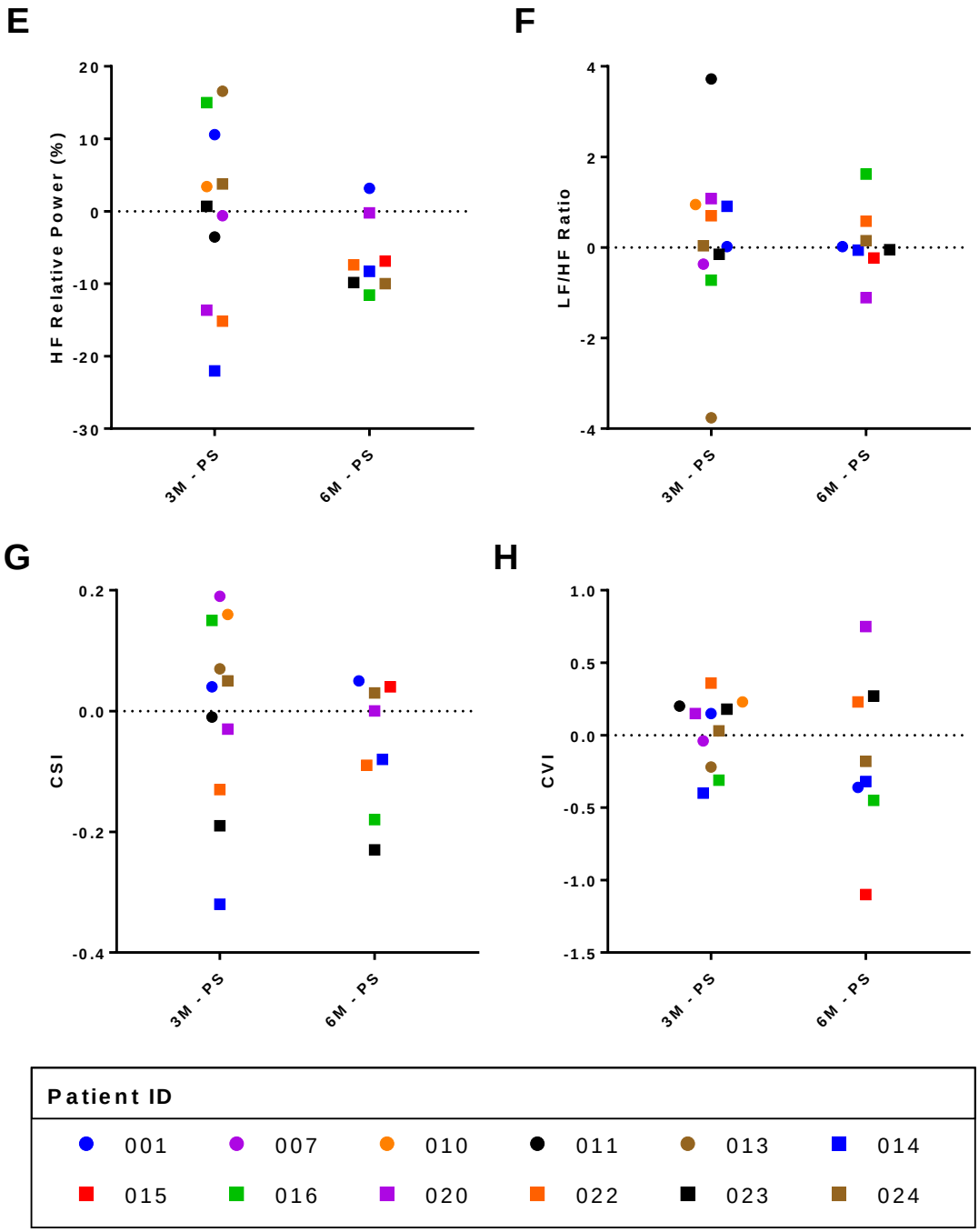

Figure 4-3 Changes in measures of HRV in individual patients (cont'd)
Summary of differences for (A) heart rate, (B) standard deviation in heart rate, (C) HRV triangular index, (D) LF relative power, (E) HF relative power, (F) LF/HF ratio, (G) cardiac sympathetic index(CSI), and (H) cardiac vagal index (CVI). PS = Pre-stimulation, 3M = 3 months, 6M = 6 months.

Table 4-2 HRV parameter analysis

	One-way ANOVA p value (n=7)	3M – PS (n=11)			6M – PS (n=8)		
		Mean diff.	SD of diff.	T test p value	Mean diff.	SD of diff.	T test p value
Mean HR (bpm)	0.211	+1.162	10.17	0.7127	+3.885	6.628	0.1413
STD HR (bpm)	0.4489	+0.5203	1.31	0.2171	+0.1164	1.496	0.8321
HRV Triangular Index	0.3912	-1.022	3.759	0.3882	-0.0931	3.115	0.935
LF Relative Power (%)	0.235	+6.625	21.85	0.3383	-8.021	10.8	0.0738
HF Relative Power (%)	0.3939	-0.4536	12.45	0.9062	-6.381	5.149	0.0099
LF/HF Ratio	0.6487	+0.2188	1.775	0.6911	+0.1161	0.7713	0.6832
CSI	0.3974	-0.0022	0.1593	0.9637	-0.0580	0.1054	0.1638
CVI	0.9474	+0.0294	0.2456	0.6996	-0.1459	0.56	0.4852

One-way ANOVA with repeated measures and Tukey corrections did not identify any significant changes in measures of HRV over time (**Table 4-2**).

Mean heart rate (**Table 4-2**) increased by 1.162 ± 10.17 beats per minute (bpm) at 3 months and 3.885 ± 6.628 bpm at 6 months compared to PS. Patient 011 had a high resting heart rate (HR) 122 bpm at PS and reduced to 95 bpm at 3 months. Although this large decrease in HR, this was likely attributed to increased exercise and weight loss. We did not find cases of bradycardia in the current study.

6/8 patients displayed a reduced LF relative power (ms^2) (**Figure 4-2D** and **Figure 4-3D**) and the reduction in mean LF relative power by 6 months was approaching significance (-8.021±10.8 ms^2, T-test p=0.0738, **Table 4-2**). 6/8 patients also displayed a reduced HF relative power (ms^2) (**Figure 4-2E** and **Figure 4-3E**)and the reduction in mean HF relative power by 6 months was statistically significant (-6.381±5.149 ms^2, T test p=0.0099, **Table 4-2**). No other measures of HRV changed significantly over time following VNS implantation according to one-way ANOVA and T test analysis (**Table 4-2**).

Changes in HRV were independent of age and gender (data not shown). Data from individual patients for different measures of HRV were compared to questionnaire results from **Chapter 3** and cell frequency, cell function, and cytokine expression results from **Chapter 5** but no trends were observed (data not shown).

4.4. Discussion

VNS did not induce bradycardia in the current study. On the contrary, we report an increased mean heart rate (HR) at 3 and 6-month post-VNS implantation but was not significantly different to HR at pre-stimulation. No significant changes in HR at 3 and 12 months were reported in a large clinical trial (n=195) observing the long term effects of VNS (Handforth *et al.*, 1998; DeGiorgio *et al.*, 2000).

We report a significant reduction in mean HF relative power 6 months following VNS implantation and the sample size was sufficiently powered to detect this (**Appendix Table 3**). LF is traditionally considered a measure of vagal tone while HF is more complex and assumed to reflect mainly sympathetic tone (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996). The significance of changes in HRV and how VNS is affecting balance between the sympathetic and vagal tone is unclear however. While some have reported an increased HF power (ms^2) and standard deviation of RR intervals (SDNN) following VNS (Libbus *et al.*, 2015), others report reduced HF power (ms^2) (Liu *et al.*, 2017), LF power (ms^2) and LF/HF ratio (Schomer *et al.*, 2014), or no changes in HRV (Garamendi *et al.*, 2017) following VNS implantation. Additionally, the current study uses 5-min recordings while other studies have used 24 hour ambulatory ECGs to remove circadian influence on HRV (Schomer *et al.*, 2014; Garamendi *et al.*, 2017; Liu *et al.*, 2017). Billman reviewed the interpretation of LF/HF ratio and summarised data on LF/HF ratio to sympathetic and parasympathetic nerve activity. Due to the non-linear relationship between these variables, Billman concluded that LF/HF ratio does not accurately quantify sympathetic and parasympathetic nerve activity (Billman, 2013).

No trends were observed when comparing HRV data to questionnaire scores, cell frequency, measures of cell function, and cytokine expression from individual patients (data not shown). This could be due to a number of limitations including: heterogeneity in patient population, maximum tolerated stimulation intensity not yet achieved, inadequate sample size, influence of the circadian rhythm on HRV and medications being taken.

In summary, although we identify a significant increase in HF power following 6 months of VNS treatment, we could not identify other changes which may be associated with changes in HRV.

Chapter 5. The Immune System

5.1. Introduction

Inflammation is the response to damage or infections to the body and is often the first step in the process of clearing and repairing any damaged tissues. However, when this process is not controlled, excessive or chronic inflammation may damage surrounding healthy tissue. Most methods of controlling inflammation in autoimmune diseases have a pharmacological approach which may have unwanted adverse effects or interactions with other drugs. Vagal nerve activity modulates the inflammatory response and electrical stimulation of this nerve dampens the inflammatory response. VNS could potentially treat inflammatory conditions such as RA, stroke, and inflammatory GI diseases without adverse effects. However, its underlying mechanisms are unclear and warrant deeper investigation.

Tregs are potent modulators of inflammation. Although treatment with VNS in rats increases Treg frequency (Morishita *et al.*, 2015; Jeremias *et al.*, 2016), changes in Tregs following VNS treatment have not been investigated in humans and may be key to the anti-inflammatory response seen in treatment with VNS. In this study, flow cytometry was performed to investigate quantitative changes in Tregs and other putative immune cells.

VNS could potentially alter the immune system in other ways such as the functional ability of cells in response to an inflammatory stimulus. Cell division is a surrogate marker of cell function (Roederer, 2011); the higher number of divisions a cell undergoes, the higher its functional capacity in response to a stimulus which can be quantified as division and proliferation indices. While the division index is the average number of divisions a cell undergoes within a sample

and is an overall measure of functional capacity for the sample. The proliferation index is the average number of divisions a cell undergoes excluding undivided cells and is a measure of the function of responding cells within a sample. In this study, flow cytometry was used to assess these functional changes in Tregs and other putative immune cells against *in vitro* methods of stimulation to mimic antigen presentation (activation of CD3 receptors) and activation of TLRs (via enterotoxin).

Some studies have investigated differences in cytokine expression following VNS (Corcoran *et al.*, 2005; Veerle De Herdt *et al.*, 2009; Majoie *et al.*, 2011; Aalbers *et al.*, 2012; Koopman *et al.*, 2016), however to our knowledge, no studies have reported the use of cytokine arrays to observe these changes. In this study, we use a semi-quantitative membrane-based array to observe relative changes in inflammatory cytokines to assess identify any changes associated with VNS treatment and may indicate an enhanced anti-inflammatory or a dampened "inflammatory tone". Changes in basal levels of inflammatory and anti-inflammatory cytokines.

Aim 3: To determine changes in anti-inflammatory cell frequency and function and in pro and anti-inflammatory cytokines after VNS

Hypothesis: VNS will alter cell frequencies and expression of cytokines in blood to promote immune tolerance. Furthermore, VNS will enhance the functional capacity of Tregs to promote immune tolerance.

5.2. General Methodology

A total of 15 patients with refractory epilepsy recruited in the study were asked to provide a blood sample at each visit before and at 3- and 6-months after VNS implantation. Whole blood was collected from the cubital vein into vacutainers containing heparin.

PBMCs were isolated by a ficoll-density gradient method according to the manufacturer's protocol. Briefly, whole blood was diluted with sterile PBS and layered gently on top of LSM. Samples were centrifuged at 400 x *g* for 40 minutes with soft start and no brake to isolate plasma and PBMCs.

Isolated PBMCs were divided to assess frequency of immune cell populations on the day of blood sampling and to assess functional changes before and 3- and 6-months after VNS. Antibodies against cell surface markers: CD4, CD8a, CD14, CD19, CD25, and intracellular marker foxp3 were used to observe changes in monocyte, B cell, CD8 T cells, CD4 T cells, $CD25^+$ cells, and $CD4^+$ $CD25^+$ $foxp3^+$ Tregs via flow cytometry and analysed in FlowJo. In the functional assay, PBMCs were stained with a proliferation dye and incubated for 96 hours under 3 conditions: control (unstimulated), anti-CD3 antibody-coated wells to mimic activation of T cells via APC pathways, and SEB to mimic activation of T cells via enterotoxin pathways in response to bacteria. Following incubation, population frequencies were assessed by flow cytometry, and the division and proliferation indices of each population were calculated using the FlowJo Proliferation platform.

Raybiotech cytokine arrays were used to investigate changes in the levels of 80 inflammatory cytokines from plasma according to manufacturer protocol. Briefly, cytokine array membranes were incubated with plasma samples, secondary

antibody cocktail, then HRP-streptavidin. The relative expression of cytokines was measured via chemiluminescence imaging and given as a percentage relative to the average of positive controls. This was analysed using paired T test with Bonferroni correction.

5.3. Results

5.3.1. Recruitment

A total of 5 healthy volunteers were recruited from the University of Sheffield and blood samples were collected for the optimisation of flow cytometry and cytokine array assays. A summary of patient recruitment for analysing the anti-inflammatory effects of VNS can be found below (**Figure 5-1**).

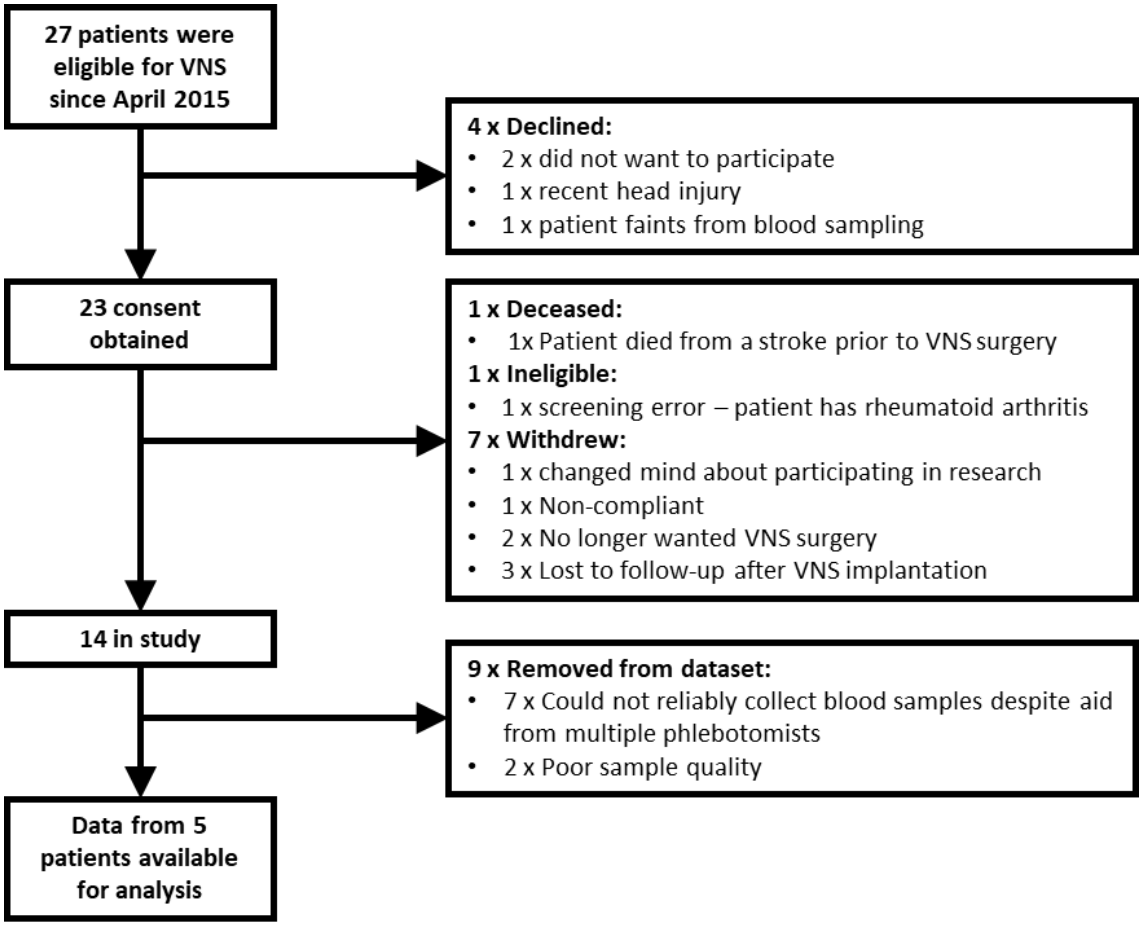

Figure 5-1 Summary of recruitment for analysing anti-inflammatory effects of VNS

Table 5-1 Summary of patients for analysing anti-inflammatory effects of VNS

Patient	Age (years)	Gender	Cell frequency	Functional assay	Cytokine array	Stimulator model	Intensity (mA)	Frequency (Hz)	Pulse width (µs)	On time (sec)	Off time (min)
011	22	M	✓	✓	✕	106	1.13	20	250	30	1.8
014	46	F	✓	✓	✓	105	1.00	20	250	30	1.8
016	25	M	✓	✓	✓	105	0.75	30	250	30	3
020	21	M	✓	✓	✓	105	1.75	25	250	30	1.8
022	42	F	✓	✓	✓	105	1.50	20	250	30	3
✓=full set of data available, ✕=data not available											

Blood samples from 5 of the 15 recruited patients were obtained prior to VNS implantation PS and at 3- and 6-months post- VNS implantation. A full list of medications taken for patients 011, 014, 016, and 022 are available in **Appendix Section 8.3.** The list of medications taken for patient 020 was not available.

5.3.2. Optimisation of flow cytometry assay

The ThermoFisher Fluorescence SpectraViewer was used to identify fluorophore and fluorescent dyes compatible with the available 4-laser flow cytometer (LSRII) and its configuration at the University of Sheffield (**Appendix Figure 2**). Commercially available antibodies conjugated to compatible fluorophores and fluorescent dyes were selected, and a panel consisting of 6 fluorescent antibodies with 6 fluorescent isotype controls, a UV cell viability dye and a fluorescent cell proliferation marker were used in the study (**Table 5-2**).

Table 5-2 Flow cytometry antibody and dye panel with corresponding controls

Antibody/Dye	Control
Mouse anti-human CD4:FITC	Mouse IgG2b K Isotype Control FITC
Mouse anti-human CD25:PE	Mouse IgG1 K Isotype Control PE
Mouse anti-human CD8a APC-eFluor780	Mouse IgG1 K Isotype Control APC-eFluor780
Mouse anti-human CD14:PerCP-Cy5.5	Mouse IgG1 K Isotype Control PerCP-Cy5.5
Mouse anti-human CD19:PE-Cy7	Mouse IgG1 K Isotype Control PE-Cy7
Rat anti-human Foxp3:APC	Rat IgG2a K Isotype Control APC
UV cell viability dye	Unstained PBMCs
Cell Proliferation Dye eFluor® 450	Unstained PBMCs

Blood samples collected from 5 adult healthy volunteers (2 male, 3 female) were used to isolate PBMCs using the manufacturer's protocol for the LSM (Lonza). PBMCs. Isolated PBMCs were incubated and stained according to the methods outlined in (**Section 2.2.7**).

Unstained PBMCs were used to calibrate voltages for forward and side scatter detection plates (used to detect size and granularity of cells) which also served as a negative control. PBMCs stained only with cell viability dye, PBMCs stained only with cell proliferation dye, and flow cytometry beads incubated with individual fluorescent antibodies (positive beads – affinity to mouse or rat antigen, negative beads – no affinity to mouse or rat antigens) were used to calibrate the voltages

of each coloured detector for the first run. PBMCs were incubated with fluorescent isotype antibodies as another negative control to ensure specific binding of fluorescent antibodies against CD4, CD25, foxp3, CD8a, CD14 and CD19. Isotype controls and unstained cells were used to determine the positive or negative expression of a receptor or dye. Optimised voltages were fixed in subsequent runs and all controls mentioned above were used at each run to ensure consistency and sensitivity between runs.

5.3.3. Cell Population Frequency

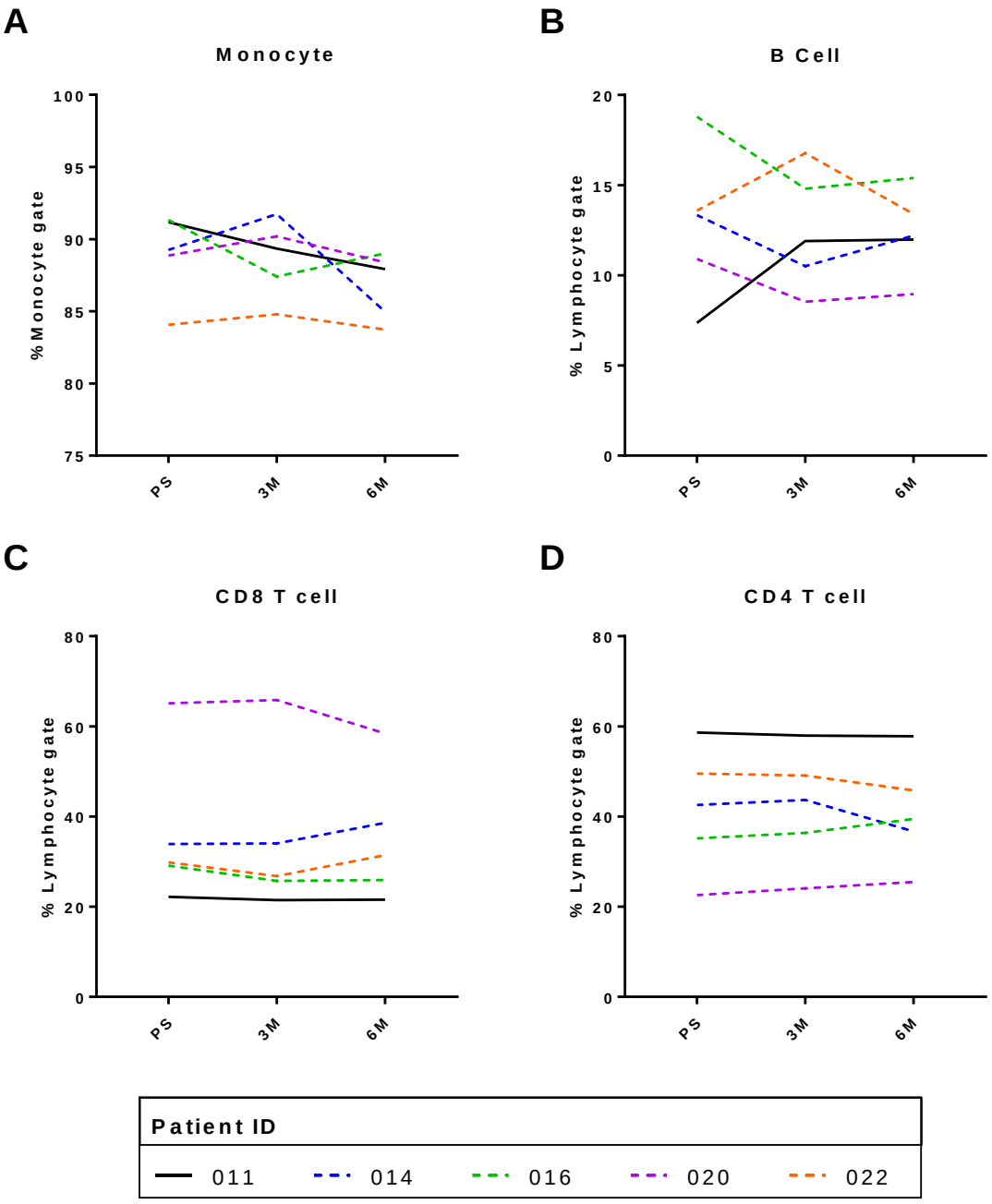

Figure 5-2 Cell frequency on day of blood sampling in individual patients
Frequency of cell types in the monocyte gate: (A) Monocyte; lymphocyte gate: (B) B cell, (C) CD8a+ cells, (D) CD4+ cells, (E) CD25+ cells, (F) CD4+ CD25+ cells, and (G) Tregs. (H) Frequency of Tregs in CD4+ cells. (I) Frequency of Tregs in CD4+ CD25+ cells. * = $p<0.05$.

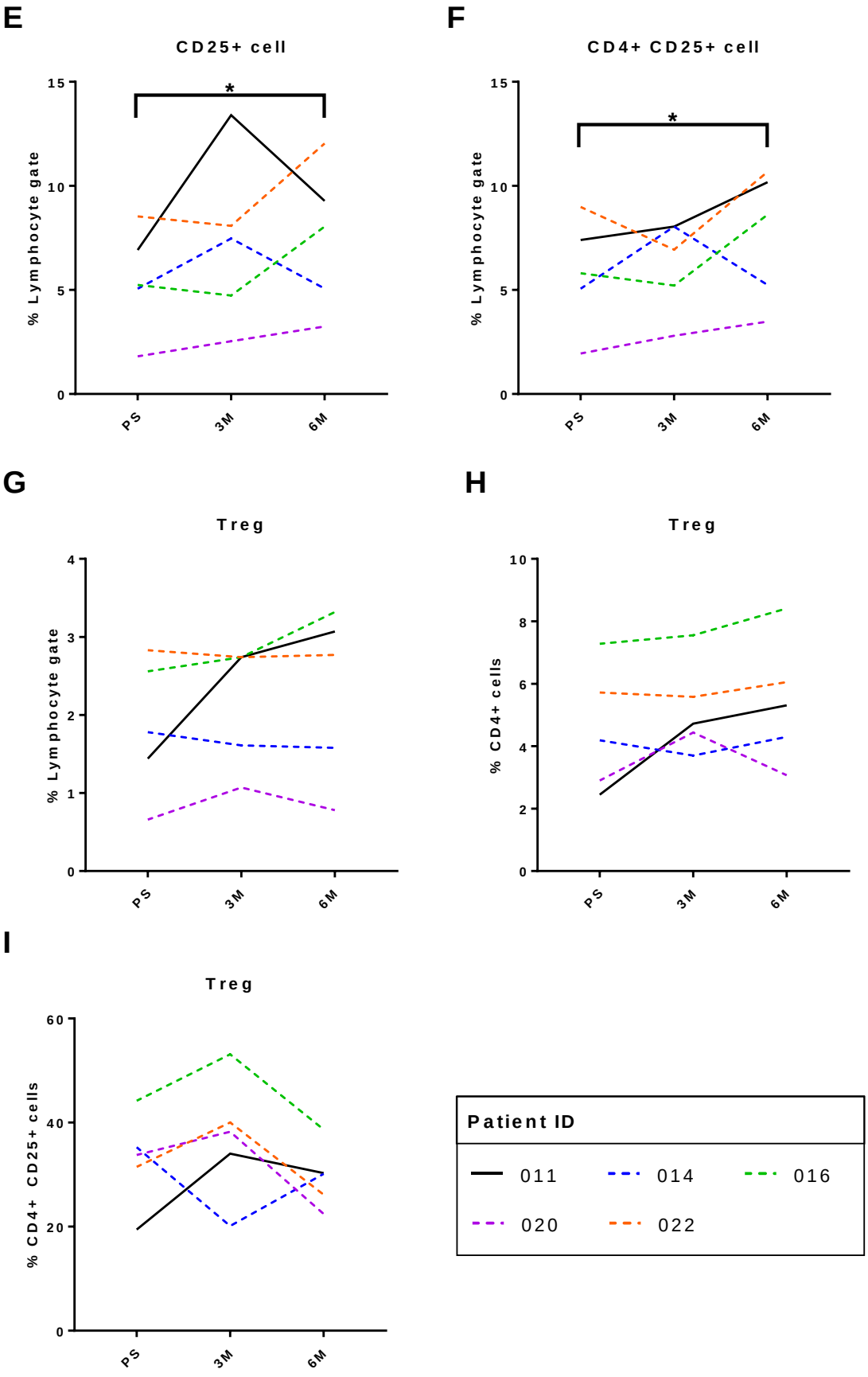

Figure 5-2 Cell frequency on day of blood sampling in individual patients (cont'd)
Frequency of cell types in the monocyte gate: (A) Monocyte; lymphocyte gate: (B) B cell, (C) CD8a+ cells, (D) CD4+ cells, (E) CD25+ cells, (F) CD4+ CD25+ cells, and (G) Tregs. (H) Frequency of Tregs in CD4+ cells. (I) Frequency of Tregs in CD4+ CD25+ cells. * = $p<0.05$.

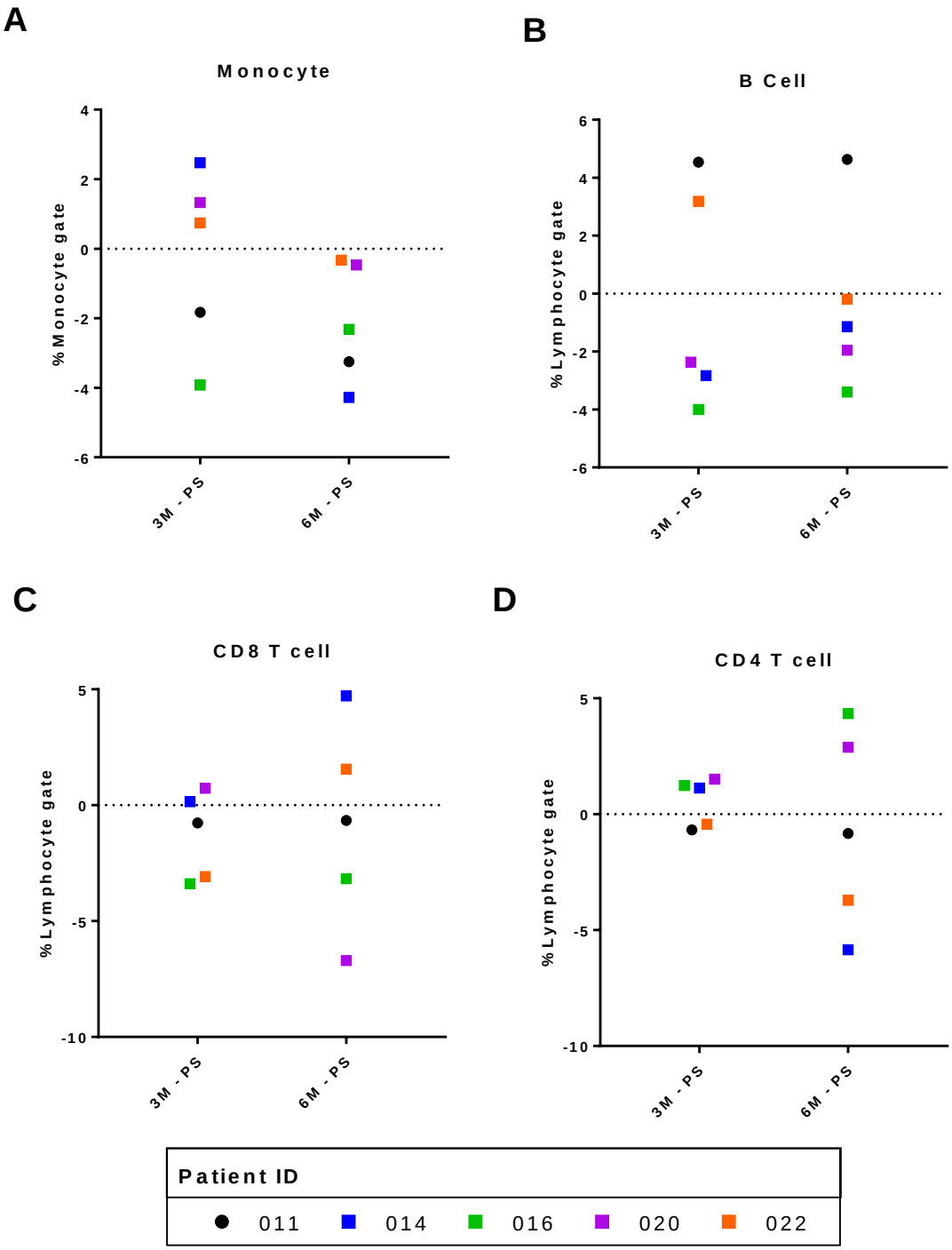

Figure 5-3 Difference in cell frequency on day of blood sampling in individual patients
Differences in cell population frequencies for (A) Monocyte, (B) B cell, (C) CD8a+ cells, (D) CD4+ cells, (E) CD25+ cells, (F) CD4+ CD25+ cells, and (G) Tregs. Differences in frequency of Tregs expressed as a % of (H) CD4+ cells and (I) CD4+ CD25+ cells.

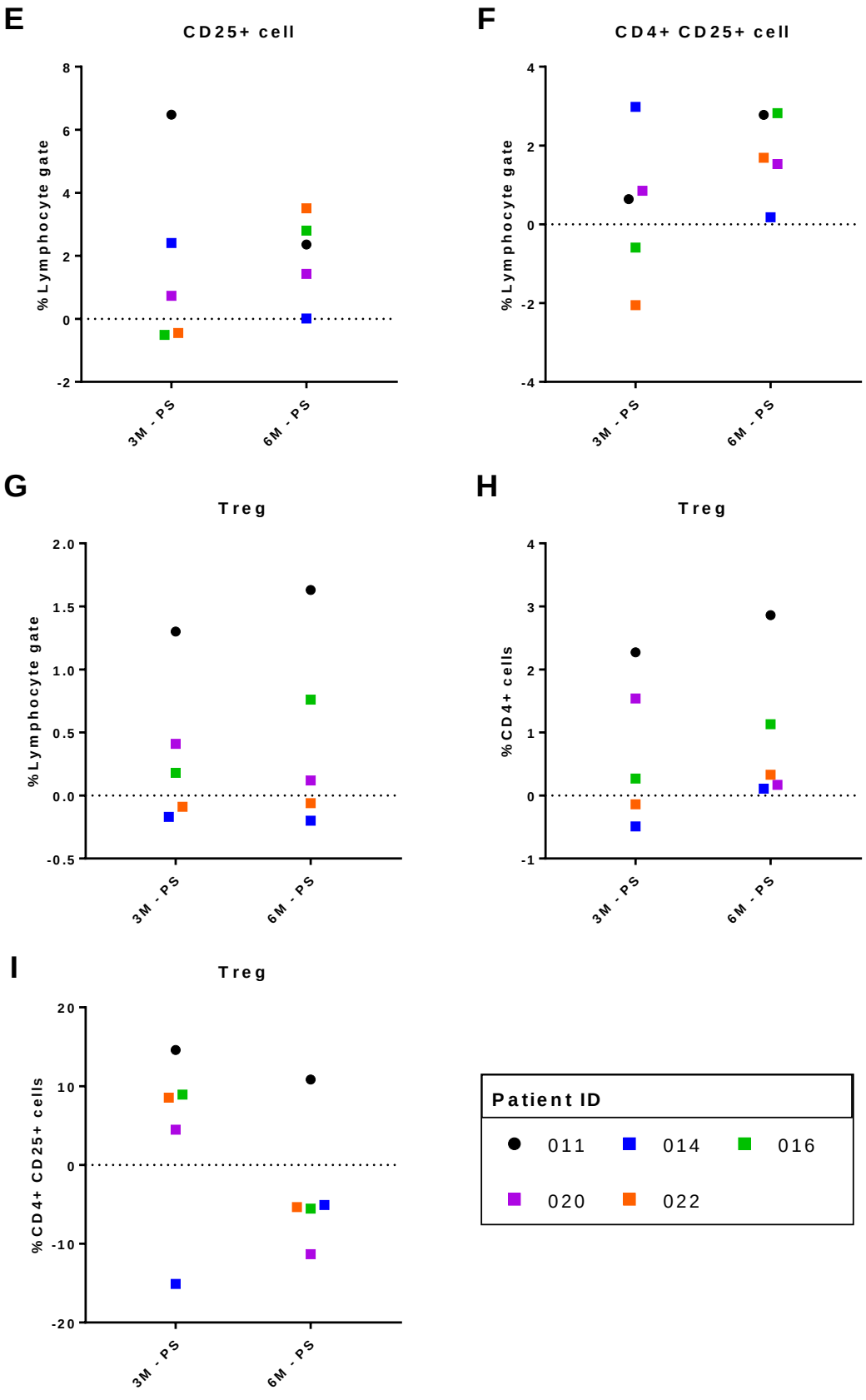

Figure 5-3 Difference in cell frequency on day of blood sampling in individual patients (cont'd)
Differences in cell population frequencies for (A) Monocyte, (B) B cell, (C) CD8a+ cells, (D) CD4+ cells, (E) CD25+ cells, (F) CD4+ CD25+ cells, and (G)Tregs. Differences in frequency of Tregs expressed as a % of (H) CD4+ cells and (I) CD4+ CD25+ cells.

Table 5-3 Analysis of cell frequency on day of blood sampling

Cell	One-way ANOVA p value (n=5)	3M – PS (n=5)			6M – PS (n=5)		
		Mean diff. (%)	SD of diff. (%)	T test p value	Mean diff. (%)	SD of diff. (%)	T test p value
Monocyte	0.1958	-0.242	2.59	0.8447	-2.13	1.725	0.0508
B cell	0.896	-0.294	3.872	0.8734	-0.41	3.053	0.7789
CD8+	0.6717	-1.272	1.873	0.2035	-0.852	4.369	0.6853
CD4+	0.6297	+0.554	1.026	0.2936	-0.634	4.299	0.7581
CD25+	0.2753	+1.732	2.907	0.2536	+2.022	1.354	0.0289
CD4+CD25+	0.1966	+0.366	1.864	0.6832	+1.8	1.085	0.0207
Treg	0.2768	+0.326	0.5908	0.2848	+0.45	0.755	0.2535
Treg/CD4+	0.21	+0.69	1.17	0.2577	+0.92	1.159	0.1506
Treg/CD4+CD25+	0.3129	+4.28	11.42	0.4492	-3.288	8.322	0.4269

Comparing differences in cell population frequencies in individuals revealed a reduction in monocytes for 5/5 patients (**Figure 5-2A** and **Figure 5-3A**) and increase in CD25+ cells for 4/5 patients (**Figure 5-2E** and **Figure 5-3E**) and CD4+ CD25+ cells for 4/5 patients (**Figure 5-2F** and **Figure 5-3F**) at 6 months. There was an increased expression of Tregs in CD4+ CD25+ cells for 4/5 patients at 3 months (**Figure 5-2I** and **Figure 5-3I**), and CD4+ cells for 5/5 patients at 6 months (**Figure 5-2H** and **Figure 5-3H**). No other trends in cell frequencies were found.

One-way ANOVA analysis (**Table 5-3**) did not detect significant change over time in any of the tested cell populations. However, paired T test analysis (**Table 5-3**) revealed a significant increase of +2.022±1.354% in CD25+ ($p=0.0289$) and +1.8±1.085% in CD4+ CD25+ cells ($p=0.0207$) from the lymphocyte gate at 6 months post-VNS implantation compared to pre-stimulation in 5/5 patients. A reduction of -2.13±1.725% in monocyte population from the monocyte gate was detected by 6-months post-VNS implantation in 5/5 patients and was approaching statistical significance ($p=0.0508$).

5.3.4. Functional Assay

5.3.4.1. Frequency

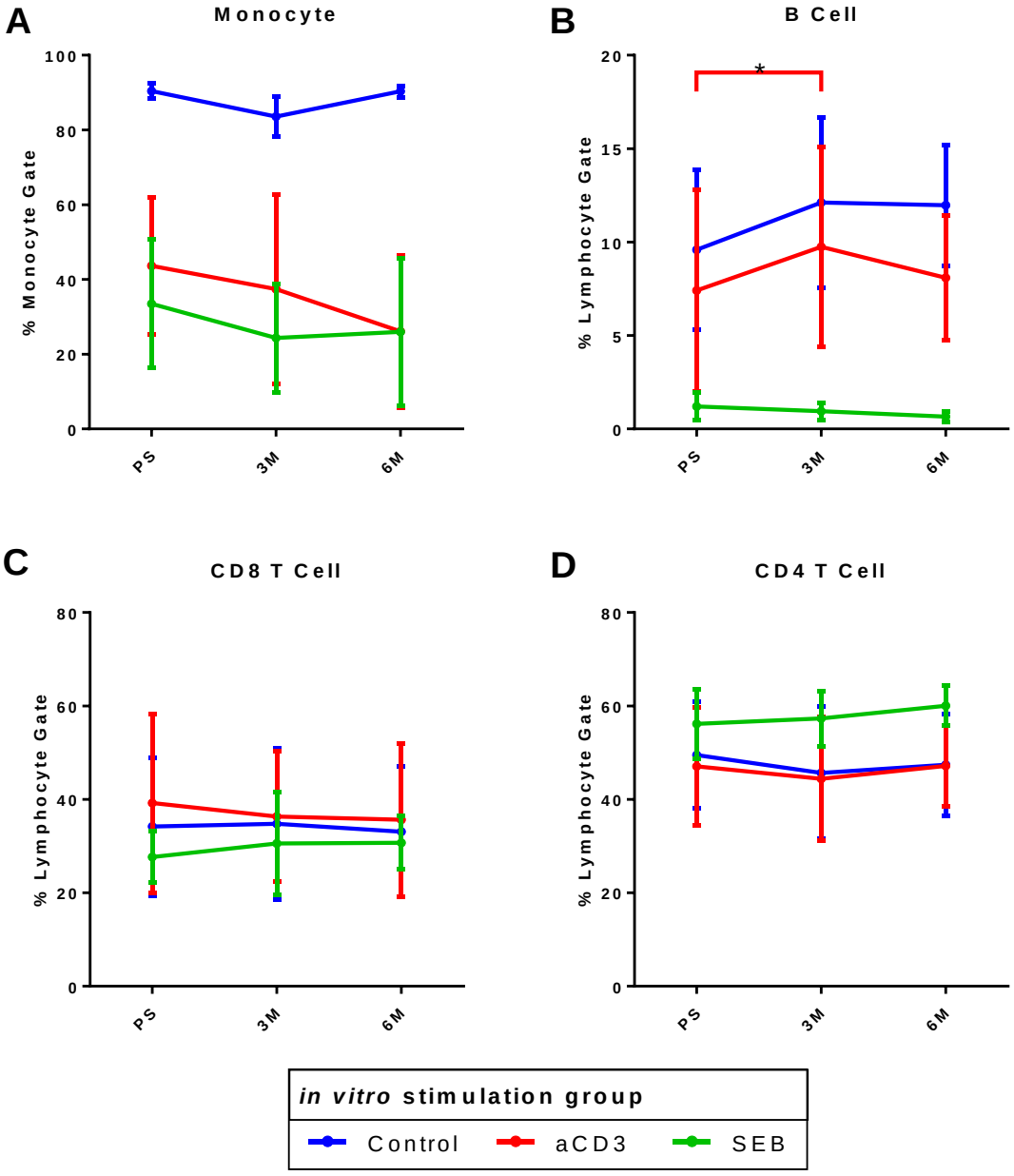

Figure 5-4 Cell frequencies following functional assay
Mean cell population frequencies for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs. Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05 between timepoints within the aCD3 (red) or SEB (green) groups.

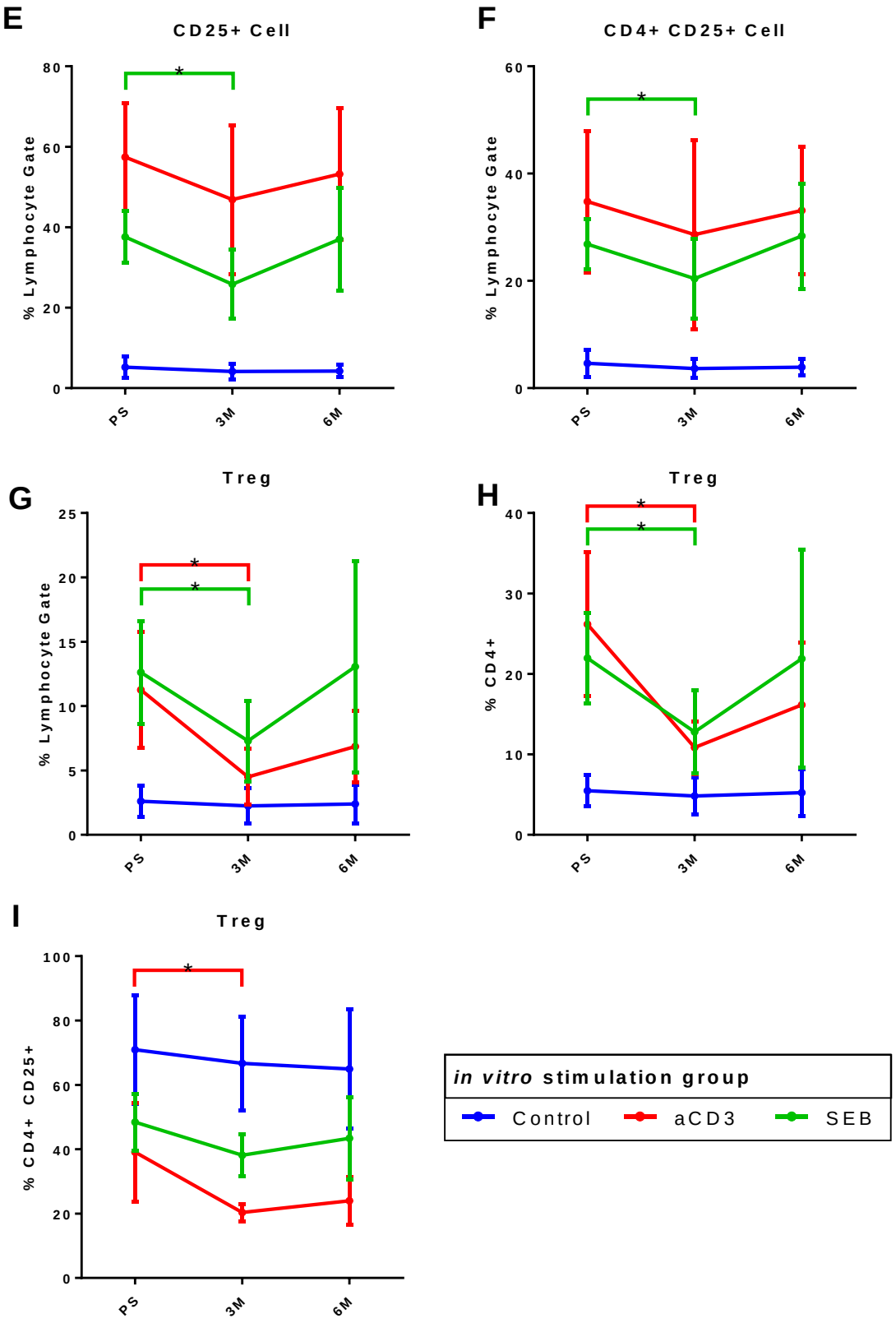

Figure 5-4 Cell frequencies following functional assay (cont'd)
Mean cell population frequencies for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs. Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05 between timepoints within the aCD3 (red) or SEB (green) groups.

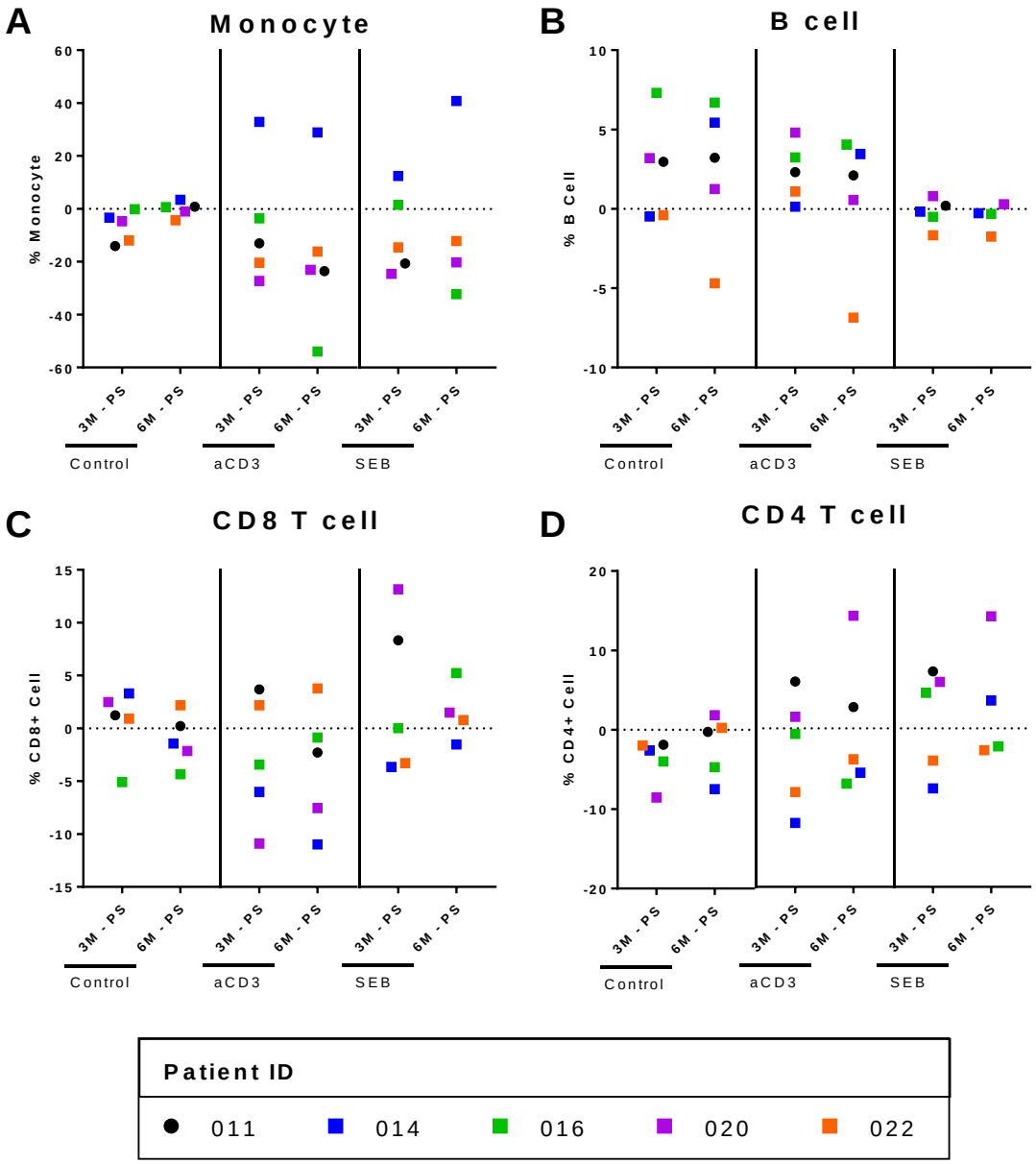

Figure 5-5 Difference in cell frequency following functional assay in individual patients
Differences in cell frequency for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

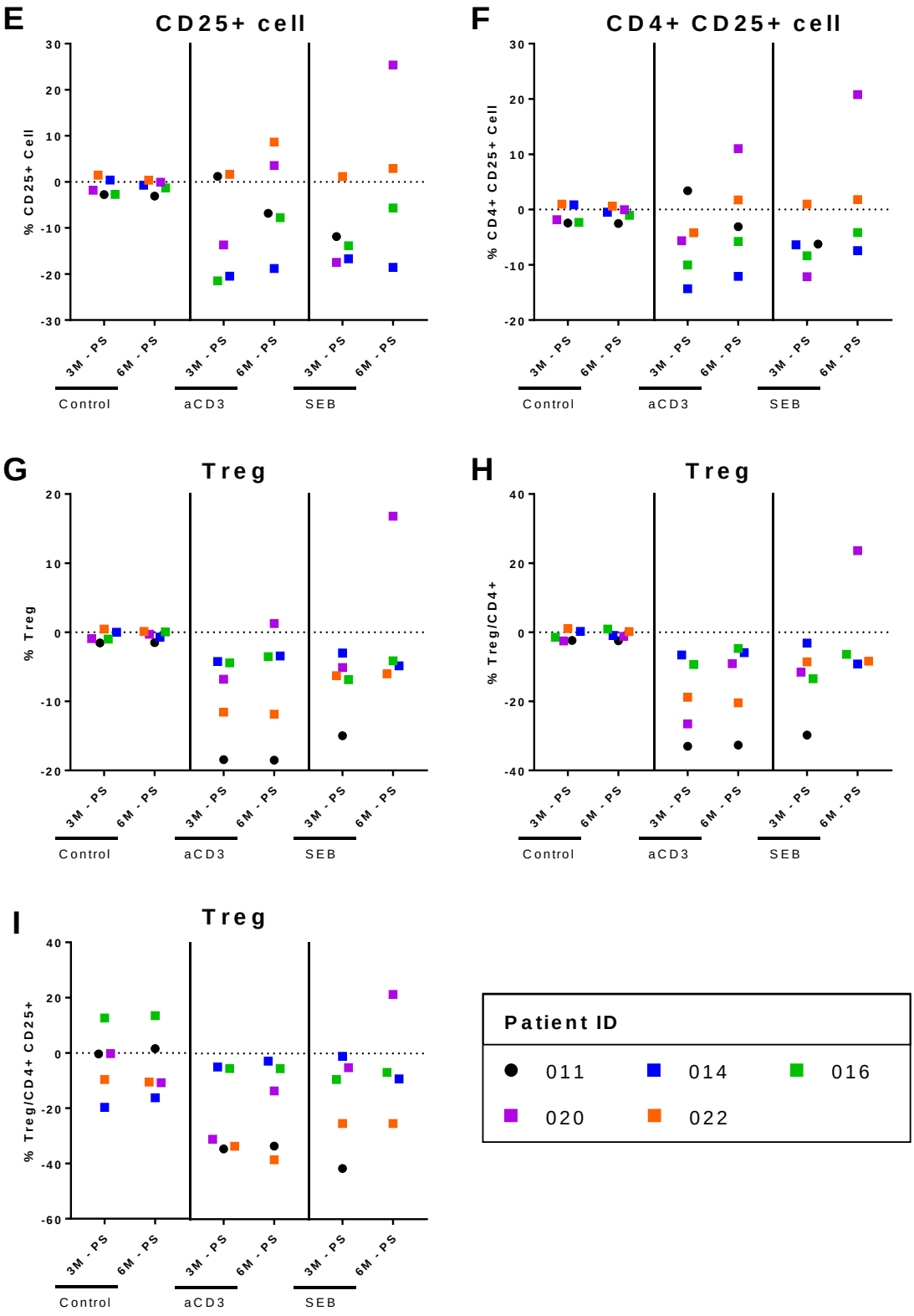

Figure 5-5 Difference in cell frequency following functional assay in individual patients (cont'd)

Differences in cell frequency for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

Table 5-4 Two-way ANOVA of cell frequency following functional assay

	Two-way ANOVA p values		
	Time	Condition	Time x Condition
Monocyte	0.7265	0.0002	0.593
B cell	0.6395	0.0003	0.3636
CD8+	0.6873	0.1372	0.5009
CD4+	0.4842	0.0037	0.5109
CD25+	0.1768	0.0003	0.2276
CD4+CD25+	0.2361	<0.0001	0.2645
Treg	0.2158	0.0002	0.2643
Treg/CD4+	0.1181	0.001	0.1465
Treg/CD4+ CD25+	0.1122	0.0043	0.5385

Table 5-5 Paired T test for cell frequency following functional assay

Cell	Condition	Comparison	Mean Diff. (%)	SD of Diff. (%)	Paired T test P Value
Monocyte	Control	3M-PS	-6.848	5.932	0.061
		6M-PS	-0.098	2.867	0.943
	aCD3	3M-PS	-6.224	23.586	0.587
		6M-PS	-17.516	29.829	0.259
	SEB	3M-PS	-9.126	15.666	0.263
		6M-PS	-5.903	32.273	0.739
B Cell	Control	3M-PS	+2.520	3.207	0.154
		6M-PS	+2.378	4.472	0.300
	aCD3	3M-PS	+2.328	1.822	0.046
		6M-PS	+0.674	4.416	0.750
	SEB	3M-PS	-0.264	0.920	0.556
		6M-PS	-0.505	0.869	0.329
CD8 T Cell	Control	3M-PS	+0.570	3.307	0.720
		6M-PS	-1.106	2.451	0.370
	aCD3	3M-PS	-2.894	5.985	0.340
		6M-PS	-3.582	5.781	0.238
	SEB	3M-PS	+2.912	7.483	0.433
		6M-PS	+1.488	2.801	0.366
CD4 T Cell	Control	3M-PS	-3.804	2.778	0.038
		6M-PS	-2.078	3.878	0.297
	aCD3	3M-PS	-2.656	7.226	0.457
		6M-PS	+0.090	8.717	0.983
	SEB	3M-PS	+1.176	6.577	0.710
		6M-PS	+3.153	7.857	0.481

Table 5-4 Paired T test for cell frequency following functional assay (cont'd)

Cell	Condition	Comparison	Mean Diff. (%)	SD of Diff. (%)	Paired T test P Value
CD25+ Cell	Control	3M-PS	-1.088	1.913	0.272
		6M-PS	-0.958	1.342	0.186
	aCD3	3M-PS	-10.558	11.343	0.106
		6M-PS	-4.228	10.701	0.427
	SEB	3M-PS	-11.748	7.549	0.025
		6M-PS	+1.013	18.475	0.920
CD4+ CD25+ Cell	Control	3M-PS	-0.950	1.723	0.285
		6M-PS	-0.694	1.190	0.262
	aCD3	3M-PS	-6.164	6.649	0.107
		6M-PS	-1.640	8.663	0.694
	SEB	3M-PS	-6.430	4.776	0.040
		6M-PS	+2.748	12.628	0.693
Treg	Control	3M-PS	-0.602	0.818	0.175
		6M-PS	-0.466	0.661	0.190
	aCD3	3M-PS	-9.090	6.003	0.028
		6M-PS	-7.216	7.893	0.110
	SEB	3M-PS	-7.248	4.566	0.024
		6M-PS	+0.440	10.934	0.941
Treg/CD4+	Control	3M-PS	-1.002	1.592	0.232
		6M-PS	-0.686	1.309	0.306
	aCD3	3M-PS	-18.858	11.173	0.020
		6M-PS	-14.560	11.884	0.052
	SEB	3M-PS	-13.294	10.007	0.041
		6M-PS	-0.075	15.854	0.993
Treg/CD4+ CD25+	Control	3M-PS	-3.456	12.036	0.556
		6M-PS	-4.498	11.977	0.448
	aCD3	3M-PS	-21.910	15.331	0.033
		6M-PS	-18.794	16.300	0.061
	SEB	3M-PS	-16.534	16.772	0.092
		6M-PS	-5.020	19.403	0.641

A trend in reduced monocyte frequency was seen in PBMCs incubated with anti-CD3 antibody and SEB (**Figure 5-4A** and **Figure 5-5A**), and increased frequencies for B cells (**Figure 5-4B** and **Figure 5-5B**). Treg frequency reduced when incubated with anti-CD3 antibody 3 months following VNS implantation (**Figure 5-4G and H**, and **Figure 5-5G and H**). No other trends in cell frequency were found.

Two-way ANOVA for cell population frequencies (**Table 5-5**) showed significant differences between conditions in all cell populations except CD8+ cells. No significant effects were seen over time and between time and conditions.

Pair-wise comparisons (**Table 5-5**) revealed significant increase in B cell frequency ($p=0.046$) and reduction in Treg frequency (when calculated as % of lymphocytes: $p=0.028$, % of CD4+ cells: $p=0.020$, and % of CD4+ CD25+ cells $p=0.033$) in response to anti-CD3 antibody at 3 months. Significant decreases in were also found for CD25+ cell ($p=0.025$), CD4+ CD25+ cell ($p=0.040$), and Treg frequency (when calculated as % of lymphocytes: $p=0.024$) in response to SEB at 3 months. The expression of Tregs within the CD4+ and CD4+ CD25+ population decreased at 3 months post-VNS implantation in response to anti-CD3 antibody.

5.3.4.2. Division Index

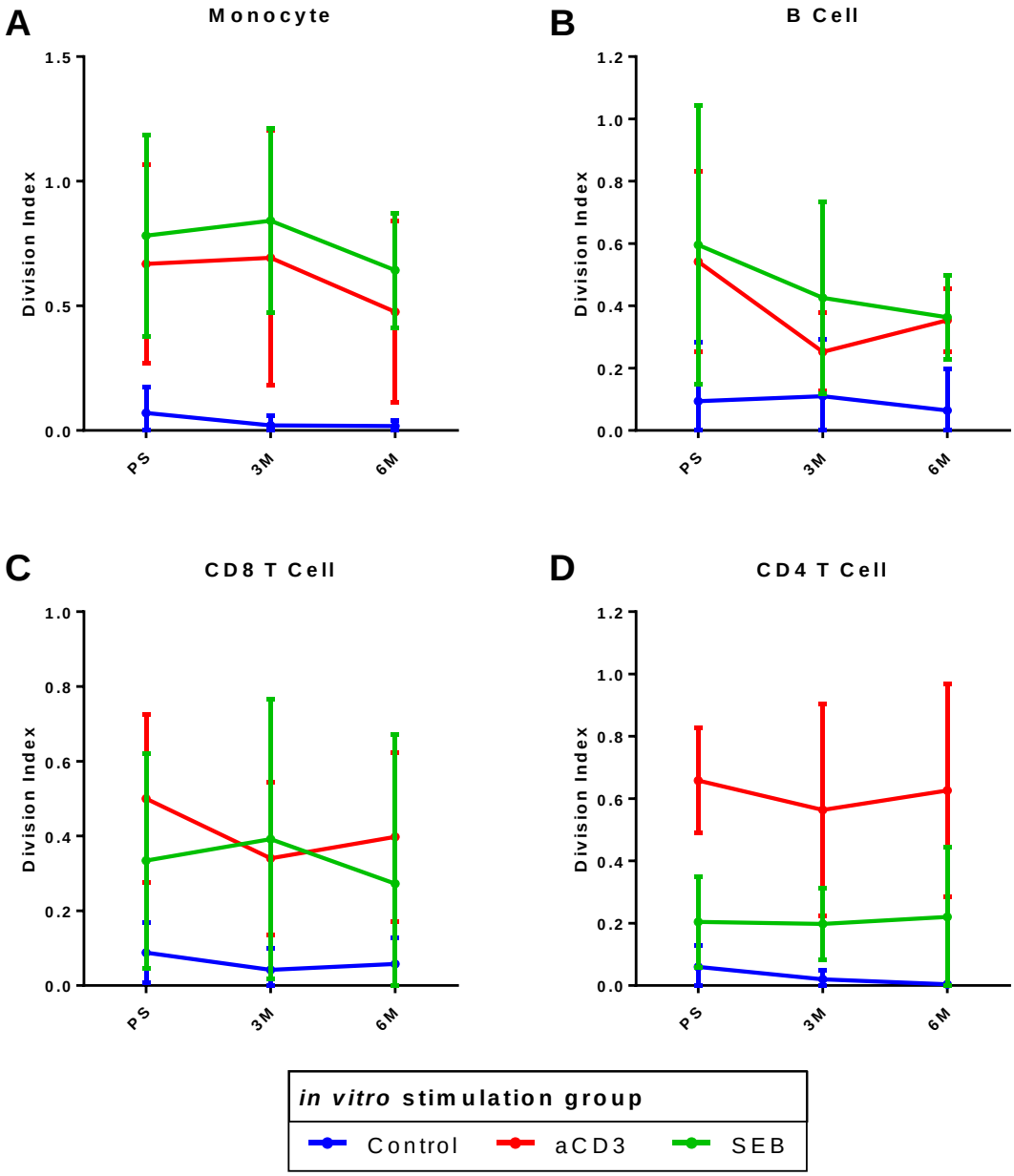

Figure 5-6 Division index of cells following functional assay
Mean division indices for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G) Tregs. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05, **p<0.001 between timepoints within the aCD3 (red) or SEB (green) groups.

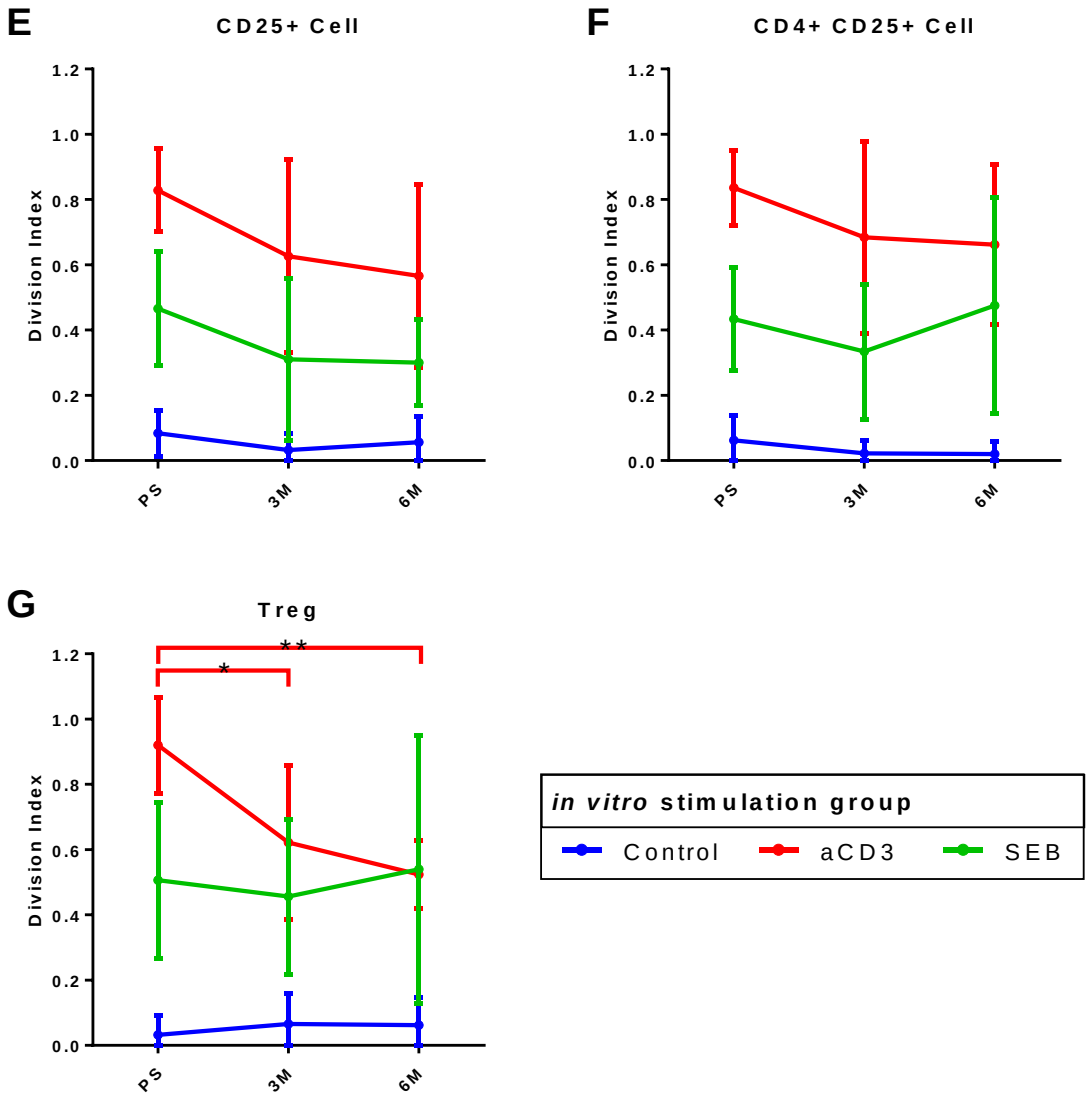

Figure 5-6 Division index of cells following functional assay (cont'd)
Mean division indices for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G) Tregs. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05, **p<0.001 between timepoints within the aCD3 (red) or SEB (green) groups.

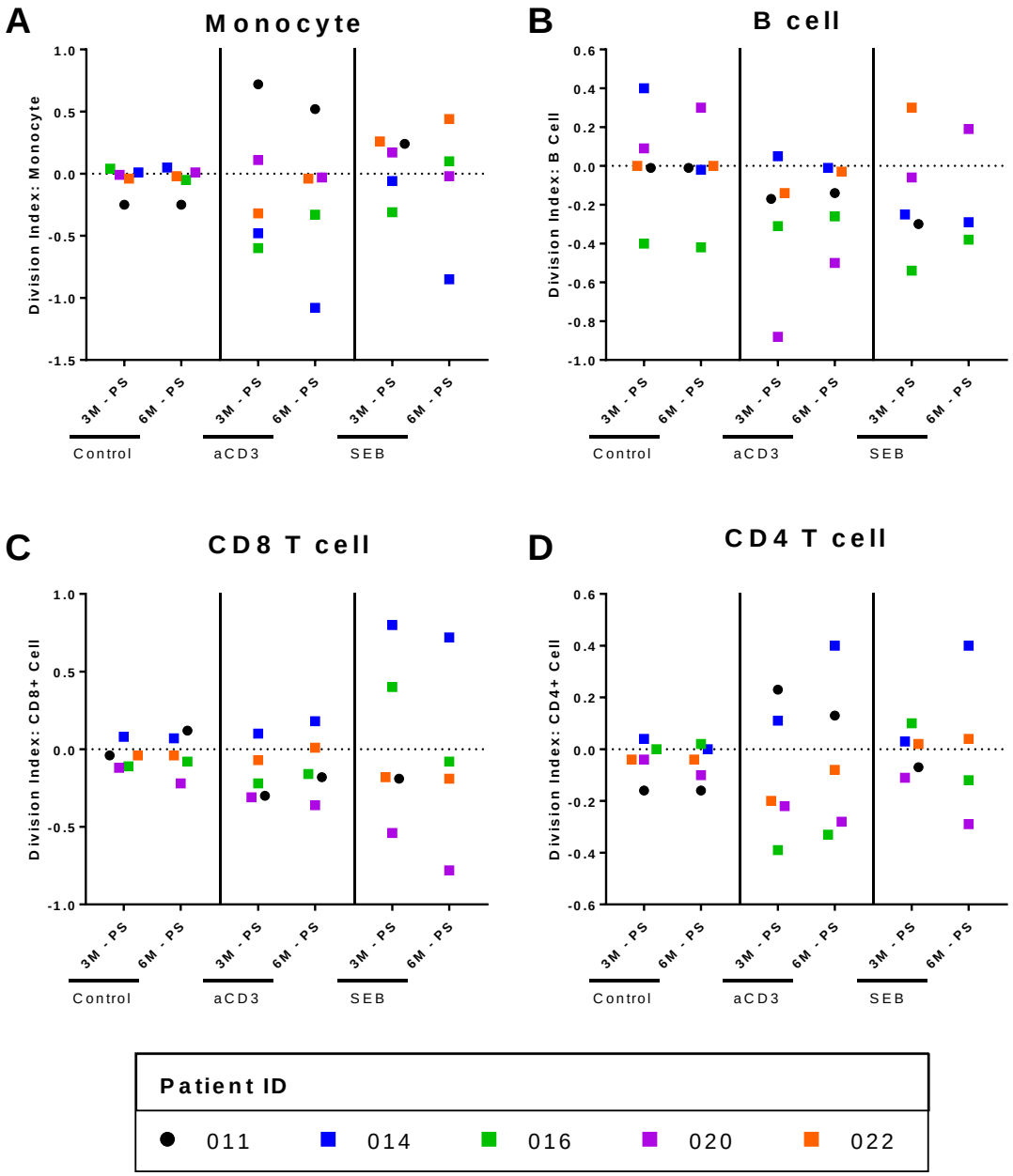

Figure 5-7 Differences in division index of cells following functional assay in individual patients

Differences in division index for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

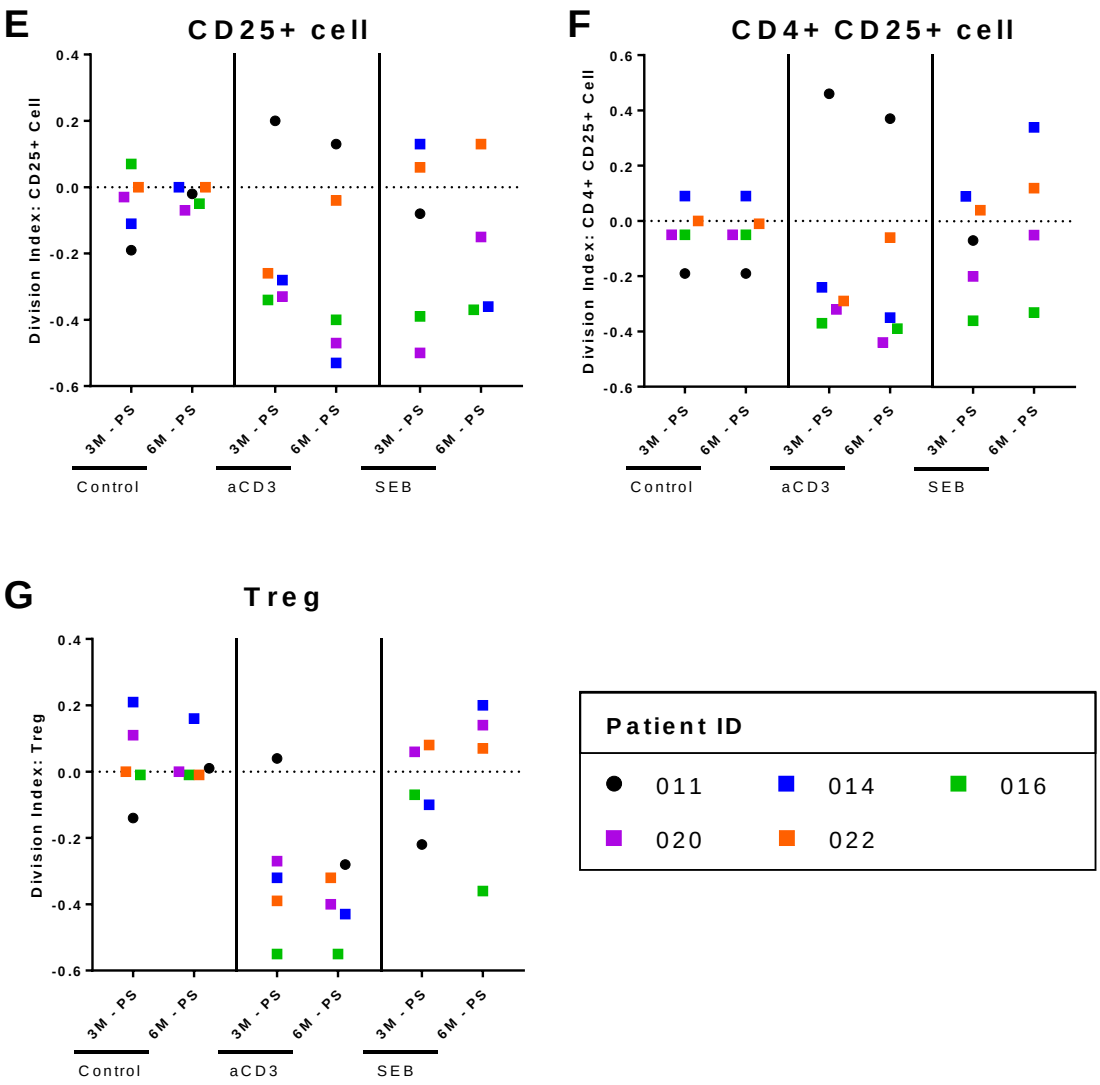

Figure 5-7 Differences in division index of cells following functional assay in individual patients (cont'd)

Differences in division index for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

Table 5-6 Two-way ANOVA of division index following functional assay

	Two-way ANOVA p values		
	Time	Condition	Time x Condition
Monocyte	0.561	0.0016	0.339
B cell	0.345	0.0712	0.526
CD8+	0.809	0.0121	0.592
CD4+	0.759	0.0019	0.464
CD25+	0.054	<0.0001	0.211
CD4+CD25+	0.093	0.0006	0.013
Treg	0.071	0.0018	0.001

Table 5-7 Paired T test for division index following functional assay

Cell	Condition	Comparison	Mean Diff.	SD of Diff.	P Value
Monocyte	Control	3M-PS	-0.050	0.116	0.388
		6M-PS	-0.052	0.117	0.375
	aCD3	3M-PS	-0.114	0.538	0.850
		6M-PS	-0.192	0.584	0.503
	SEB	3M-PS	+0.060	0.243	0.610
		6M-PS	-0.083	0.548	0.783
B Cell	Control	3M-PS	+0.016	0.286	0.906
		6M-PS	-0.030	0.256	0.806
	aCD3	3M-PS	-0.290	0.354	0.141
		6M-PS	-0.188	0.201	0.105
	SEB	3M-PS	-0.170	0.314	0.292
		6M-PS	-0.160	0.306	0.461
CD8+ Cell	Control	3M-PS	-0.046	0.080	0.267
		6M-PS	-0.030	0.133	0.642
	aCD3	3M-PS	-0.160	0.174	0.109
		6M-PS	-0.102	0.205	0.328
	SEB	3M-PS	+0.058	0.535	0.820
		6M-PS	-0.083	0.617	0.806
CD4+ Cell	Control	3M-PS	-0.040	0.075	0.298
		6M-PS	-0.056	0.074	0.166
	aCD3	3M-PS	-0.094	0.256	0.457
		6M-PS	-0.032	0.302	0.825
	SEB	3M-PS	-0.006	0.084	0.881
		6M-PS	+0.008	0.294	0.963

Table 5-6 Paired T test for division index following functional assay (cont'd)

Cell	Condition	Comparison	Mean Diff.	SD of Diff.	P Value
CD25+ Cell	Control	3M-PS	-0.052	0.101	0.312
		6M-PS	-0.028	0.031	0.115
	aCD3	3M-PS	-0.202	0.227	0.118
		6M-PS	-0.262	0.290	0.114
	SEB	3M-PS	-0.156	0.277	0.277
		6M-PS	-0.188	0.235	0.208
CD4+ CD25+ Cell	Control	3M-PS	-0.040	0.101	0.428
		6M-PS	-0.042	0.101	0.403
	aCD3	3M-PS	-0.152	0.345	0.381
		6M-PS	-0.174	0.338	0.314
	SEB	3M-PS	-0.100	0.183	0.289
		6M-PS	+0.020	0.283	0.896
Treg	Control	3M-PS	+0.034	0.132	0.597
		6M-PS	+0.030	0.073	0.411
	aCD3	3M-PS	-0.298	0.216	0.037
		6M-PS	-0.396	0.105	0.001
	SEB	3M-PS	-0.050	0.123	0.416
		6M-PS	+0.013	0.254	0.928

Comparing mean division indices over time (**Figure 5-6**), the control group had division indices of <0.1 for all cell types. PBMCS incubated with anti-CD3 antibodies displayed a trend in reducing division indices for monocytes, B cells, CD4 T cells, CD25+ cells, CD4+ CD25+ cells and Tregs (**Figure 5-6B, D-G**). PBMCS incubated with SEB displayed a trend in reducing division indices for monocyte, B cell and CD25+ cells (**Figure 5-6A, B, E**).

Comparing the changes in division index from individuals (**Figure 5-7**), we found a trend in reducing division indices for B cells, CD25+ cells, and CD4+ CD25+ cells in response to anti-CD3 antibodies but not in response to SEB.

Two-way ANOVA for division indices (**Table 5-7**) revealed a significant difference between conditions over time for all cell types except B cells. Significant differences across time and conditions were detected for CD4+ CD25+ cells and Tregs.

Pairwise comparisons (**Table 5-7**) revealed a significant decrease in Treg division in response to anti-CD3 antibodies at 3- ($p=0.037$) and 6-months ($p=0.001$) following VNS implantation.

5.3.4.3. Proliferation Index

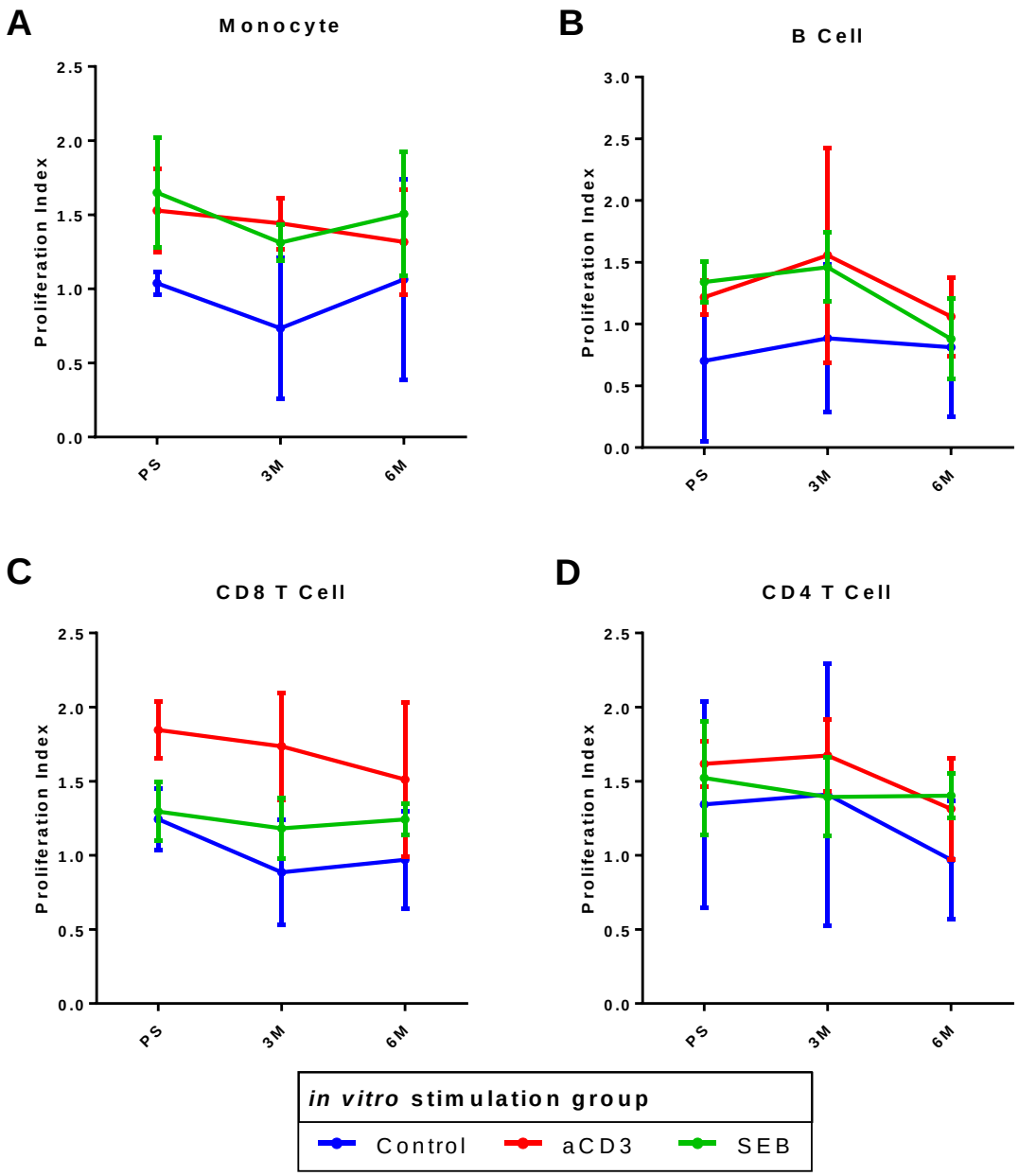

Figure 5-8 Proliferation Index of cells following functional assay
Mean proliferation indices for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G) Tregs. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05 between timepoints within the aCD3 (red) or SEB (green) groups.

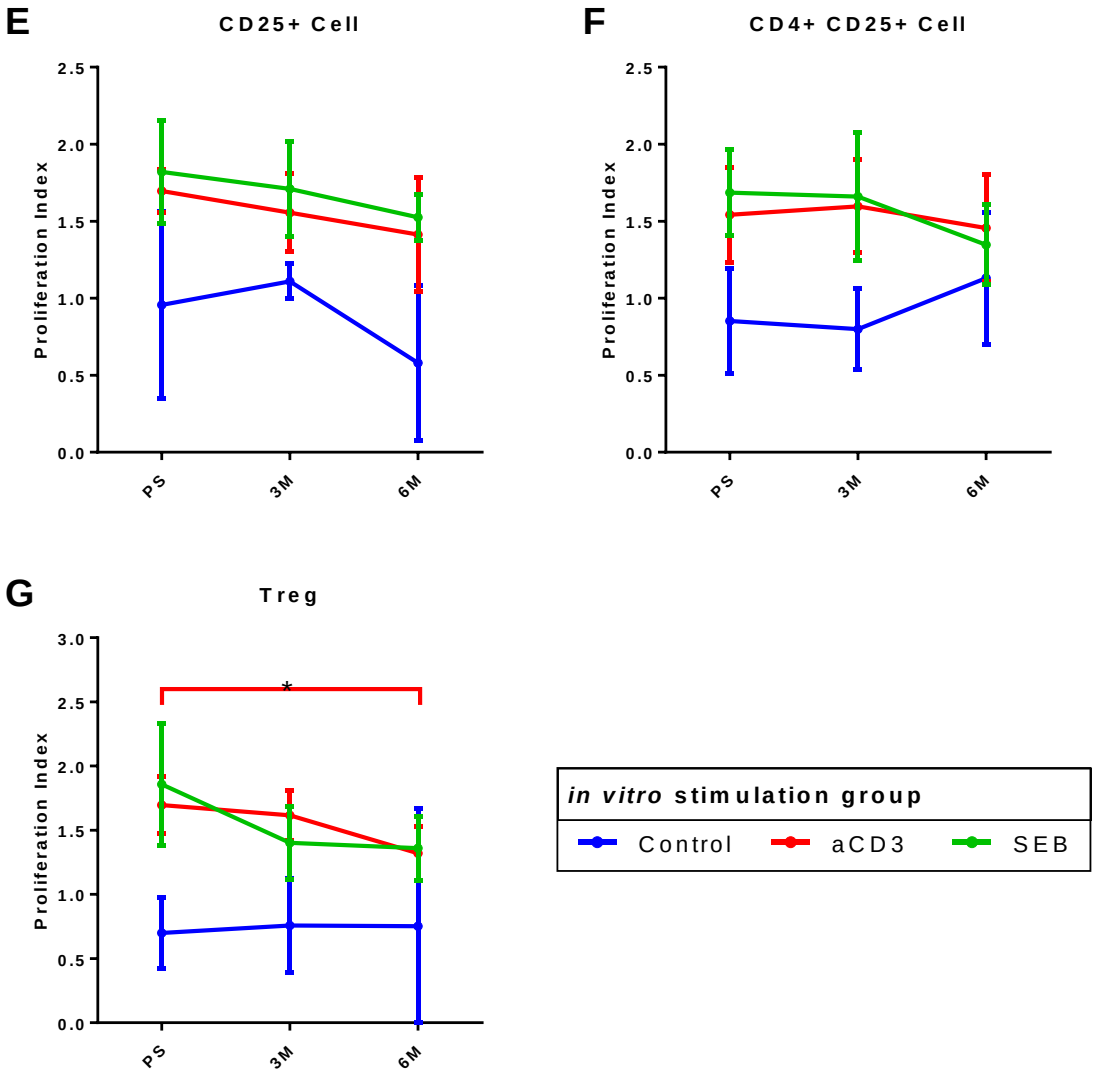

Figure 5-8 Proliferation Index of cells following functional assay (cont'd)
Mean proliferation indices for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G) Tregs. PS = pre-stimulation, 3M = 3 months -post VNS implantation, 6M = 6-months post VNS implantation. Error bars = standard deviation. Paired T test comparison with Tukey correction p value: *p<0.05 between timepoints within the aCD3 (red) or SEB (green) groups.

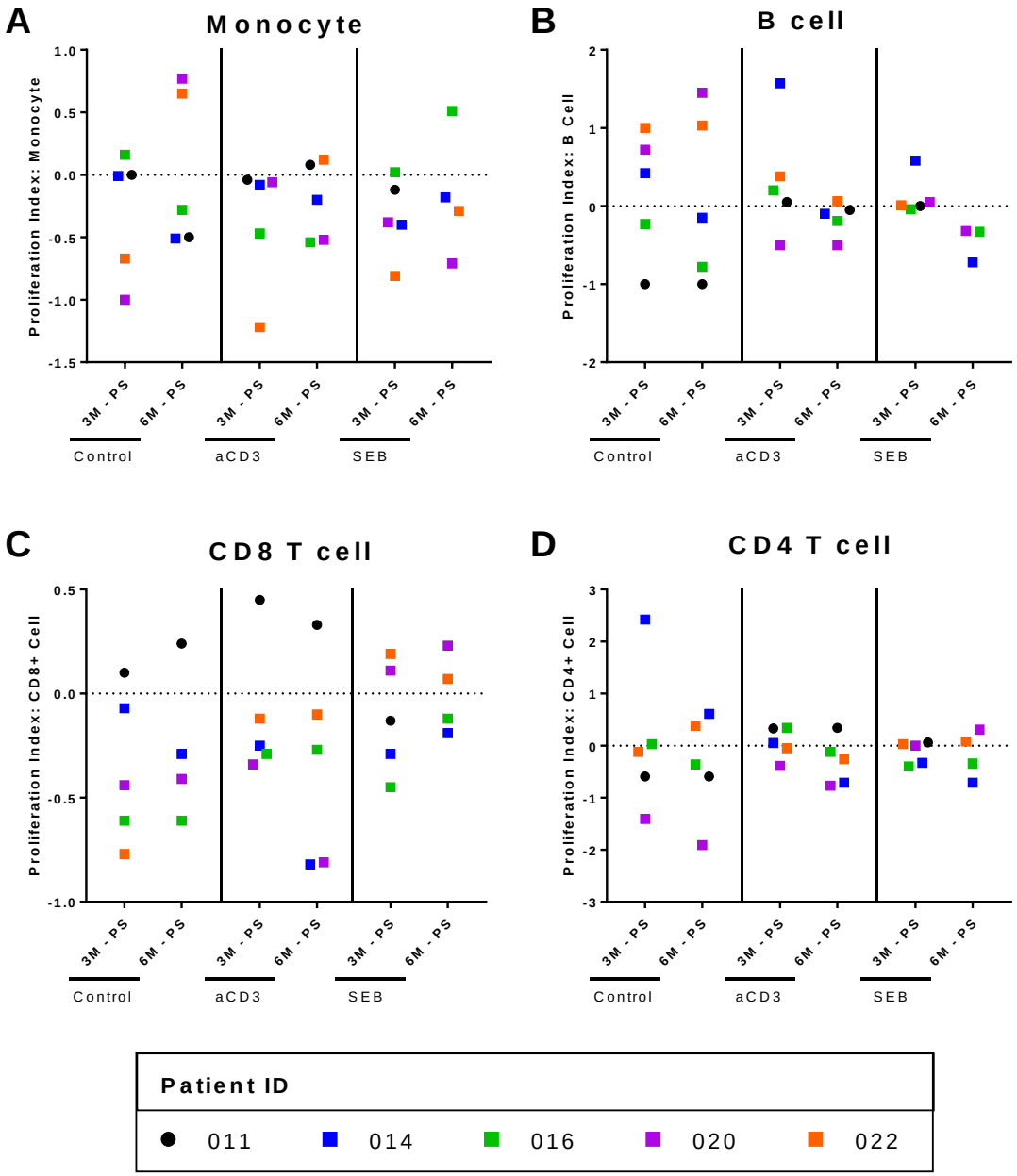

Figure 5-9 Differences in proliferation index of cells following functional assay in individual patients

Differences in proliferation index for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

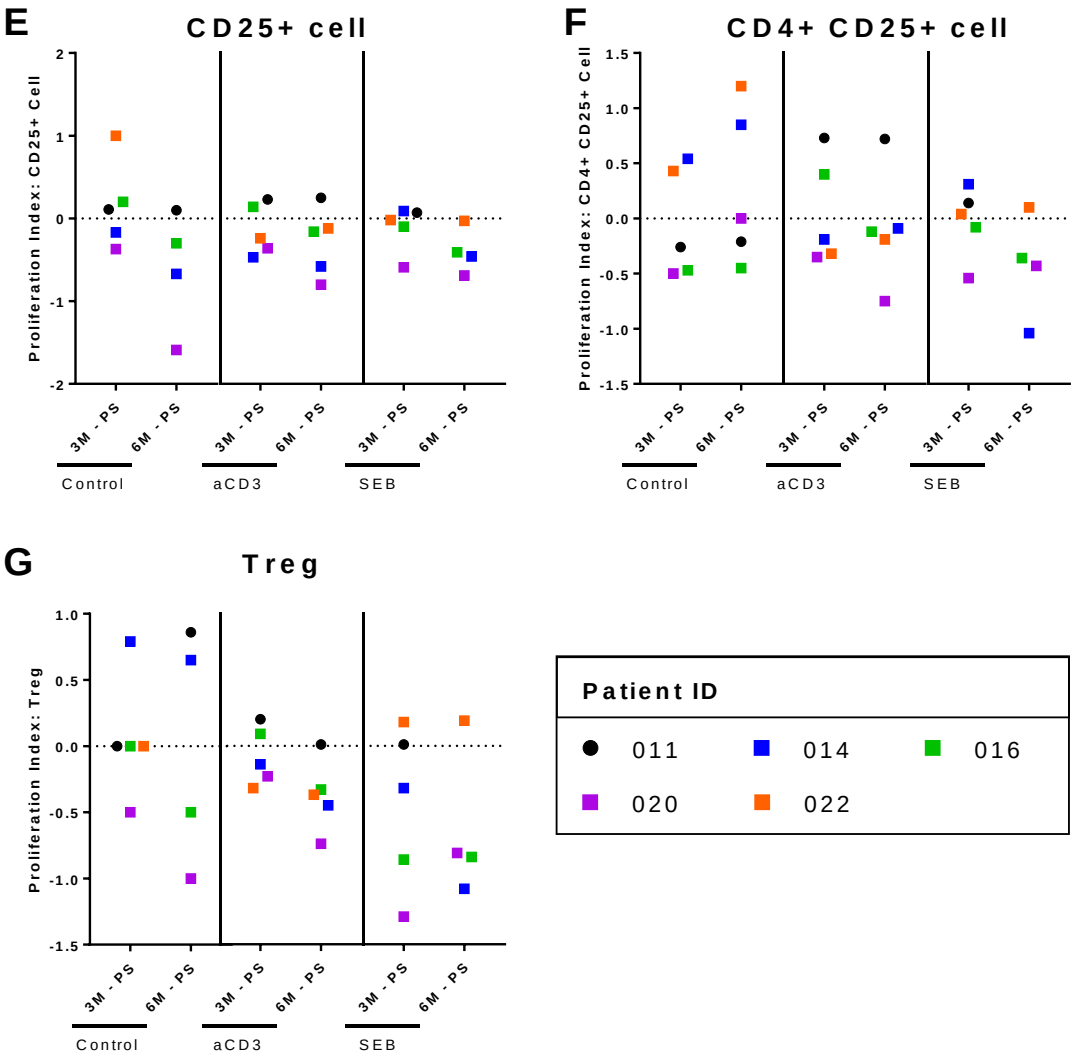

Figure 5-9 Differences in proliferation index of cells following functional assay in individual patients (cont'd)

Differences in proliferation index for (A) Monocyte, (B) B cell, (C) CD8+, (D) CD4+, (E) CD25+, (F) CD4+ CD25+ and (G-I) Tregs in individuals between 3 months post-VNS implantation compared to pre-stimulation (3M-PS) and 6-months post VNS implantation compared to pre-stimulation (6M-PS). Treg expression also displayed as a percentage of (H) CD4+ and (I) CD4+ CD25+ cells. Samples for patient 011 at 6M in response to SEB stimulation became infected during incubation and were not included in data analysis.

Table 5-8 Two-way ANOVA of proliferation index following functional assay

	Two-way ANOVA p values		
	Time	Condition	Time x Condition
Monocyte	0.1712	0.1167	0.855
B cell	0.1261	0.1615	0.2123
CD8+	0.0016	0.0529	0.1157
CD4+	0.3324	0.2782	0.8344
CD25+	0.0398	0.0018	0.1025
CD4+CD25+	0.7958	0.0008	0.1206
Treg	0.0293	0.0002	0.438

Table 5-9 Paired T test for proliferation index following functional assay

Cell	Condition	Comparison	Mean Diff.	SD of Diff.	P Value
Monocyte	Control	3M-PS	-0.304	0.503	0.248
		6M-PS	+0.026	0.633	0.931
	aCD3	3M-PS	-0.374	0.505	0.212
		6M-PS	-0.212	0.315	0.207
	SEB	3M-PS	-0.338	0.318	0.076
		6M-PS	-0.168	0.506	0.555
B Cell	Control	3M-PS	+0.182	0.803	0.639
		6M-PS	+0.110	1.088	0.832
	aCD3	3M-PS	+0.340	0.762	0.375
		6M-PS	-0.156	0.212	0.176
	SEB	3M-PS	+0.120	0.259	0.359
		6M-PS	-0.457	0.228	0.074
CD8+ Cell	Control	3M-PS	-0.358	0.365	0.093
		6M-PS	-0.268	0.363	0.237
	aCD3	3M-PS	-0.110	0.323	0.489
		6M-PS	-0.334	0.491	0.203
	SEB	3M-PS	-0.114	0.268	0.395
		6M-PS	-0.002	0.190	0.981
CD4+ Cell	Control	3M-PS	+0.066	1.431	0.923
		6M-PS	-0.374	0.993	0.447
	aCD3	3M-PS	+0.056	0.302	0.700
		6M-PS	-0.304	0.456	0.210
	SEB	3M-PS	-0.128	0.219	0.261
		6M-PS	-0.165	0.452	0.518

Table 5-8 Paired T test for proliferation index following functional assay (cont'd)

Cell	Condition	Comparison	Mean Diff.	SD of Diff.	P Value
CD25+ Cell	Control	3M-PS	+0.154	0.524	0.547
		6M-PS	-0.615	0.722	0.187
	aCD3	3M-PS	-0.140	0.309	0.369
		6M-PS	-0.282	0.413	0.201
	SEB	3M-PS	-0.110	0.279	0.428
		6M-PS	-0.398	0.274	0.062
CD4+ CD25+ Cell	Control	3M-PS	-0.052	0.500	0.828
		6M-PS	+0.278	0.711	0.431
	aCD3	3M-PS	+0.054	0.485	0.815
		6M-PS	-0.086	0.525	0.733
	SEB	3M-PS	-0.026	0.321	0.865
		6M-PS	-0.433	0.468	0.162
Treg	Control	3M-PS	+0.058	0.463	0.793
		6M-PS	+0.003	0.897	0.996
	aCD3	3M-PS	-0.080	0.219	0.459
		6M-PS	-0.376	0.269	0.035
	SEB	3M-PS	-0.456	0.612	0.171
		6M-PS	-0.635	0.563	0.109

Mean proliferation index of samples decreased over time for CD8 T cell, CD4 T cell, and CD25+ cells under all conditions (**Figure 5-8C-E**). Trends in decreasing proliferation index for monocyte and Treg were found in PBMCs incubated with anti-CD3 antibody (**Figure 5-8A and G**). Trends in decreasing proliferation indices for B cell, CD4+ CD25+ cell, and Tregs were found in PBMCs incubated with SEB (**Figure 5-8B, F, and G**).

Comparing the changes in proliferation indices from individuals (**Figure 5-9**), we found a trend in reducing proliferation index for Treg following *in vitro* stimulation with anti-CD3 antibody and SEB (**Figure 5-9G**).

Two-way ANOVA for proliferation indices (**Table 5-9**) revealed a significant difference across time in CD8+ cells, CD25+ cells and Tregs, and between conditions for CD25+ cells, CD4+ CD25+ cells, and Tregs. No significant interaction was found between time and condition in proliferation index.

Pairwise comparisons revealed a significant decrease in Treg proliferation index at 6 months post-VNS implantation when PBMCs were incubated with anti-CD3 antibodies ($p=0.035$) (**Table 5-9**).

5.3.5. Cytokine Array

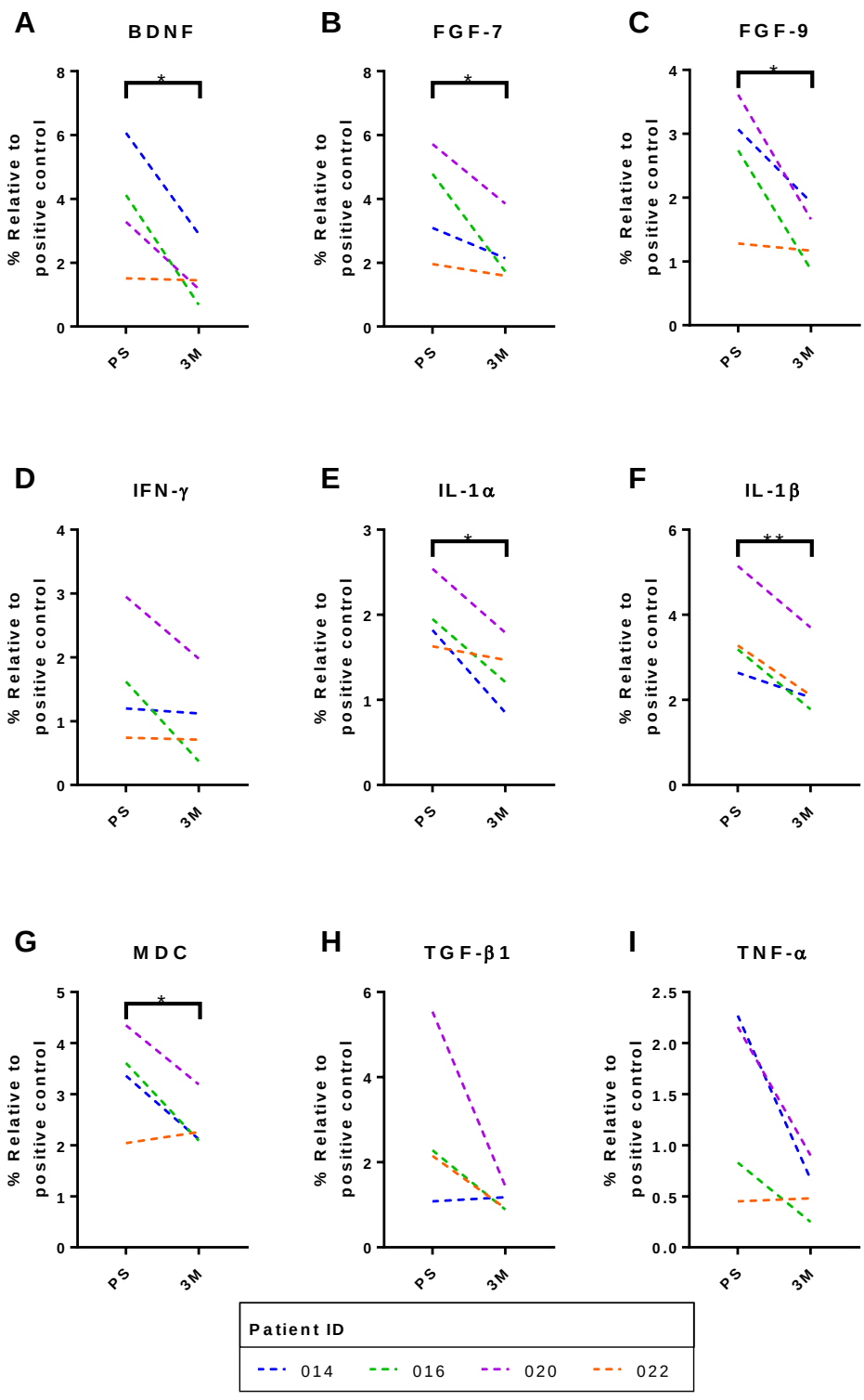

Figure 5-10 Cytokine levels before and 3 months after VNS implantation in individual patients.
Changes in levels of (A) BDNF, (B) FGF-7, (C) FGF-9, (D) IFN-γ, (E) IL-1α, (F) IL-1β, (G) MDC, (H) TGF-β1 and (I) TNF-α in individuals from cytokine arrays before VNS implantation (pre-stimulation, PS) compared to 3 months after VNS implantation (3M). Paired T test with Bonferroni correction, *p<0.05, **p<0.001.

Table 5-10 Paired T test for cytokine array

Cytokine	Mean change relative to PS	SD of mean change	Paired T test p value
IL-1β	-46.78%	32%	0.0054
IL-1α	-48.32%	34%	0.0160
FGF-9	-50.43%	34%	0.0293
BDNF	-56.01%	35%	0.0322
FGF-7	-46.37%	33%	0.0380
MDC	-38.52%	40%	0.0491
TNF-α	-55.71%	39%	0.0504
IFN-γ	-36.26%	39%	0.0784
TGF-β1	-55.94%	41%	0.0785

Note: P values for paired T test was Bonferonni corrected.
Table containing analysis for all 80 cytokines found in **Appendix Table 5**

Paired T test comparisons of 80 cytokines identified significant decrease the expression of brain-derived neurotrophic factor (BDNF), fibroblast growth factor 7 (FGF-7), fibroblast growth factor 9 (FGF-9), interleukin-1 alpha (IL-1α), interleukin-1beta (IL-1β), and macrophage derived chemokine (MDC) (**Table 5-10** and **Figure 5-10**). Reduction in the expression of TNF-α, interferon gamma (IFN-γ), and transforming growth factor beta 1 (TGF-β1) were approaching statistical significance. No other significant changes in cytokine expressions were observed (**Appendix Table 5**).

5.4. Discussion

5.4.1. Cell Population Frequencies

Flow cytometry analysis of cell population frequencies from PBMCs on the day of blood sampling revealed a trend in decreasing monocyte frequency following VNS treatment. A significant increased frequency of CD25+ cells and CD4+ CD25+ cells were observed 6 months following VNS treatment. Furthermore, a trend in increasing Treg frequency was observed. Treg frequency following VNS has not been previously investigated in humans, our finding is in line with the study by Morishita and colleagues. Here, they reported a 1% increase in Treg frequency following treatment with VNS in rats (Morishita *et al.*, 2015).

Patients in the current study had displayed higher frequency of Tregs compared to frequencies reported in healthy volunteers (Tregs: 0.78 ± 0.09% of lymphocytes) (Zahran and Elsayh, 2014). Only one study has investigated the frequency of Treg in epilepsy where frequency was abnormally low in children with epilepsy compared to healthy children (Li, Ma and Wang, 2011). One possible explanation for the variability in reporting Treg frequencies could be due to unreported differences in gating strategies during flow cytometry analysis. Magness and colleagues studied the reproducibility of flow cytometry data for murine intestinal epithelial cells and aimed to standardise its reporting for future studies (Magness *et al.*, 2013). A similar approach to standardise the reporting of Tregs would help improve the quality of future studies.

5.4.2. Functional Assay

Two-way ANOVA did not reveal significant changes in cell frequencies receiving *in vitro* stimulation after VNS treatment in response to *in vitro* stimulation (**Table 5-4**). However, T-test comparisons revealed significant reductions in Treg frequencies in response to aCD3 and SEB at 3 months post-VNS implantation (**Table 5-5**). We also found a significant difference in division index between time and condition for CD4+ CD25+ cells and Tregs (**Table 5-6**). Subsequent T-test comparisons revealed a significant reduction in Treg function up to 6 months after VNS implantation (**Table 5-7**). When comparing the proliferation index, there was a significant difference over time for CD8 T cells, CD25+ cells and Tregs (**Table 5-8**) and paired T test comparisons revealed a significant reduction in Treg proliferation at 6 months in response to aCD3 (**Table 5-9**). Taken together, Treg function in response to anti-CD3 and SEB were blunted following VNS stimulation. These findings contradict the increase in Treg frequency observed on the day of blood sampling. Cell division is only one aspect of function, and the observed changes in Treg function warrants further investigations into other aspects of Treg function following treatment with VNS.

5.4.3. Cytokine Array

A significant reduction in the relative expressions of BDNF, FGF-7, FGF-9, IL-1α, IL-1β, and MDC were identified following cytokine arrays from 4 patients prior to and 3 months following VNS implantation. A reduction in TNF-α was also detected and approaching statistical significance.

Although the role of BDNF in inflammation is unknown, it role in depression has been heavily investigated. Meta-analysis of 11 studies correlated decreased concentrations of serum BDNF with major depression (Sen, Duman and

Sanacora, 2008), and <2.3 ng/ml BDNF may also be associated with OCD, anxiety (Wang *et al.*, 2015) and schizophrenia (Qin *et al.*, 2017).

FGF-7 and FGF-9 are fibroblast growth factors for skin (Rubin *et al.*, 1989) and blood vessels (Singla and Wang, 2016). Studies have investigated its roles in differentiation of epithelial cells during development (Belleudi *et al.*, 2014), controlling proliferation of epithelial cells in the GI tract (Playford *et al.*, 1998), wound healing (Marti *et al.*, 2008; Zheng *et al.*, 2014).

IL-1α and IL-1β are pro-inflammatory cytokines which activate IL-1 receptors to upregulate inflammatory pathways (England *et al.*, 2014; Schett, Dayer and Manger, 2015). While IL-1α is released from necrotic cells and act as alarmins (Kim *et al.*, 2013; England *et al.*, 2014), IL-1β is synthesised and secreted by cells of the innate immune system in response to apoptotic signals (England *et al.*, 2014).

MDC is highly expressed by macrophages and dendritic cells with chemokine functions for dendritic cells, activated NK cells (Godiska *et al.*, 1997), and T helper 2 lymphocytes (Andrew *et al.*, 1998) via activation of C-C chemokine receptor type 4 (CCR4) receptors (Imai *et al.*, 1998).

Although other groups have investigated changes in plasma cytokine levels following VNS treatment in patients, the effects of VNS on cytokine profile are unclear. One study reported a significant reduction in IL-1β (Koopman *et al.*, 2016), IL-6, and TNF-α (Koopman *et al.*, 2016), while another study reports significant increase in IL-6, TGF-β and TNF-α (Corcoran *et al.*, 2005). Three other studies did not find significant changes in cytokine levels in adults (Barone *et al.*, 2007; Majoie *et al.*, 2011) or children (Aalbers *et al.*, 2012) following VNS treatment. One limitation of cytokine arrays used in the current study is that it

provides relative and not absolute change in cytokine levels thus additional assays are required to deduce the concentration of cytokines. This would allow direct comparison with the studies above which have investigated changes in cytokine concentration.

In summary, this study identified changes in the frequency for monocytes and CD25+ and CD4+ CD25+ cells with a trend in increasing Tregs. However, the functional capacity of Tregs in response to anti-CD3 antibodies may be reduced. Additional functional tests to assess the effects of VNS on Tregs are necessary. Furthermore, we identify changes in BDNF, FGF-7, FGF-9, and MDC, and future studies quantifying the expression of these cytokines with larger sample sizes are needed to validate this.

Chapter 6. Discussion

The vagus nerve has many different functions (**Section 1.1**), however its underlying mechanisms remain unclear. Despite this, electrical stimulation of the vagus nerve has shown therapeutic benefits for conditions including: refractory epilepsy (**Section 1.2.1**), depression (**Section 1.3.1**), as well as inflammatory conditions including rheumatoid arthritis, stroke, and inflammatory gastrointestinal diseases (**Section 1.5.3.3**). This thesis aimed to explore additional effects of VNS treatment on mood, HRV, and inflammation to gain a deeper understanding behind the mechanisms of VNS, and to identify potential biomarkers for optimising treatment and to act as surrogate clinical endpoints.

The key findings from this thesis were:

- VNS may reduce perceived stress.
- HRV reduces HF power which suggests a reduced sympathetic tone. However, this was not associated with the other beneficial effects of VNS observed in this study.
- While increases in Treg frequency from fresh blood suggests promotion of immune tolerance, Tregs also displayed reduced function suggesting reduction in immune tolerance.
- Cytokine array analysis identified significant changes in: BDNF, FGF-7, FGF-9, IL-1α, IL-1β, and MDC following VNS treatment.

6.1. Combinatory Effects of VNS

The vagus nerve interacts with many systems including the HPA-axis, neuroendocrine system, and immune system but much of the underlying mechanisms remain unknown. Interactions between these systems could potentially orchestrate complex responses and VNS may produce equally complex effects which may be difficult to isolate and study.

For example, psychological stress can be a major factor for some diseases (Kendler, Karkowski and Prescott, 1999; Hammen, 2005; Booth *et al.*, 2015; Targownik *et al.*, 2015) with physiological responses including stress hormone production via the HPA-axis which may exacerbate disease through a positive-feedback loop (Tsigos and Chrousos, 2002). In **Chapter 3**, we report a trend in reducing perceived stress 6 months following VNS implantation. Although no studies have previously reported changes in perceived stress following VNS, this finding is in line with the reduction in plasma cortisol levels in refractory epilepsy patients following VNS treatment (Majoie *et al.*, 2011). The interaction between stress and VNS is currently unknown, however the innervation of vagus afferents to the hypothalamus via the NTS suggests a potential mechanism for the vagus nerve to modulate the HPA-axis. Therefore, VNS may have the potential to treat stress disorders through modulation of the HPA axis. Additional tools to monitor both perceived and physiological stress, as well as larger and longer studies will be required to confirm the therapeutic effects of VNS on stress.

The HPA axis is able to exert cardiac effects including altering HRV (Thayer and Sternberg, 2006). Measures of HRV can be used as an indicator of the body's ability to self-regulate physiological functions and autonomic tone (McCraty and Shaffer, 2015) and therefore may serve as aa non-invasive biomarker. In

Chapter 4, a significant reduction in HF relative power was found 6-months after VNS implantation but did not correlate to other measures tested in this thesis. As discussed in **Section 4.4**, While changes in HF power have been observed previously in other studies (Libbus *et al.*, 2015; Liu *et al.*, 2017), LF and HF measures of HRV do not correlate linearly to autonomic activity and cannot be used to infer autonomic tone (Billman, 2013). Despite this, such measures of HRV could still be relevant as biomarkers such as predicting response to VNS treatment in refractory epilepsy patients (Liu *et al.*, 2017). Five-minute ECG recordings were used in the current study in a similar fashion to the study by Ponnusamy and colleagues (Ponnusamy, Marques and Reuber, 2012). However, short ECG recordings may not be suitable for monitoring changes in HRV following VNS treatment due to influence from circadian effects. Future studies deploying 24-hour ambulatory ECGs would exclude circadian effects on HRV. Additionally, following patients for up to 2 years post-VNS implantation may allow the identification of trends between HRV and changes in mood or immune system once a therapeutic level of stimulation for reducing seizure frequency has been achieved.

The underlying mechanisms for the anti-inflammatory reflex was first hypothesised by Tracey and colleagues (**Section 1.5.3.1**). However, this does not explain how VNS exerts its anti-inflammatory effect systemically as observed in rat studies. In **Chapter 5**, immune cell frequencies and functions were observed before and after VNS implantation. A trend in increasing Treg frequency was observed and is in line with the increase in Tregs found in rat following VNS treatment (Morishita *et al.*, 2015). However, the current study also found a significant reduction in Treg function in response to anti-CD3 antibody. While the interactions between VNS and Tregs remain unknown, additional assays

assessing functional changes in Tregs would aid in the understanding between VNS and Tregs.

To our knowledge, no studies have reported the use of cytokine arrays to investigate the effects of VNS. In **Chapter 5**, cytokine array analysis of fresh plasma samples before and 3 months following VNS implantation identified significant reductions in the expression for BDNF, FGF-7, FGF-9, IL-1α, IL-1β, and MDC.

While the role of BDNF in inflammation is currently unknown, its mechanism for its anti-depressive effects are known (Duman and Monteggia, 2006), and enhancing BDNF concentrations and activity have been an aim for treating depression. Most studies on FGF-7 and FGF-9 have investigated their roles in differentiation of epithelial cells (Playford *et al.*, 1998; Belleudi *et al.*, 2014) and wound healing (Marti *et al.*, 2008; Zheng *et al.*, 2014). IL-1α and IL-1β are classical markers of inflammation which upregulate inflammatory pathways (England *et al.*, 2014; Schett, Dayer and Manger, 2015). Macrophage derived chemokines MDCs (aka CCL22) have been reported to play a role in the recruitment of Tregs to inflamed tissue and tumours (Nishikawa and Sakaguchi, 2010). While the normal role of Tregs is to resolve inflammation, in the context of tumour immunity, it may act to promote immune tolerance and allow the proliferation of cancer cells (Li *et al.*, 2013).

Additional studies are required to validate these findings and quantify the changes in expression of these cytokines. This would provide us with a greater understanding behind the mechanism and effects of VNS.

6.2. General Limitations

As stated in the introduction (**Section 1.6**), we were limited by the heterogenous patient population with different co-morbidities and patients taking a combination of many medications. Although some patients took medication with anti-inflammatory effects, these were not prescribed routinely and thus were not excluded from the study (**Appendix Section 8.3**). The study was also restricted to a small sample size as only patients receiving VNS implantation at RHH were approached and recruited. A multi-centre approach in the future would increase sample size to aid the validation of findings from the current study.

Data was collected up to 6-months following VNS implantation in the current study. This duration may not have been long enough as it can take up to 2 years to optimise device settings to achieve a meaningful reduction in seizure frequency. By extending the study to collect data from patients at 2 years post-VNS implantation, further analysis comparing changes in mood, HRV and immune system between responders and non-responders to VNS treatment will be possible.

6.3. Alternative Methods of VNS

In addition to overcoming the limitations in study above, alternative methods of VNS could potentially be used to enhance research in VNS. Implantable VNS currently requires surgical intervention and therefore its associated surgical risks. This may pose unnecessary risks for the majority of refractory epilepsy patients who do not respond to VNS treatment. To address this, transcutaneous forms of VNS have been explored and offered as an alternative to invasive VNS. Transcutaneous VNS (tVNS) does not require surgical intervention and treatment

can be halted easily should the patient not respond to tVNS treatment. Furthermore, as the device is not implanted, stimulation settings may be adjusted easily without hospital visits. Currently there are two forms of tVNS available: Gammacore by Electrocore and NEMOS by Cerbomed, both of which are safe and well tolerated in patients (**Figure 6-1**). Gammacore is a handheld device to stimulate the cervical nerve and has been primarily used to treat headaches and migraines (Goadsby *et al.*, 2014; Barbanti *et al.*, 2015; Grazzi, Padovan and Barbanti, 2015; Nesbitt *et al.*, 2015). The NEMOS system stimulates an auricular branch of the vagus nerve at the concha of the ear to produce anti-epileptic effects. Furthermore, fMRI analysis has shown its ability to stimulate the NTS in a similar fashion to invasive VNS (Dietrich *et al.*, 2008; Frangos, Ellrich and Komisaruk, 2014). The therapeutic potential of tVNS is yet to be fully explored and could be used in future studies to examine its effects on stress, and explore the underlying mechanisms in the relationship between VNS and Tregs.

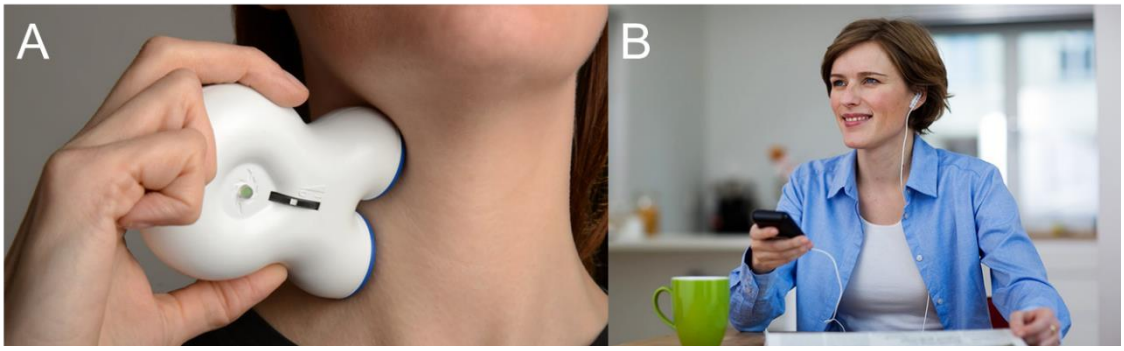

Figure 6-1 commercially available tVNS devices
(**A**) Gammacore stimulator manufactured by Electrocore (www.gammacore.co.uk), (**B**) NEMOS stimulator manufactured by Cerbomed (www.nemos.uk.com).

6.4. Concluding Remarks

This thesis explored the effects of VNS on mood, HRV, and immune system in patients with refractory epilepsy and identified potential avenues to further VNS research. First, we observed a trend in reducing perceived stress following VNS implantation suggesting its therapeutic potential for treating stress disorders. Secondly, we observed a significant increase in Treg frequency but also found a reduction in Treg function 6 months following VNS implantation. Further work is required to study the underlying mechanisms of VNS on Treg function. Lastly, we identified significant reductions in expression of BDNF, FGF-7, FGF-9, IL-1α, IL-1β, and MDC 3 months following VNS implantation, with future work being required to validate and quantify the expression of these cytokines.

The interactions between the vagus nerve and other systems is complex. While much of the therapeutic applications and mechanisms of VNS remain to be explored, it has the potential to benefit patients who may otherwise remain refractory to treatment.

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Chapter 8. Appendix

8.1. Approvals

8.1.1. Independent Scientific Approval

The University Of Sheffield.

Harvey Leung <hleung1@sheffield.ac.uk>

Results of Independent Scientific Review for STH18326

1 message

Gaurika.Kapoor@sth.nhs.uk <Gaurika.Kapoor@sth.nhs.uk> 17 July 2014 at 16:17
To: hleung1@sheffield.ac.uk, Arshad.Majid@sth.nhs.uk
Cc: Jodie.Keyworth@sth.nhs.uk, Samantha.Heaton@sth.nhs.uk

Dear Prof Majid,

The following project was sent for Independent Scientific Review

Project Title: The Influence of vagal nerve stimulation on blood markers of inflammation

STH Registration Number: 18326

Directorate affiliation: Neurosciences

Investigator: Arshad Majid

Outcome

The outcome of the review was as follows (see attached reviewers comments):

Lead Reviewer

A. No changes required; minor suggestions at discretion of the researcher; take the study forward

Second Reviewer

A. No changes required; minor suggestions at discretion of the researcher; take the study forward

Noting the Reviewers' gradings, your study has been granted scientific review approval.

Please insert a copy of this and any previous scientific review correspondence to your site file, and contact your designated Research Co-ordinator for further guidance on submission to ethics and Research Governance Authorisation for your project.

Assistance

If you have any questions about the scientific review, please contact Dr Dipak Patel (dipak.patel@sth.nhs.uk). Advice on the rest of the registration procedure will be provided by your designated Coordinator.

Kind regards,

Gaurika

Gaurika Kapoor, M.A.

Research Management & Governance Facilitator, Clinical Research Office Sheffield

1st Floor, 11 Broomfield Road, Sheffield, S10 2SE

Tel: 0114 22 65939 **Fax:** 0114 226 5937 **E-mail:** gaurika.kapoor@sth.nhs.uk **Website:** www.sheffieldclinicalresearch.org

Please note our new address w.e.f. 11 Aug 2014: Research Department, D Floor, Royal Hallamshire Hospital, Glossop Road, Sheffield, S10 2JF. Telephone numbers will remain the same.

University of Sheffield and Sheffield Teaching Hospitals NHS FT - working in partnership to promote excellence in clinical research

2 attachments

STH18326 First Review.docx
29K

STH18326 Second Review.doc
59K

8.1.2. Research Ethics Committee Approval

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7827
Fax: 0161 625 7299

29 May 2015

Mr Harvey Leung
The University of Sheffield
53 Spooner Road
Sheffield
S10 5BL

Dear Harvey,

Study title:	**The Influence of vagal nerve stimulation on blood markers of inflammation**
REC reference:	**14/NW/1221**
Protocol number:	**NA**
Amendment number:	**Amendment number 3, 11/05/2014**
Amendment date:	**11 May 2015**
IRAS project ID:	**157533**

The above amendment was reviewed by the Sub-Committee in correspondence.

Favourable opinion

Approval was sought to add a fatigue assessment scale to the study to observe fatigue in epilepsy patients before and after VNS.

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*
Notice of Substantial Amendment (non-CTIMP)	Amendment number 3, 11/05/2014	11 May 2015
Participant information sheet (PIS) [Tracked]	3	11 May 2015
Participant information sheet (PIS) [Clean]	3	11 May 2015

Research protocol or project proposal [Tracked]	4	11 May 2015
Research protocol or project proposal [Clean]	4	11 May 2015
Validated questionnaire	1	11 May 2015

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.

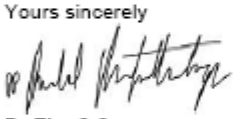
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/NW/1221: Please quote this number on all correspondence

Yours sincerely



Dr Tim S Sprosen
Chair

E-mail: nrescommittee.northwest-haydock@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Professor Arshad Majid, The University of Sheffield
Miss Sam Heaton, Sheffield Teaching Hospitals

NRES Committee North West - Haydock

Attendance at Sub-Committee of the REC meeting on 26 May 2015

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Chris Haywood	Director of Nursing	Yes	
Dr Tim S Sprosen	Epidemiologist	Yes	Chair

Also in attendance:

Name	*Position (or reason for attending)*
Ms Rachel Katzenellenbogen	REC Manager

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7827
Fax: 0161 625 7299

15 January 2015

Arshad Majid
Professor and Chair of Cerebrovascular Neurology
The University of Sheffield
SITraN
385A Glossop Road
Sheffield
S10 2HQ

Dear Arshad,

Study title:	**The Influence of vagal nerve stimulation on blood markers of inflammation**
REC reference:	**14/NW/1221**
Protocol number:	**NA**
Amendment number:	**1**
Amendment date:	**14 November 2014**
IRAS project ID:	**157533**

The above amendment was reviewed by the Sub-Committee in correspondence.

Favourable opinion

Approval was sought for changes to the study to observe before and after vagal nerve stimulation.

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 or letters		10 December 2014
Letters of invitation to participant		10 December 2014

Notice of Substantial Amendment (non-CTIMP)	1	14 November 2014
Other [NDDI-E]		10 December 2014
Participant consent form [Consent Form]		10 December 2014
Participant information sheet (PIS) [Patient]		10 December 2014
Participant information sheet (PIS) [volunteer]		10 December 2014
Research protocol or project proposal [Protocol]		10 December 2014
Validated questionnaire		10 December 2014
Validated questionnaire		10 December 2014
Validated questionnaire		10 December 2014

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 and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/NW/1221: Please quote this number on all correspondence

Yours sincerely

On behalf of
Dr Tim S Sprosen
Chair

E-mail: nrescommittee.northwest-haydock@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Ms Sam Heaton, Sheffield Teaching Hospitals NHS Foundation Trust

NRES Committee North West - Haydock

Attendance at Sub-Committee of the REC meeting on 13 January 2015

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Ezzat Kozman	Consultant Gynaecologist	Yes	
Dr Tim S Sprosen	Epidemiologist	Yes	Chair

Also in attendance:

Name	*Position (or reason for attending)*
Ms Rachel Katzenellenbogen	REC Manager

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7827
Fax: 0161 625 7299

13 August 2014

Professor Arshad Majid
Professor and Chair of Cerebrovascular Neurology
The University of Sheffield
SITraN
385A Glossop Road
Sheffield
S10 2HQ

Dear Professor Majid

Study title:	**The Influence of vagal nerve stimulation on blood markers of inflammation**
REC reference:	**14/NW/1221**
Protocol number:	**NA**
IRAS project ID:	**157533**

The Proportionate Review Sub-committee of the NRES Committee North West - Haydock reviewed the above application on 12 August 2014.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager Rachel Katzenellenbogen, nrescommittee.northwest-haydock@nhs.net.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

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.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study 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 a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

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).

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").

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP Information Sheet]	1	05 August 2014
Participant consent form [Volunteer consent form]	1.0	06 June 2014
Participant consent form [Patient consent form]	1.0	17 December 2013
Participant information sheet (PIS) [Patient Information Sheet]	1	05 August 2014
Participant information sheet (PIS) [Volunteer Information Sheet]	1	05 August 2014
REC Application Form [REC_Form_04082014]		04 August 2014
Research protocol or project proposal [Protocol]	1	30 July 2014
Summary CV for Chief Investigator (CI) [Majid, Arshad CV]	1	30 July 2014
Summary CV for student [Leung, Harvey CV]	1	30 July 2014

Validated questionnaire [Perceived stress scale questionnaire]		

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

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
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

14/NW/1221 **Please quote this number on all correspondence**

Yours sincerely

On behalf of
Professor Ravi S Gulati
Chair

Email: nrescommittee.northwest-haydock@nhs.net

A Research Ethics Committee established by the Health Research Authority

Enclosures:	List of names and professions of members who took part in the review "After ethical review – guidance for researchers"
Copy to:	Ms Sam Heaton, Sheffield Teaching Hospitals NHS Foundation Trust

NRES Committee North West - Haydock

Attendance at PRS Sub-Committee of the REC meeting on 12 August 2014

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Moyra Ann Baldwin	Retired Senior Lecturer - Oncology	Yes	
Professor Ravi S Gulati (Chair)	Consultant Physician	Yes	
Mr Peter Ward	Lay Member	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Rachel Katzenellenbogen	REC Manager
Katie Southeard	REC Assistant

8.2. Study related documents

8.2.1. Ethics Application

NHS REC Form | Reference: 14/NW/1221 | IRAS Version 3.5

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
The Influence of VNS on blood markers of inflammation

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
(●) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
( ) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Will the study involve the use of any medical device without a CE Mark, or a CE marked device which has been modified or will be used outside its intended purposes?

( ) Yes (●) No

2b. Please answer the following question(s):

a) Does the study involve the use of any ionising radiation?	( ) Yes	(●) No
b) Will you be taking new human tissue samples (or other human biological samples)?	(●) Yes	( ) No
c) Will you be using existing human tissue samples (or other human biological samples)?	( ) Yes	(●) No
d) Will the study involve any other clinical procedures with participants (e.g. MRI, ultrasound, physical examination)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

Date: 04/08/2014 | 1 | 157533/648098/1/447

☑ England
☐ Scotland
☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Are all the research costs and infrastructure costs for this study provided by an NIHR Biomedical Research Centre, NIHR Biomedical Research Unit, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC) or NIHR Research Centre for Patient Safety & Service Quality in all study sites?

○ Yes ◉ No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP).

5b. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN) support and inclusion in the NIHR Clinical Research Network (CRN) Portfolio? Please see information button for further details.

○ Yes ◉ No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP) and you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form immediately after completing this project filter and before completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of

identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):
PhD student will be collecting samples from patients and analysing data.

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

◉ Yes ○ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

○ Yes ◉ No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

○ Yes ◉ No

NHS REC Form | Reference: 14/NW/1221 | IRAS Version 3.5

Integrated Research Application System
Application Form for Basic science study involving procedures with human participants

NHS Health Research Authority

Application to NHS/HSC Research Ethics Committee

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
The Influence of VNS on blood markers of inflammation

Please complete these details after you have booked the REC application for review.

REC Name:
North West - Haydock

REC Reference Number:
14/NW/1221

Submission date:
04/08/2014

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

The Influence of vagal nerve stimulation on blood markers of inflammation

A2-1. Educational projects

Name and contact details of student(s):

Student 1

Title	Forename/Initials	Surname
Mr	Harvey	Leung

Address [redacted]

Post Code [redacted]
E-mail [redacted]
Telephone [redacted]
Fax

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD Neuroscience

Name of educational establishment:
The University of Sheffield

Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Prof	Arshad	Majid
Address	SITraN		
	385A Glossop Road		
	Sheffield		
Post Code	S10 2HQ		
E-mail	arshad.majid@sheffield.ac.uk		
Telephone	01142222249		
Fax			

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mr Harvey Leung	☑ Prof Arshad Majid

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- ○ Student
- ◉ Academic supervisor
- ○ Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Prof	Arshad	Majid
Post	Professor and Chair of Cerebrovascular Neurology		
Qualifications	MB, ChB		
Employer	The University of Sheffield		
Work Address	SITraN		
	385A Glossop Road		
	Sheffield		
Post Code	S10 2HQ		
Work E-mail	arshad.majid@sheffield.ac.uk		
* Personal E-mail	arshad.majid@sheffield.ac.uk		

Work Telephone	01142222249
* Personal Telephone/Mobile	[redacted]
Fax	

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

	Title	Forename/Initials	Surname
	Ms	Sam	Heaton
Address	CRO		
	11 Broomfield Road		
	Sheffield		
Post Code	S10 2SE		
E-mail	samantha.heaton@sth.nhs.uk		
Telephone	01142265942		
Fax			

A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):	STH18326
Sponsor's/protocol number:	NA
Protocol Version:	1.0
Protocol Date:	12/05/2014
Funder's reference number:	
Project website:	

Registry reference number(s):
The Department of Health's Research Governance Framework for Health and Social Care and the research governance frameworks for Wales, Scotland and Northern Ireland set out the requirement for registration of trials. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

International Standard Randomised Controlled Trial Number (ISRCTN):
ClinicalTrials.gov Identifier (NCT number):

Additional reference number(s):

Ref.Number Description	Reference Number

A5-2. Is this application linked to a previous study or another current application?

○ Yes ◉ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, this summary will be published on the website of the National Research Ethics Service following the ethical review.*

Stroke is associated with an intense initial inflammatory phase, which has profound detrimental effects in the brain. In experimental models of stroke, immunosuppression mediated by regulatory T cells (Tregs) can limit the extent of brain damage in mice. Other evidence in mice suggest that it's numbers and activity can be influenced by the nervous system via a nerve called the vagus nerve, and it's stimulation produces immunosuppressive effects.

Vagus nerve stimulation (VNS) is already safely used clinically to treat drug-resistant epilepsy patients and provides us with a unique opportunity, for the first time; to observe its effects on Tregs in humans. We hypothesize that VNS in humans will result in the increased number of cells and activity of Tregs in blood. We will also measure levels of inflammation biomarkers such as cytokines in blood because changes in these can also affect inflammation. Data generated from this study will guide future studies that will explore the effectiveness of VNS in treating stroke and other inflammatory diseases. The data generated in humans will be compared to the data currently being generated in a similar study using sheep.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

Potential unwanted findings may indicate disease from blood or ECGs
Unwanted findings will be discussed with patients and patient's GP so that appropriate medical management can be made.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply:*

- ☐ Case series/ case note review
- ☐ Case control
- ☑ Cohort observation
- ☐ Controlled trial without randomisation
- ☐ Cross-sectional study
- ☐ Database analysis
- ☐ Epidemiology
- ☑ Feasibility/ pilot study
- ☑ Laboratory study
- ☐ Metanalysis
- ☑ Qualitative research
- ☑ Questionnaire, interview or observation study
- ☐ Randomised controlled trial
- ☐ Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

We hypothesise that VNS has profound anti-inflammatory effects in the body.
We will test our hypothesis through the following specific aims:
1. To determine Treg proliferation and activity in humans before and after VNS.
2. To determine the influence of VNS on pro and anti-inflammatory cytokines.

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

Do the biomarkers from blood correlate with seizure control following vagus nerve stimulation?

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

In stroke, there is an initial inflammatory phase, which is linked to the extent of brain damage. Experiments in mice suggest that regulatory T cells (Tregs) have a vital role in suppressing this inflammatory response and Treg appears to protect the brain from this damage. Interestingly, when we cut the vagus nerve in mice, we see an increased inflammatory response to infections in addition to the decreased number and activity of Tregs. However, when vagus nerve stimulation (VNS) is given to mice, they become resilient to infections and other causes of inflammation.

There is strong evidence for the modulation of the inflammatory response by the vagus nerve and that VNS has an anti-inflammatory effect although we still do not know how this works. In this project, we predict that the increased number and activity of Tregs following VNS is responsible for its anti-inflammatory effects to suppress inflammation. A sheep model of VNS will be used in a similar study to compare with the patient study and aim to develop an animal model of stroke in future studies.

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

1. Methodology
Blood will be drawn for a full blood count and to isolate regulatory T cells (Tregs). Blood will be collected and analysed before vagus nerve stimulation (VNS), and at 1 and 6 months after VNS which will occur at routine clinic visits. Samples may be frozen and stored for up to 1 year after collection for repeating experiments. We will also ask patients to fill out a small questionnaire on recent infections, state of excitation etc. We will also perform an ECG at each visit.

2. Design: type of study design and justification
Initially, blood from 5 healthy volunteers will be used to optimise experiments before collecting blood from 10 epilepsy patients. These 10 patients that will be undergoing VNS treatment will be recruited initially from the epilepsy clinic in Royal Hallamshire Hospital. We will use blood from these patients to optimize our protocol and to inform statistical analysis.

During the course of study, we anticipate an additional 30 patients for a total of 5 healthy volunteers and 40 patients to be recruited for the study.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- ☐ Design of the research
- ☐ Management of the research
- ☐ Undertaking the research
- ☐ Analysis of results
- ☑ Dissemination of findings
- ☐ None of the above

Give details of involvement, or if none please justify the absence of involvement.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

- Participants must be above 18 years or older
- Participants without a history of autoimmune disorders
- Participants without a history of long term immunomodulatory treatment

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

- Participants under the age of 18 years
- Participants with a history of autoimmune disorders
- Participants who have previously received immunomodulatory treatment

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Seeking consent	1	0	1day	Neurologists in the epilepsy clinic at Royal Hallamshire Hospital
Questionnaires	3	0	5mins	Neurologists in the epilepsy clinic at Royal Hallamshire Hospital

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Blood collection	3	3	10mins	Phlebotomist, epilepsy nurse, research nurse, or if needed Professor Majid at Royal Hallamshire Hospital
ECG	3	1	10mins	Researcher Mr Leung at the Royal Hallamshire Hospital

A21. How long do you expect each participant to be in the study in total?

8 months

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Short term pain and discomfort from blood collection

A24. What is the potential for benefit to research participants?

None

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used? *For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under arrangements with the responsible care organisation(s).*

Mr Harvey Leung and Professor Arshad Majid will identify healthy volunteers in the Sheffield Institute for Translational Neuroscience (SITraN). Full details of the study will be given verbally and the volunteer information sheet supplied. A formal written consent will be taken before blood collection. Mr Leung (or Professor Majid) will be able to answer any questions about the study while the volunteer is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary. Volunteers will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in the study. Full voluntary consent will be obtained by Mr. Leung.

Professor Markus Reuber and Dr Stephen Howell will identify epilepsy patients for VNS in the epilepsy clinic in RHH. Full details of the study will be given verbally and the patient information sheet supplied. A formal written consent will be taken at the initial visit for VNS surgery with verbal confirmation at each subsequent visit. Professor Reuber (or Professor Majid) will be able to answer any questions about the study while the patient is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary and non−participation will have no bearing on their care or treatment. Patients will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in this study. Full voluntary consent will be obtained by the neurologists and completion of a consent form, at the subsequent appointment when the patient undergoes a routine blood sample before VNS and at 1 and 6 months after VNS.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

○ Yes ◉ No

Please give details below:
Patients will be identified at their routine clinical visit.

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

○ Yes ◉ No

A29. How and by whom will potential participants first be approached?

Mr Harvey Leung and Professor Arshad Majid will identify healthy volunteers in the Sheffield Institute for Translational Neuroscience (SITraN). Full details of the study will be given verbally and the volunteer information sheet supplied. A formal written consent will be taken before blood collection. Mr Leung (or Professor Majid) will be able to answer any questions about the study while the volunteer is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary. Volunteers will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in the study. Full voluntary consent will be obtained by Mr. Leung.

Professor Markus Reuber and Dr Stephen Howell will identify epilepsy patients for VNS in the epilepsy clinic in RHH. Full details of the study will be given verbally and the patient information sheet supplied. A formal written consent will be taken at the initial visit for VNS surgery with verbal confirmation at each subsequent visit. Professor Reuber (or Professor Majid) will be able to answer any questions about the study while the patient is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary and non−participation will have no bearing on their care or treatment. Patients will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in this study. Full voluntary consent will be obtained by the neurologists and completion of a consent form, at the subsequent appointment when the patient undergoes a routine blood sample before VNS and at 1 and 6 months after VNS.

A30-1. Will you obtain informed consent from or on behalf of research participants?

(●) Yes ( ) No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Mr Harvey Leung and Professor Arshad Majid will identify healthy volunteers in the Sheffield Institute for Translational Neuroscience (SITraN). Full details of the study will be given verbally and the volunteer information sheet supplied. A formal written consent will be taken before blood collection. Mr Leung (or Professor Majid) will be able to answer any questions about the study while the volunteer is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary. Volunteers will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in the study. Full voluntary consent will be obtained by Mr. Leung.

A formal written consent will be taken at the initial visit for VNS surgery with verbal confirmation at each subsequent visit. Professor Reuber (or Professor Majid) will be able to answer any questions about the study while the patient is considering the information. Emphasis will be placed on the fact that participation is entirely voluntary and non−participation will have no bearing on their care or treatment. Patients will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in this study. Full voluntary consent will be obtained by the neurologists and completion of a consent form, at the subsequent appointment when the patient undergoes a routine blood sample before VNS and at 1 and 6 months after VNS.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

(●) Yes ( ) No

A31. How long will you allow potential participants to decide whether or not to take part?

At least 24 hours, longer if desired

A32. Will you recruit any participants who are involved in current research or have recently been involved in any research prior to recruitment?

(●) Yes
( ) No
( ) Not Known

If Yes, please give details and justify their inclusion. If Not Known, what steps will you take to find out?

As long as the participant is not involved in another intervention study

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or

written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

Resources do not allow for such arrangements, therefore participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs will not be recruited.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

- ○ The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.
- ◉ The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.
- ○ The participant would continue to be included in the study.
- ○ Not applicable – informed consent will not be sought from any participants in this research.
- ○ Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

If you plan to retain and make further use of identifiable data/tissue following loss of capacity, you should inform participants about this when seeking their consent initially.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

- ☑ Access to medical records by those outside the direct healthcare team
- ☐ Electronic transfer by magnetic or optical media, email or computer networks
- ☐ Sharing of personal data with other organisations
- ☐ Export of personal data outside the EEA
- ☐ Use of personal addresses, postcodes, faxes, emails or telephone numbers
- ☐ Publication of direct quotations from respondents
- ☐ Publication of data that might allow identification of individuals
- ☐ Use of audio/visual recording devices
- ☑ Storage of personal data on any of the following:
  - ☐ Manual files including X-rays
  - ☑ NHS computers
  - ☐ Home or other personal computers
  - ☐ University computers
  - ☐ Private company computers
  - ☐ Laptop computers

Further details:
All personal data concerning study participants will be kept strictly confidential, and medical records will only be

accessed by the clinicians who routinely access them. Personal data will be anonymised at the RHH and stored on a password-protected computer database managed by Prof Majid and Mr Leung at SITraN. Identifiable data will not be published or used by the researchers, as all samples will be assigned a unique identification code, the details of which Prof Majid and Mr Leung will maintain at SITraN and store for 3 years after completion of the study to enable publications to be generated.

A38. How will you ensure the confidentiality of personal data? *Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

Identifiable data will not be published or used by the researchers, as all samples will be assigned a unique identification code.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

All personal data concerning study participants will be kept strictly confidential, and medical records will only be accessed by the clinicians who routinely access them

Storage and use of data after the end of the study

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ○ 6 – 12 months
- ◉ 12 months – 3 years
- ○ Over 3 years

If longer than 12 months, please justify:
Data will be accessed for up to 3 years after the study has ended in order to generate publications.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

○ Yes ◉ No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

(•) Yes ( ) No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

( ) Yes (•) No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50. Will the research be registered on a public database?

The Department of Health's Research Governance Framework for Health and Social Care and the research governance frameworks for Wales, Scotland and Northern Ireland set out the requirement for registration of trials. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

( ) Yes (•) No

Please give details, or justify if not registering the research.

Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- [x] Peer reviewed scientific journals
- [x] Internal report
- [x] Conference presentation
- [ ] Publication on website
- [ ] Other publication
- [ ] Submission to regulatory authorities
- [ ] Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- [ ] No plans to report or disseminate the results
- [ ] Other (please specify)

A53. Will you inform participants of the results?

(•) Yes ( ) No

Please give details of how you will inform participants or justify if not doing so.
Results will be verbally communicated to participants if requested and copies of any publications made available to them.

5. Scientific and Statistical Review

A54. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- [ ] Independent external review

- [ ] Review within a company
- [ ] Review within a multi–centre research group
- [x] Review within the Chief Investigator's institution or host organisation
- [x] Review within the research team
- [ ] Review by educational supervisor
- [ ] Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The ISR has been conducted by STH R&D department and has been given a positive outcome following some minor changes to the protocol

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- [ ] Review by independent statistician commissioned by funder or sponsor
- [ ] Other review by independent statistician
- [ ] Review by company statistician
- [ ] Review by a statistician within the Chief Investigator's institution
- [ ] Review by a statistician within the research team or multi–centre group
- [ ] Review by educational supervisor
- [ ] Other review by individual with relevant statistical expertise
- [x] No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Mr	Harvey	Leung
Department	BSc Biomedical Sciences (The University of Sheffield) MSc Translational Neuroscience (The University of Sheffield)		
Institution	The university of SHeffield		
Work Address	[redacted]		
Post Code	[redacted]		
Telephone			
Fax			
Mobile	[redacted]		
E-mail	[redacted]		

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

1. Change in Treg activity in quantity and function following VNS
2. Change in cytokine levels following VNS

A58. What are the secondary outcome measures? *(if any)*

Comparison between frequency of seizures and the changes in biomarker of VNS before and after VNS therapy.

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size: 45
Total international sample size (including UK):
Total in European Economic Area:

Further details:
5 Healthy volunteers for experiment optimisation. Initial 10 patients in a pilot study, and an expect to recruit an additional 30 patients by the end of year 1 for a total of 5 healthy volunteers and 40 VNS patients.

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

We currently cannot calculate sample size due to insufficient available data. Therefore, a pilot study with 10 patients will be carried out first

A61. Will participants be allocated to groups at random?

○ Yes ◉ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

As we will be using relatively simple statistical tests (ANOVAs/Student's two-tailed T tests), statistical opinion will initially not be sought. However, we will consult the School of Health and Related Research (ScHARR) if needed.

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co−applicants, protocol co−authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title	Forename/Initials	Surname
	Dr	Jaleel	Miyan
Post	Senior Lecturer		
Qualifications	BSc, PhD, FHEA		
Employer	The University of Manchester		
Work Address	Faculty of Life Sciences, AV Hill Building		
	Oxford Road		
	Manchester		
Post Code	M13 9PT		
Telephone	01613064205		
Fax			
Mobile			
Work Email	j.miyan@manchester.ac.uk		

	Title	Forename/Initials	Surname
	Prof	Andrew	Heath
Post Qualifications			
Employer			
Work Address	Dept of Infection and Immunity, The University of Sheffield Medical School Beech Hill Road Sheffield		
Post Code	S10 2RX		
Telephone	01142268634		
Fax	01142268898		
Mobile			
Work Email	a.w.heath@sheffield.ac.uk		

	Title	Forename/Initials	Surname
	Prof	Markus	Reuber
Post Qualifications			
Employer			
Work Address	Academic Neurology Unit, Royal Hallamshire Hospital Glossop Road Sheffield		
Post Code	S10 2JF		
Telephone	01142268763		
Fax	01142713158		
Mobile			
Work Email	markus.reuber@sth.nhs.uk		

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status:
- (●) NHS or HSC care organisation
- ( ) Academic
- ( ) Pharmaceutical industry
- ( ) Medical device industry
- ( ) Local Authority
- ( ) Other social care provider (including voluntary sector or private organisation)
- ( ) Other

Commercial status:

If Other, please specify:

Contact person

Name of organisation	Sheffield Teaching Hospitals NHS Foundation Trust
Given name	Simon
Family name	Heller
Address	STH Research Department

Town/city	1st Floor, 11 Broomfield Road
Post code	S10 2SE
Country	UNITED KINGDOM
Telephone	0114 226 5938
Fax	0114 226 5937
E-mail	s.heller@sheffield.ac.uk

Is the sponsor based outside the UK?
○ Yes ◉ No

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

A65. Has external funding for the research been secured?

☐ Funding secured from one or more funders
☐ External funding application to one or more funders in progress
☑ No application for external funding will be made

What type of research project is this?
◉ Standalone project
○ Project that is part of a programme grant
○ Project that is part of a Centre grant
○ Project that is part of a fellowship/ personal award/ research training award
○ Other

Other – please state:

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

○ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68-1. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
	Ms	Sam	Heaton
Organisation	Sheffield Teaching Hospitals NHS Foundation Trust		
Address	CRO 11 Broomfield Road Sheffield		
Post Code	S10 2SE		
Work Email	samantha.heaton@sth.nhs.uk		
Telephone	01142265942		
Fax			

Mobile

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 01/08/2014
Planned end date: 30/09/2016
Total duration:
Years: 2 Months: 1 Days: 30

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)

Last patient last visit

A71-2. Where will the research take place? *(Tick as appropriate)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland
- [ ] Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
( ) Yes (•) No

A72. What host organisations (NHS or other) in the UK will be responsible for the research sites? *Please indicate the type of organisation by ticking the box and give approximate numbers of planned research sites:*

[x] NHS organisations in England	1
[ ] NHS organisations in Wales	
[ ] NHS organisations in Scotland	
[ ] HSC organisations in Northern Ireland	
[ ] GP practices in England	
[ ] GP practices in Wales	
[ ] GP practices in Scotland	
[ ] GP practices in Northern Ireland	
[ ] Social care organisations	
[ ] Phase 1 trial units	
[ ] Prison establishments	
[ ] Probation areas	
[ ] Independent hospitals	
[ ] Educational establishments	
[ ] Independent research units	
[ ] Other (give details)	

Total UK sites in study: 1

A76. Insurance/ indemnity to meet potential legal liabilities

***Note:* in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland**

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

- [x] NHS indemnity scheme will apply (NHS sponsors only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

STH are sponsoring the study and are a NHS organisation

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

- [x] NHS indemnity scheme will apply (protocol authors with NHS contracts only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

STH are sponsoring the study and are a NHS organisation

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

- [x] NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)
- [ ] Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

STH are sponsoring the study and are a NHS organisation

Please enclose a copy of relevant documents.

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood only

2. Who will collect the samples?

Blood will be drawn by the phlebotomist, epilepsy nurse, research nurse, or if needed Professor Majid.

3. Who will the samples be removed from?

☑ Living donors
☐ The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?
◉ Yes ○ No

In future research?
◉ Yes ○ No ○ Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

○ Yes ◉ No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*
◉ Yes ○ No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
◉ Yes ○ No

If Yes, say who will have access to the code and personal information about the donor.

All personal data concerning study participants will be kept strictly confidential, and medical records will only be accessed by the clinicians who routinely access them. Personal data will be anonymised at Royal Hallamshire Hospital and stored on a password-protected computer database managed by Prof Majid and Mr Leung at Sheffield Institute for translational neuroscience.

In a form in which the donor could be identifiable to researchers?
○ Yes ◉ No

9. What types of test or analysis will be carried out on the samples?

Flow cytometry and statistical analysis will be performed on the obtained peripheral blood mononuclear cells and cytokines.

10. Will the research involve the analysis or use of human DNA in the samples?

○ Yes ◉ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Samples will be stored in SITraN, Sheffield. This will be accessed by Prof. Majid and Mr.Leung.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☐ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

☐ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☑ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☐ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.*

Research site		Investigator/ Collaborator/ Contact	
Institution name	Sheffield teaching Hospitals NHS Foundation Trust	Title	Prof
Department name	Neurosciences	First name/ Initials	Arshad
Street address	Glossop Road	Surname	Majid
Town/city	Sheffield		
Post Code	S10 2JF		

PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.

4. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendment.

5. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.

6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2006.

7. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.

8. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 1998.

9. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:

    - Will be held by the REC (where applicable) until at least 3 years after the end of the study; and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
    - May be disclosed to the operational managers of review bodies, or the appointing authority for the REC (where applicable), in order to check that the application has been processed correctly or to investigate any complaint.
    - May be seen by auditors appointed to undertake accreditation of RECs (where applicable).
    - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.
    - May be sent by email to REC members.

10. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

11. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

Contact point for publication*(Not applicable for R&D Forms)*

NRES would like to include a contact point with the published summary of the study for those wishing to seek further information. We would be grateful if you would indicate one of the contact points below.

(●) Chief Investigator

( ) Sponsor

- ◯ Study co-ordinator
- ◯ Student
- ◯ Other – please give details
- ◯ None

Access to application for training purposes *(Not applicable for R&D Forms)*
Optional – please tick as appropriate:

☐ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

This section was signed electronically by Professor Arshad Majid on 01/08/2014 14:20.

Job Title/Post:	Cons
Organisation:	STH
Email:	arshad.majid@sheffield.ac.uk
Signature:	.....
Print Name:	
Date:	*(dd/mm/yyyy)*

D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co−sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.
3. Any necessary indemnity or insurance arrangements, as described in question A76, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.
4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
6. The duties of sponsors set out in the Research Governance Framework for Health and Social Care will be undertaken in relation to this research.
7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

This section was signed electronically by Dr Dipak Patel on 30/07/2014 14:19.

Job Title/Post:	Research Manager
Organisation:	Sheffield Teaching Hospitals NHS Foundation Trust
Email:	dipak.patel@sth.nhs.uk

D3. Declaration for student projects by academic supervisor(s)

1. I have read and approved both the research proposal and this application. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level.

2. I undertake to fulfil the responsibilities of the supervisor for this study as set out in the Research Governance Framework for Health and Social Care.

3. I take responsibility for ensuring that this study is conducted in accordance with the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research, in conjunction with clinical supervisors as appropriate.

4. I take responsibility for ensuring that the applicant is up to date and complies with the requirements of the law and relevant guidelines relating to security and confidentiality of patient and other personal data, in conjunction with clinical supervisors as appropriate.

Academic supervisor 1

This section was signed electronically by Professor Arshad Majid on 01/08/2014 11:47.

Job Title/Post:	Consultant
Organisation:	STH
Email:	arshad.majid@sheffield.ac.uk

8.2.2. Protocol outline

STH RESEARCH DEPARTMENT

PROTOCOL OUTLINE Template[1]

	Contents
1	**Project details** 1. Investigator details Prof Arshad Majid (Supervisor) Email: Arshad.Majid@sheffield.ac.uk Tel: 01142 222249 Mr Harvey Leung (Student) Email: HLeung1@sheffield.ac.uk Tel: [redacted] Miss Rebecca Daniels (MSc Student) Email: rcadaniels1@sheffield.ac.uk Tel: [redacted] 2. Sponsor details Sheffield Teaching Hospitals NHS Foundation Trust 3. Project title The Influence of vagal nerve stimulation on blood markers of inflammation 4. STH Project Reference number STH18326 5. Protocol version number and date Version 5 , Date: **11/05/2015** 6. Signatures of Chief Investigator and Sponsor* 7. EUDRACT & CTA Number* NA 8. STH Directorate affiliation Neurosciences
2	**Research question**: clearly defined and answerable We hypothesise that vagal nerve stimulation (VNS) has clinically relevant anti-inflammatory effects. In the proposed study, we will observe the influence of VNS on inflammatory markers, in epilepsy patients undergoing VNS. Blood will be sampled before and after stimulation and inflammatory markers will be measured to determine changes in these factors after VNS. The main focus of this project will be on a class of anti-inflammatory T cells called regulatory T cells (Tregs) which have been shown to play an important role in supressing inflammation in a number of diseases. Secondary objectives include observing the correlation between VNS and its effects on physiological and perceived-stress.
3	**Abstract** Stroke is associated with an intense initial inflammatory phase, which has profound detrimental effects in the brain. In experimental models of stroke, immunosuppression mediated by regulatory T cells (Tregs) can limit the extent of brain damage in mice. Other evidence in mice suggest that its numbers and activity can be influenced by the nervous system via a nerve called the vagus nerve, and its stimulation produces immunosuppressive effects. We therefore hypothesise that vagal nerve stimulation (VNS) will have an anti-inflammatory effect that could be beneficial to post-stroke outcome. VNS in the form of an implanted device is already safely used clinically to treat drug-resistant epilepsy patients and provides us with a unique opportunity, for the first time; to observe its effects on Tregs in humans. We hypothesize that VNS in humans will result in the increased number of Treg cells and increased Treg activity in the blood. We will also measure levels of inflammation biomarkers such as cytokines in blood because changes in these can also affect inflammation. Data generated from this study will guide future studies that will explore the effectiveness of VNS in treating stroke and other inflammatory diseases. The data generated in humans will be compared to the data currently being generated in a similar study using sheep. This study will contribute to Harvey Leung's PhD thesis and Rebecca Daniel's MSc thesis.

4	**Aim of the study** We hypothesise that VNS has profound anti-inflammatory effects in the body. We will test our hypothesis through the following specific aims: 1. To determine Treg proliferation and activity in humans before and after VNS. 2. To determine the influence of VNS on pro and anti-inflammatory cytokines. Secondary aims include: 1. Observe the correlations of inflammatory markers, seizure severity, mood disorder, anxiety, fatigue, and markers of physiological arousal before and after VNS.
5	**Background**: clinical and scientific justification Stroke is a major cause of death and disability worldwide. In the UK alone, over 150,000 strokes occur in a year with 1.1 million survivors of stroke still alive. Although there has been a decrease in number of stroke cases and an increase in survival over the past 10 years, it is still one of the major causes of adult disability with varying severity. The costs of this is estimated to be between £3.7 billion and £8.0 billion and is set to grow with the increase in survival rate (Stroke.org.uk). With so many survivors of stroke there is a need to reduce the severity of disabilities following stroke to provide a better quality of life and reduce the cost associated with stroke. In stroke, there is an initial inflammatory phase which is highly detrimental to the brain. This initial inflammatory phase has been identified as a factor for the extent of brain damage and studies have looked for methods of immunosuppression to reduce this (Dirnagl et al., 2007). Increasing evidence suggests regulatory T cells (Tregs) have a vital role in the suppression of this inflammatory response in an experimental model of stroke in mice (Liesz et al., 2009). Furthermore, Treg therapy in mouse models of stroke appears to be protective (Li et al., 2013). Interestingly, removal of neural input to the spleen (where lymphocytes mature) by cutting the vagus nerve (vagotomy) produces an uncontrolled inflammatory response in vagotomised mice that are unable to suppress the immune response. Analysis of the population of Tregs following this showed a significant decrease in Treg populations and decreased immunosuppressive activity (Mahony et al., 2009). Another study showed that vagal nerve stimulation (VNS) in mice increased their ability to suppress the inflammatory response and increase the survivability of mice from heatstroke as the cause of inflammation (Yamakawa et al., 2013). However, Treg populations and activity following electrical VNS were not analysed. Taking these observations together, we hypothesise that VNS will increase immunosuppressive ability in humans through the increased proliferation and activity of Tregs. In this study, we will have the unique opportunity to observe the effects of VNS on Treg populations and activity. VNS has been in safe clinical use in humans for over 20 years as a treatment for drug-resistant epilepsy (Fisher, 2012). The effect of VNS on Tregs has not been previously been examined in human blood and could provide insight into possible additional applications of VNS to limit brain damage in acute brain ischaemia and other inflammatory diseases. We will compare findings from VNS a similar study in sheep (a large animal model of VNS) and will form the basis of future studies to test the ability of VNS to limit brain damage following a stroke and other diseases. This could ultimately lead to alternative applications for VNS in stroke and other diseases.
6	**Plan of the investigation** 1. Methodology Blood will be drawn into purple topped vials containing EDTA tubes for a full blood count and a tube containing heparin to isolate peripheral blood mononuclear cells (PBMCs). These samples will be analysed on the same day through flow cytometry to observe populations of lymphocytes and other markers of inflammation such as pro and anti-inflammatory cytokines. Samples will be collected and analysed before VNS and up to 6 months after VNS which will occur at routine clinical visits for epilepsy patients undergoing VNS and have been seizure-free for at least 48 hours. Isolated PBMCs and plasma may be frozen and stored for up to 1 year after sample collection to repeat experiment. We will also ask patients to fill out brief questionnaires to

collect data on factors which could cause immune system activation including, infections, stress, anxiety or depression. A three minute ECG recording will also be performed to allow us calculate heart rate variability measures as physiological stress indicators. Epilepsy patients will also fill out an additional questionnaire on recent seizures and fatigue.

2. Design: type of study design and justification
Initially, blood from 5 healthy volunteers will be used to optimise experiments before collecting blood from 10 epilepsy patients. These 10 patients who will be undergoing device insertion for VNS treatment will be recruited from the epilepsy and neurosurgical clinics at the Royal Hallamshire Hospital. We will use blood from these patients to inform statistical analysis for a prospective study. During the course of study, we anticipate an additional 30 epilepsy patients due to undergo device insertion of VNS treatment for a total of 5 healthy volunteers and 40 patients to be recruited for the VNS study.

3. Setting:
Participants for the VNS study will be recruited in the epilepsy clinic of Royal Hallamshire Hospital (RHH) in Sheffield with blood samples processed and analysed within the medical school and in SITraN. If needed, participants may also be recruited in Salford Royal Hospital with samples processed and analysed at the University of Manchester in collaboration with the University of Sheffield.

4. Participants:
Initially, 5 healthy volunteers will be recruited to optimise protocols before recruiting 10 patients for a pilot study to determine statistical power and sample size needed for this study. We estimate that an additional 30 patients will need to be recruited for a total of 5 healthy volunteers and 40 patients to be recruited in total by the end of the first year for the VNS study.

Inclusion criteria:
•Participants must be above 18 years or older
•Participants without a history of autoimmune disorders
•Participants without a history of long term immunomodulatory treatment

Exclusion criteria:
•Participants under the age of 18 years
•Participants with a history of autoimmune disorders
•Participants who have previously received immunomodulatory treatment
•Who already have an electrical implant such as a pacemaker
•Already taking part in another intervention study
Patients that have experienced seizure(s) 48 hours prior or presenting with signs of clinical infection on the day of blood sampling may be asked to have the routine consultation and sampling postponed as a measure of control for the study.

5. Sample size: Power of the study. Viability and representativeness of the sample
We currently cannot calculate sample size due to insufficient available data. Therefore, samples from 5 healthy volunteers will be used to optimise experiments and a pilot study with 10 patients will be carried out first for the VNS study. We estimate an additional 30 patients will be recruited by the end of the first year for the VNS study.

6. Recruitment: method used to identify, approach, recruit and consent
Epilepsy patients for the VNS study will be identified and recruited by neurologists or members of the research team in the epilepsy clinic in RHH. Patients will be sent a patient information sheet by post at least 48 hours before their appointment in hospital. A formal written consent will be taken at the initial visit for VNS surgery. A member of the research team will be able to answer any questions about the study when the patient attends for their appointment and before they are asked to provide consent. Emphasis will be placed on the fact that participation is entirely voluntary and non–participation will have no bearing on their care or treatment. Patients will be given sufficient time (at least 24 hours) to decide whether or not they want to take part in this study. Full voluntary consent will be obtained by the neurologists or a member of the research team via completion of a consent form, at the initial appointment when the patient undergoes a routine check-up before VNS, and a verbal confirmation at subsequent visits up to 6 months after VNS.
The recruitment of healthy volunteers for the optimisation of the VNS will be carried out at the University of Sheffield by a member of the research team. Participants will be given at least 24 hours to decide whether they would like to participate in the study and full voluntary consent will be obtained in writing by a member of the research team prior to the start of sample collection or experiments.

7. Outcome measure(s)
We will determine the quantity of Tregs and their anti-inflammatory potential as well as markers of inflammation before and after VNS. This will determine changes in lymphocyte populations and expression of inflammatory markers. Statistical analysis will be performed to determine changes before VNS and at routine check-ups up to 6 months post-insertion of the stimulator. This study findings will be compared with a similar study conducted in sheep.
Secondary aims observing stress, depression, and epilepsy severity from questionnaires and ECGs will also undergo statistical analysis to determine any correlation between the inflammatory effect and VNS.

8. Analysis including statistical methods, where appropriate will use ANOVA with Bonferroni correction and student's two tailed T test will be performed to compare lymphocyte populations and markers of inflammation before VNS and up to 6 months after VNS.

9. Intervention: flow chart indicating participant's involvement throughout the course of the study

Patients for VNS study

Potential participants are identified by neurologists or a member of the research team in the epilepsy clinic at Royal Hallamshire Hospital

↓

Potential participants are given patient information sheet by post about the study and given at least 48 hours to consider the information

↓

Informed consent obtained by neurologists or a member of the research team at subsequent appointment

↓

Patients will provide an extra volume (25ml) of blood, ECG recording, and answer brief questionnaires at the same time as their routine clinic visit

No further contact is required.

10. Safety assessment: safety parameters and adverse event reporting for interventional studies
There is simple blood sampling with no intervention planned for the VNS study. Only adverse effects will include bruising, pain, and discomfort from drawing blood. This risk will be minimised, however, through coordination of blood sampling on routine check-up before and after VNS. Blood will be drawn by the phlebotomist, epilepsy nurse, research nurse, or a member of the research team.

11. Justification of use of screening tools/questionnaires, etc: include data collection tools, eg screening tools, questionnaires and Case Report Forms
Data will be collected through flow cytometry to quantify lymphocyte populations and markers of inflammation obtained from blood. ECG, perceived stress scale, anxiety, fatigue, and depression screening questionnaires will provide data on both physiological and psychological indictors of stress. Inflammation and stress markers will be observed to identify any correlation between these factors and seizure control according to the Liverpool seizure severity scale (LSSS). Data from 10 patients will be used for a pilot study for VNS study the project.

12. Quality control: Monitoring and audit procedures*

Improvements in seizure control will be monitored to evaluate whether VNS is having a beneficial effect on the patient. Additionally, if a clinical infection is present on the day of a routine check-up or if the patient has had a seizure in the last 48 hours prior to the check-up, blood sampling may be postponed until the patient or volunteer has recovered as a control measure to ensure quality for the research.

13. Project plan with timescale and clearly delineated milestones

Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Recruitment																										
Optimisation																										
Pilot study																										
Data collection																										
Statistical analysis																										
Thesis preparation																										

•Optimisation completed by end of month 1
•Pilot study with 10 patients for VNS by end of month 3.
•All participants recruited by the end of 16 months to be able to conduct longitudinal study that span to up to 8 months each for the VNS study.
•Data collection and statistical analysis complete by 24 months
•Thesis complete by 26 months

7 **Statistical opinion:** recommended for quantitative studies; include evidence and discuss as applicable

As we will be using relatively simple statistical tests (ANOVAs/Student's two-tailed T tests), statistical opinion will initially not be sought. However, we will consult the School of Health and Related Research (ScHARR) if needed.

8 **Project management:** describe what arrangements have been made

Regular weekly meetings between Prof Majid and Mr Leung. Prof Majid will be responsible for monitoring progress and supervising Mr Leung for the length of the study with the aim of scientific publication.

Regular meetings between Prof Majid, Mr Leung and Miss Daniels. Both Prof Majid and Mr Leung will be responsible for monitoring progress and supervising Miss Daniels for the length of her MSc Clinical Neurology project.

9 **Expertise:** of the researcher and associated team

Prof Majid is an experienced neurologist with extensive training in pre-clinical and clinical research. He has worked as a consultant neurologist in several large tertiary care centres for over 11 years. He has extensive experience running large clinical trials.

Mr Leung is a PhD student at the University of Sheffield with a background in BSc Biomedical Science and MSc Translational Neuroscience.

Miss Daniels is a MSc Clinical Neurology student at the University of Sheffield with a background in BSc Natural Sciences

10 **Ethical issues:** description of issues and methods used to address them; include Subject Information Sheet(s) and Consent Form(s) where applicable

The risk to participants is not considered to be significantly greater than that normally associated with routine diagnostic procedures, and if possible blood for research will be taken at the same time as those taken for routine diagnostic purposes. The small risks of complications such as scratching, bruising and minor discomfort arising from taking blood are fully outlined in the patient information sheets. ECGs and questionnaires used this project are non-intrusive and used routinely for screening patients for around the world.

	Vulnerable adults and people under 18 years of age will be excluded from taking part. Consent will be obtained via signed consent forms for all participants by the consultant providing care for patients in the epilepsy clinic. Information will be provided orally and supported by the patient information sheets. Professor Majid will be available to answer any questions about the study. All personal data concerning study participants will be kept strictly confidential, and medical records will only be accessed by the clinicians who routinely access them. Personal data will be anonymised at the RHH and stored on a password-protected computer database managed by Prof Majid and Mr Leung at SITraN. Identifiable data will not be published or used by the researchers, as all samples will be assigned a unique identification code, the details of which Prof Majid and Mr Leung will maintain at SITraN and store for 3 years after completion of the study to enable publications to be generated.
11	**Service users:** involvement during study design Service users were not involved in the study design. Results will be verbally communicated to participants if requested and copies of any publications made available to them.
12	**Dissemination:** methods for dissemination of the research It is anticipated that results of this study will be reported in peer-reviewed journals, in internal reports at SITraN, presented at relevant local, national and international conferences related to stroke and neuroimmunology research and to associated community groups.
13	**Taking the work forward:** describe the strategy for development if the research project is productive This study will form the basis for further study to investigate the potential of vagal nerve stimulation as a treatment in limiting brain damage after a stroke in large animal models. Funding for this will be sought through external funding bodies in the relevant fields. Continued collaboration with Cyberonics, Texas to provide stimulators will be ideal to further explore the potential of nerve stimulators.
14	**Intellectual Property:** describe what arrangements have been made No arrangements have been made as intellectual property issues do not arise with this project.
15	**Costing schedule:** specify the costs associated with the project ECG machine and costs - £1000 Flow cytometry machine time - £500 Antibodies and other supplies - £3000 Miscellaneous - £2000
16	**Funding arrangements:** If there is no funding associated with the project, explain the agreement with the host research team/ clinical area for the use of resources. The funding will come from Professor Majid's research account.
17	**References** Dirnagl, U., Klehmet, J., Braun, J.S., Harms, H., Meisel, C., Ziemssen, T., Prass, K., and Meisel, A. (2007). Stroke-induced immunodepression: experimental evidence and clinical relevance. Stroke. 38, 770–773. Fisher, R. (2012). Therapeutic devices for epilepsy. Ann. Neurol. 71, 157–168. Li, P., Mao, L., Zhou, G., Leak, R.K., Sun, B.-L., Chen, J., and Hu, X. (2013). Adoptive Regulatory T-Cell Therapy Preserves Systemic Immune Homeostasis After Cerebral Ischemia. Stroke. Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., Giese, T., and Veltkamp, R. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat. Med. 15, 192–199. Mahony, C.O., Kleij, H. Van Der, Bienenstock, J., Shanahan, F., Mahony, L.O., O'Mahony, C., van der Kleij, H., and O'Mahony, L. (2009). Loss of vagal anti-inflammatory effect: in vivo visualization and adoptive transfer. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R1118–26. Stroke.org.uk http://www.stroke.org.uk/resource-sheet/stroke-statistics [accessed 14/11/2013].

	Yamakawa, K., Matsumoto, N., Imamura, Y., Muroya, T., Yamada, T., Nakagawa, J., Shimazaki, J., Ogura, H., Kuwagata, Y., and Shimazu, T. (2013). Electrical vagus nerve stimulation attenuates systemic inflammation and improves survival in a rat heatstroke model. PLoS One 8, e56728.
18	**Curriculum Vitae:** include brief CV **Name:** Prof Arshad Majid **Work address:** Sheffield Institute for Translational Neuroscience (SITraN) Department of Neuroscience The University of Sheffield 385a Glossop Road Sheffield S10 2HQ Tel: 01142 222249 Email: Arshad.Majid@sheffield.ac.uk **Name:** Mr Harvey Leung **Work address:** Sheffield Institute for Translational Neuroscience (SITraN) Department of Neuroscience The University of Sheffield 385a Glossop Road Sheffield S10 2HQ Tel: [redacted] Email: HLeung1@sheffield.ac.uk **Qualifications:** BSc Biomedical Science University of Sheffield 2012 MSc Translational Neuroscience University of Sheffield 2013 **Name:** Miss Rebecca Daniels **Work address:** Sheffield Institute for Translational Neuroscience (SITraN) Department of Neuroscience The University of Sheffield 385a Glossop Road Sheffield S10 2HQ Tel: [redacted] Email: rcadaniels1@sheffield.ac.uk **Qualifications:** **BSc Natural Sciences**
19	**Other:** Contact details*

8.2.3. Volunteer information sheet

Royal Hallamshire Hospital
Glossop Road
Sheffield, S10 2JF
Tel: 0114 271 1900

VOLUNTEER INFORMATION SHEET

Study title: The influence of Vagal Nerve Stimulation on inflammatory markers in blood

Principal investigators: *Professor Arshad Majid*

You are invited to participate in a research project that will investigate whether vagal nerve stimulation alters the amount of inflammation in the body. This will be done by measuring markers of inflammation the blood.

Before you decide whether to take part it is important that you understand why the research is being done and what it will involve. Please take time to read the following information sheet carefully and discuss it with friends, or relatives, if you wish.

Do ask us if there is anything that is not clear to you or if you would like more information.

1. **What is the purpose of the study?**

The study is to discover whether vagal nerve stimulation reduces inflammation in the body. If it is found that vagal nerve stimulation reduces inflammation, then vagal nerve stimulation could be used to treat diseases that are caused by excessive inflammation.

2. **Why have I been chosen?**

You have been chosen because you are a healthy adult that is not due to be treated with vagus nerve stimulation. Your contribution to this study will help to optimise the experiments performed on patient samples in the future.

3. **Do I have to take part?**

Your participation in the study is entirely voluntary. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part you are still free to withdraw from the study at any time without giving a reason.

4. **What will happen to me if I take part?**

You will be asked to provide a sample of blood at the earliest convenience of both yourself and the researcher.

Your medical records will not be looked at by the researcher.

5. **What are the possible disadvantages and risks of taking part?**

Disadvantages and risks are kept to a minimum as a trained clinician/**researcher** will be collecting the blood in the same manner as that of collecting blood from patients in the hospital.

6. **What are the possible benefits of taking part?**

Although this study will not benefit you directly, it will contribute to research in identifying additional therapeutic uses of vagal nerve stimulation that may benefit others in the future.

7. **What if something goes wrong?**

Any complaint about the way you have been dealt with during the study will be addressed. If you have a concern about any aspect of the study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this by writing to the study principal investigator Professor Arshad Majid, Sheffield Institute for Translational Neurosciences, Glossop Road, Sheffield, S10 2HQ or telephone the NHS Complaints Procedure on 020 8672 1255.

8. **Will my taking part in this study be kept confidential?**

All samples will be anonymised at the point of collection and no information regarding your medical history will be collected for this research. All data and information regarding your age and gender will be treated as strictly confidential and securely stored in a locked office at the University of Sheffield. If any information is released outside of this office, this will be done in an anonymised form with your name removed to maintain confidentiality. Results of the study will be stored in a password protected computer database for a maximum of five years.

9. **Who is organising and funding the study?**

The University of Sheffield and Sheffield Teaching Hospitals NHS foundation Trust are organising and funding this study.

Further information can be obtained from: *Professor Arshad Majid*, Sheffield Institute for Translational Neurosciences, Glossop Road, Sheffield, S10 2HQ Tel: 0114 222 2249

8.2.4. Volunteer consent form template

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Royal
Hallamshire Hospital
Glossop Road
Sheffield, S10 2JF
Tel: 0114 271 1900

Study ID Number: **STH18326**

VOLUNTEER CONSENT FORM

Study title: The influence of Vagal Nerve Stimulation on inflammatory markers in blood

Principal Investigator: Professor Arshad Majid

Please initial box

1) I confirm that I have read and understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2) I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason. ☐

3) I agree to provide 25 ml of blood to be taken for the purposes of this research study. ☐

4) I understand that collected samples will be anonymised at the point of collection. ☐

5) I understand that some personally identifiable information (age and gender) will be collected, but will be assigned to a unique identifier. Data will be stored in a locked office in the university. ☐

6) I agree to take part in the above study. ☐

Name of Volunteer	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature

1 copy for volunteer, 1 copy for researcher

8.2.5. Patient information sheet

Royal Hallamshire Hospital
Glossop Road
Sheffield, S10 2JF
Tel: 0114 271 1900

PATIENT INFORMATION SHEET

Study title: The Influence of Vagal Nerve Stimulation on Inflammatory Markers in Blood

Principal investigator: *Professor Arshad Majid*

You are invited to participate in a research project that will investigate whether vagal nerve stimulation alters the amount of inflammation in the body. This will be done by measuring markers of inflammation the blood.

Before you decide whether to take part it is important that you understand why the research is being done and what it will involve. Please take time to read the following information sheet carefully and discuss it with friends, or relatives, if you wish.

Do ask us if there is anything that is not clear to you or if you would like more information.

1. **What is the purpose of the study?**

The study is to discover whether vagal nerve stimulation reduces inflammation in the body. If it is found that vagal nerve stimulation reduces inflammation, then vagal nerve stimulation could be used to treat diseases that are caused by excessive inflammation. The study will also investigate if the stimulation affects the levels of stress on the body.

2. **Why have I been chosen?**

You have been chosen because you will be having a vagal nerve stimulator inserted.

3. **Do I have to take part?**

Your participation in the study is entirely voluntary. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part you are still free to withdraw from the study at any time without giving a reason. If you decide *not* to take part, the standard of medical care you receive will not be affected.

4. **What will happen to me if I take part?**

You will be asked to fill out brief questionnaires on: seizures, stress, anxiety, fatigue, and depression, undergo an ECG to measure stress, and provide a blood at each routine visit before and up to 6 months after insertion of the vagal nerve stimulator. You may be asked to return at a later date if you have had a seizure 2 days before or presenting signs of clinical infections on the day of the routine visit.

Your medical records will be looked at by the researcher so we can obtain information about your symptoms, medication and past medical history.

5. What are the possible disadvantages and risks of taking part?

An extra blood test is needed for this study. This blood test may therefore involve an "extra needle". The needle used will be the same size as is normally used to take blood from patients in hospitals.

6. What are the possible benefits of taking part?

Although this study will not benefit you directly, it will contribute to research in identifying additional therapeutic uses of vagal nerve stimulation that may benefit others in the future.

7. What if something goes wrong?

Any complaint about the way you have been dealt with during the study will be addressed. If you have a concern about any aspect of the study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this by writing to the study principal investigator Professor Arshad Majid, Sheffield Institute for Translational Neurosciences, Glossop Road, Sheffield, S10 2HQ or telephone the NHS Complaints Procedure on 020 8672 1255.

8. What if I am unhappy about any aspects of my medical care?

If you have any concerns about your medical care, we will, with your permission, inform your clinical care team who can advise you and provide details of further action you can take if necessary.

9. Will my taking part in this study be kept confidential?

Information relevant to your medical condition will be collected as part of the study. These data along with your responses to the questionnaire will be treated as strictly confidential and securely stored in a locked office at the Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS foundation Trust.

If any information is released outside the study office this will be done in a coded form with your name removed from the records so that confidentiality is strictly maintained. The results of this study will be stored in a password protected computer database for a maximum of five years.

10. Who is organising and funding the study?

The University of Sheffield and Sheffield Teaching Hospitals NHS foundation Trust are organising and funding this study.

Further information can be obtained from: *Professor Arshad Majid*, Sheffield Institute for Translational Neurosciences, Glossop Road, Sheffield, S10 2HQ Tel: 0114 222 2249

8.2.6. Patient consent form template

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Royal Hallamshire Hospital
Glossop Road
Sheffield, S10 2JF
Tel: 0114 271 1900

Study ID Number | | | |

PATIENT CONSENT FORM

Study title: The influence of Vagal Nerve Stimulation on inflammatory markers in blood

Principal Investigator: Professor Arshad Majid

Please initial box

1) I confirm that I have read and understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2) I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected. ☐
3) I agree to provide 25 ml of blood to be taken either at the same time as other blood tests or for the purposes of this research study. I will also undergo and ECG and will fill out short questionnaires. ☐
4) I understand that relevant sections of my medical notes may be looked at by individuals from the research group where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
5) I understand that some personally identifiable information (my name, address, date of birth and telephone number) will be collected but these data will be stored in a locked office in the hospital or university and will be kept separate from any medical information. ☐
6) I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature

1 copy for patient, 1 copy for researcher, 1 copy to be kept with hospital notes

8.3. Patient information

Patient ID 011	**Age** 22	**Gender** Male
Diagnosis Refractory epilepsy		
List of medications prescribed during study	**Use and anti-inflammatory effects if any**	
Beclometasone	Treatment for oral ulcertaion / asthma / prophylaxis	
Carbamazepine	AED	
Epipen	Prescribed for food allergy – no cases of allergy documented during period of study	
Lamotrigine	AED	
Salbutamol sulfate *	Bronchospasm treatment	

Patient ID 014	**Age** 46	**Gender** Female
Diagnosis Refractory epilepsy		
List of medications prescribed during study	**Use and anti-inflammatory effects if any**	
Aciclovir	Treatment for suspected shingles between 16-21/6/16	
Mizolastine*	Anti-inflammatory (anti-histamine) drug for hayfever	
Omeprazole	Typically used to prevent/treat stomach ulcers and treatment for indigestion and acid reflux.	
OTC cranberry remedies for cystitis symptoms	Cystitis symptom relief	
Phenobarbitol	AED	
Zapain	Contains paracetamol and codeine phosphate.	

Patient ID 016	**Age** 25	**Gender** Male
Diagnosis Medically refractory focal epilepsy with aphasia, previous multiple subpial transections left temporal love and left MTS		
List of medications prescribed during study	**Use and anti-inflammatory effects if any**	
Clobazam	AED	
Flucloxacillin *	Antibiotic, 1 dose only	
Keppra	AED	
Midazolam	AED	
Nitrofurantoin*	Antibiotic, 1 dose only	
Rufinamide	Anticonvulsant medication, 3 doses (not routine)	

Patient ID 022	**Age** 42	**Gender** Female
Diagnosis Focal temporal lobe epilepsy		
List of medications prescribed during study	**Use and anti-inflammatory effects if any**	
Alzain (brand of pregabalin)	Treatment for epilepsy, neuropathic pain, and generalised anxiety disorder	
Ibuprofen*	NSAID	
Perampanel	AED	
Pregabalin	AED	
Zonegran	AED	

8.4. Copies of Questionnaires

8.4.1. Liverpool Seizure Severity Scale 2.0 (LSSS)

Liverpool Seizure Severity Scale 2.0

So we can better understand the severity of your seizures, please complete the following questionnaire thinking about the most severe seizure you experienced during the past 4 weeks. (This may be different for each individual, but is based on your most severe seizures over the past 4 weeks.) Your responses are a very important part of this study and will be kept strictly CONFIDENTIAL. No one but the research staff will see your responses. If results of this study are published, only aggregate data will be used; names and any other identifying information will not be reported.

How many seizures have you experienced during the past 4 weeks?	_____ seizures	**Note**: Please enter '0' if you have not experienced any seizures in the last 4 weeks and do not complete the remainder of the questionnaire. If you cannot remember the exact number of seizures you've experiences, please estimate based on the number you usually had during a single day or week.

1	**I feel that my most severe seizures have mostly been:**	Very severe	Severe	Mild	Very mild		
2	**Most commonly when I blank out/lose consciousness:**	I blank out for less than 1 minute	I blank out for between 1 and 2 minutes	I blank out for between 3 and 5 minutes	I blank out for more than 5 minutes	I never blank out/ lose consciousness	
3	**When I have my most severe seizures, I smack my lips, fidget, or behave in an unusual way:**	Always	Usually	Sometimes	Never		
4	**After my most severe seizures:**	I feel very confused	I feel fairly confused	I feel slightly confused	I do not feel confused at all		
5	**After my most severe seizures my confusion lasts for:**	Less than 1 minute	Between 1 and 5 minutes	Between 6 minutes and 1 hour	1 to 2 hours	More than 2 hours	I never feel confused
6	**When I have my most severe seizures:**	I always fall to the ground	I usually fall to the ground	I sometimes fall to the ground	I never fall to the ground		

7	**After my most severe seizures:**	I always have a headache	I usually have a headache	I sometimes have a headache	I never have a headache	
8	**After my most severe seizures:**	I always feel sleepy	I usually feel sleepy	I sometimes feel sleepy	I never feel sleepy	
9	**After my most severe seizures:**	I always find I have wet myself	I usually find that I have wet myself	I sometimes find that I have wet myself	I never find that I have wet myself	
10	**After my most severe seizures:**	I always find that I have bitten my tongue	I usually find that I have bitten my tongue	I sometimes find that I have bitten my tongue	I never find that I have bitten my tongue	
11	**After my most severe seizures:**	I always find that I have injured myself (other than biting my tongue)	I usually find that I have injured myself (other than biting my tongue)	I sometimes find that I have injured myself (other than biting my tongue)	I never find that I have injured myself (other than biting my tongue)	
12	**After my most severe seizures I can usually return to what I am doing in:**	Less than 1 minute	Between 1 and 5 minutes	Between 6 minutes and 1 hour	1 to 2 hours	More than 2 hours

Reference

Scott-Lennox, J., Bryant-Comstock, L., Lennox, R., & Baker, G. A. (2001). Reliability, validity and responsiveness of a revised scoring system for the Liverpool Seizure Severity Scale. *Epilepsy Research*, *44*(1), 53–63. doi:10.1016/S0920-1211(01)00186-3

Liverpool Seizure Severity Scale 2.0 scoring procedure

Scoring procedures for the Liverpool Seizure Severity Scale 2.0. The Liverpool Seizure Severity Scale 2.0 questionnaire produces a single unit-weighted scale that measures severity of the most severe seizures the patient experienced during the recall period. To score the Liverpool Seizure Severity Scale 2.0, the following steps are required:

Step 1: Assign '0' scores for patients without seizures during the recall period. If patients report 0 seizures during the past 4 weeks, assign a score of 0 ICTAL scale. If the number of seizures during the past 4 weeks is greater than 0 or is missing, then proceed to Step 2.

Step 2: Reverse code responses to questions 1, 3, 4, 6, 7, 8, 9, 10, and 11 so that lower values reflect less severity.

Step 3: Score the ICTAL scale:

- If 4 or more of the questions 1 through 12 have missing responses, assign a missing score for the ICTAL scale.
- If 1–3 of the questions 1–12 have missing responses, create an average of the non-missing responses for questions 1–12 and replace the missing responses with the average of the non-missing responses.
- Sum the responses to questions 1–12 (after reverse coding).
- Divide by 40.
- Multiply the dividend by 100.

Individual patients' ICTAL scores should range from 0 to 100.

Reference

Scott-Lennox, J., Bryant-Comstock, L., Lennox, R., & Baker, G. A. (2001). Reliability, validity and responsiveness of a revised scoring system for the Liverpool Seizure Severity Scale. *Epilepsy Research, 44*(1), 53–63. doi:10.1016/S0920-1211(01)00186-3

8.4.2. Neurological Disorders Depression Inventory for Epilepsy (NDDI-E)

Background:

Depression is a common disorder in epilepsy but is not routinely assessed in clinics. The Neurological disorders depression inventory for epilepsy (NDDI-E) is used to rapid screening of major depression in people with epilepsy. Symptoms of major depression in this test is able to differentiate that from adverse effects of AED. The baseline of a score <15 was determined and verified from 229 patients. An NDDI-E score of more than 15 had a specificity of 90%, sensitivity of 81%, and positive predictive value of 0·62 for the identification of major depression.

Neurological Disorders Depression Inventory for Epilepsy (NDDI-E)

Please circle the number that best describes how often you have felt over the last two weeks including today:

	Always or often	Sometimes	Rarely	Never
1. Everything is a struggle	4	3	2	1
2. Nothing I do is right	4	3	2	1
3. Feel guilty	4	3	2	1
4. I'd be better off dead	4	3	2	1
5. Frustrated	4	3	2	1
6. Difficulty finding pleasure	4	3	2	1

Once completed, please return the form back to the researcher.

Disclaimer: Although screening instruments are not intended to replace clinical judgment in the diagnosis of major depression, they are recommended by many professional and governmental health organizations as aids for identification.

References

Gilliam, F. G., Barry, J. J., Hermann, B. P., Meador, K. J., Vahle, V., & Kanner, A. M. (2006). Rapid detection of major depression in epilepsy: a multicentre study. *The Lancet. Neurology*, *5*(5), 399–405. doi:10.1016/S1474-4422(06)70415-X

www.epilepsyfoundation.org

8.4.3. General Anxiety Disorder Assessment (GAD-7)

Patient.co.uk emis

Generalised Anxiety Disorder Assessment (GAD 7)

This easy to use self-administered patient questionnaire is used as a screening tool and severity measure for generalised anxiety disorder. [1] [2]

Generalised Anxiety Disorder Questionnaire (GAD-7)

Over the last 2 weeks, how often have you been bothered by any of the following problems?

Feeling nervous, anxious or on edge?	Not at all / Several days / More than half the days / Nearly every day
Not being able to stop or control worrying?	Not at all / Several days / More than half the days / Nearly every day
Worrying too much about different things?	Not at all / Several days / More than half the days / Nearly every day
Trouble relaxing?	Not at all / Several days / More than half the days / Nearly every day
Being so restless that it is hard to sit still?	Not at all / Several days / More than half the days / Nearly every day
Becoming easily annoyed or irritable?	Not at all / Several days / More than half the days / Nearly every day
Feeling afraid as if something awful might happen?	Not at all / Several days / More than half the days / Nearly every day
Total= /21	

The GAD-7 originates from Spitzer RL, Kroenke K, Williams JB, et al; A brief measure for assessing generalized anxiety disorder: the GAD-7. Arch Intern Med. 2006 May 22;166(10):1092-7. GAD-7 © Pfizer Inc. all rights reserved; used with permission.

The GAD-7 score is calculated by assigning scores of 0, 1, 2, and 3, to the response categories of "not at all," "several days," "more than half the days," and "nearly every day," respectively, and adding together the scores for the seven questions. [3]

Scores of 5, 10, and 15 are taken as the cut off points for mild, moderate, and severe anxiety, respectively. When used as a screening tool, further evaluation is recommended when the score is 10 or greater.
Using the threshold score of 10, the GAD-7 has a sensitivity of 89% and a specificity of 82% for generalised anxiety disorder. It is moderately good at screening three other common anxiety disorders – panic disorder (sensitivity 74%, specificity 81%), social anxiety disorder (sensitivity 72%, specificity 80%), and post-traumatic stress disorder (sensitivity 66%, specificity 81%). [4]

Further reading & references

1. Swinson RP; The GAD-7 scale was accurate for diagnosing generalised anxiety disorder. Evid Based Med. 2006 Dec;11(6):184.
2. Spitzer RL, Kroenke K, Williams JB, et al; A brief measure for assessing generalized anxiety disorder: the GAD-7. Arch Intern Med. 2006 May 22;166(10):1092-7.
3. IAPT Outcomes Toolkit 2008/9 NHS website
4. Kroenke K, Spitzer RL, Williams JB, et al; Anxiety disorders in primary care: prevalence, impairment, comorbidity, and detection. Ann Intern Med. 2007 Mar 6;146(5):317-25.

Original Author:	Current Version:	
Dr Huw Thomas	Dr Huw Thomas	
Document ID:	Last Checked:	Next Review:
8736 (v10)	26/10/2010	25/10/2015

8.4.4. Fatigue Assessment Scale (FAS)

Fatigue Assessment Scale (FAS)

The following 10 statements refer to how you usually feel. For each statement you can choose one out of five answer categories, varying from *never* to *always*. 1=*never*, 2=*sometimes*; 3=*regularly*; 4=*often*; and 5=*always*.

	Never	Sometimes	Regularly	Often	Always
1. I am bothered by fatigue	1	2	3	4	5
2. I get tired very quickly	1	2	3	4	5
3. I don't do much during the day	1	2	3	4	5
4. I have enough energy for everyday life	1	2	3	4	5
5. Physically, I feel exhausted	1	2	3	4	5
6. I have problems starting things	1	2	3	4	5
7. I have problems thinking clearly	1	2	3	4	5
8. I feel no desire to do anything	1	2	3	4	5
9. Mentally, I feel exhausted	1	2	3	4	5
10.When I am doing something, I can concentrate quite well	1	2	3	4	5

Reference: Michielsen, H.J., De Vries, J. & Van Heck, G.L., 2003. Psychometric qualities of a brief self-rated fatigue measure: The Fatigue Assessment Scale. Journal of psychosomatic research, 54(4), pp.345–52.

8.4.5. Perceived Stress Scale (PSS)

PERCEIVED STRESS SCALE

Sheldon Cohen

The *Perceived Stress Scale* (PSS) is the most widely used psychological instrument for measuring the perception of stress. It is a measure of the degree to which situations in one's life are appraised as stressful. Items were designed to tap how unpredictable, uncontrollable, and overloaded respondents find their lives. The scale also includes a number of direct queries about current levels of experienced stress. The PSS was designed for use in community samples with at least a junior high school education. The items are easy to understand, and the response alternatives are simple to grasp. Moreover, the questions are of a general nature and hence are relatively free of content specific to any subpopulation group. The questions in the PSS ask about feelings and thoughts during the last month. In each case, respondents are asked how often they felt a certain way.

Evidence for Validity: Higher PSS scores were associated with (for example):

- failure to quit smoking
- failure among diabetics to control blood sugar levels
- greater vulnerability to stressful life-event-elicited depressive symptoms
- more colds

Health status relationship to PSS: Cohen et al. (1988) show correlations with PSS and: Stress Measures, Self-Reported Health and Health Services Measures, Health Behavior Measures, Smoking Status, Help Seeking Behavior.

Temporal Nature: Because levels of appraised stress should be influenced by daily hassles, major events, and changes in coping resources, predictive validity of the PSS is expected to fall off rapidly after four to eight weeks.

Scoring: PSS scores are obtained by reversing responses (e.g., 0 = 4, 1 = 3, 2 = 2, 3 = 1 & 4 = 0) to the four positively stated items (items 4, 5, 7, & 8) and then summing across all scale items. A short 4 item scale can be made from questions 2, 4, 5 and 10 of the PSS 10 item scale.

Norm Groups: L. Harris Poll gathered information on 2,387 respondents in the U.S.

Norm Table for the PSS 10 item inventory

Category	N	Mean	S.D.
Gender			
Male	926	12.1	5.9
Female	1406	13.7	6.6
Age			
18-29	645	14.2	6.2
30-44	750	13.0	6.2
45-54	285	12.6	6.1
55-64	282	11.9	6.9
65 & older	296	12.0	6.3
Race			
white	1924	12.8	6.2
Hispanic	98	14.0	6.9
black	176	14.7	7.2
other minority	50	14.1	5.0

Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts **during the last month**. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.

Name _____ Date _____

Age _____ Gender (*Circle*): **M F** Other _____

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
2. In the last month, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
3. In the last month, how often have you felt nervous and "stressed"?	0	1	2	3	4
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	0	1	2	3	4
5. In the last month, how often have you felt that things were going your way?	0	1	2	3	4
6. In the last month, how often have you found that you could not cope with all the things that you had to do?	0	1	2	3	4
7. In the last month, how often have you been able to control irritations in your life?	0	1	2	3	4
8. In the last month, how often have you felt that you were on top of things?	0	1	2	3	4
9. In the last month, how often have you been angered because of things that were outside of your control?	0	1	2	3	4
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

Please feel free to use the *Perceived Stress Scale* for your research.

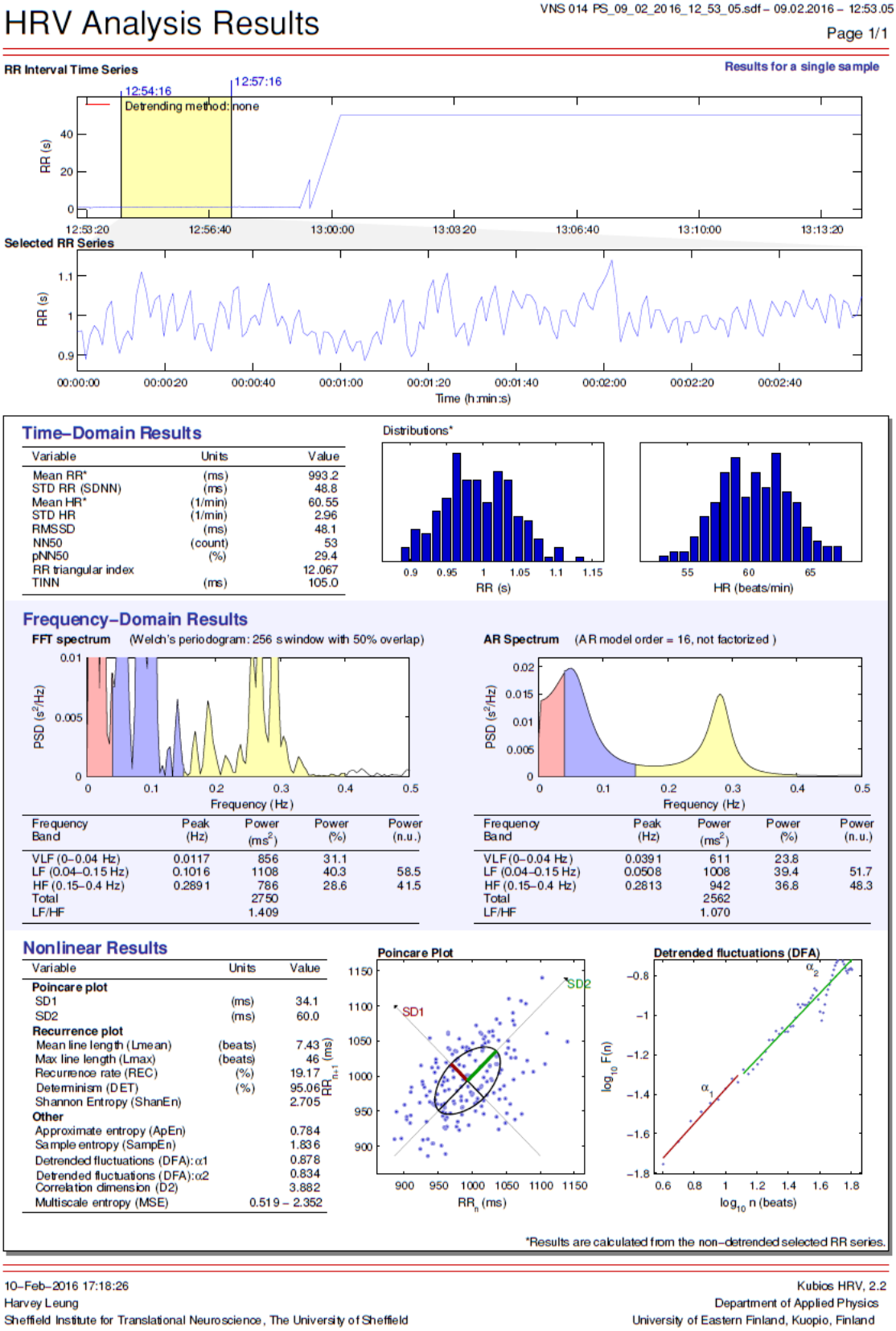
Mind Garden, Inc.
info@mindgarden.com
www.mindgarden.com

References

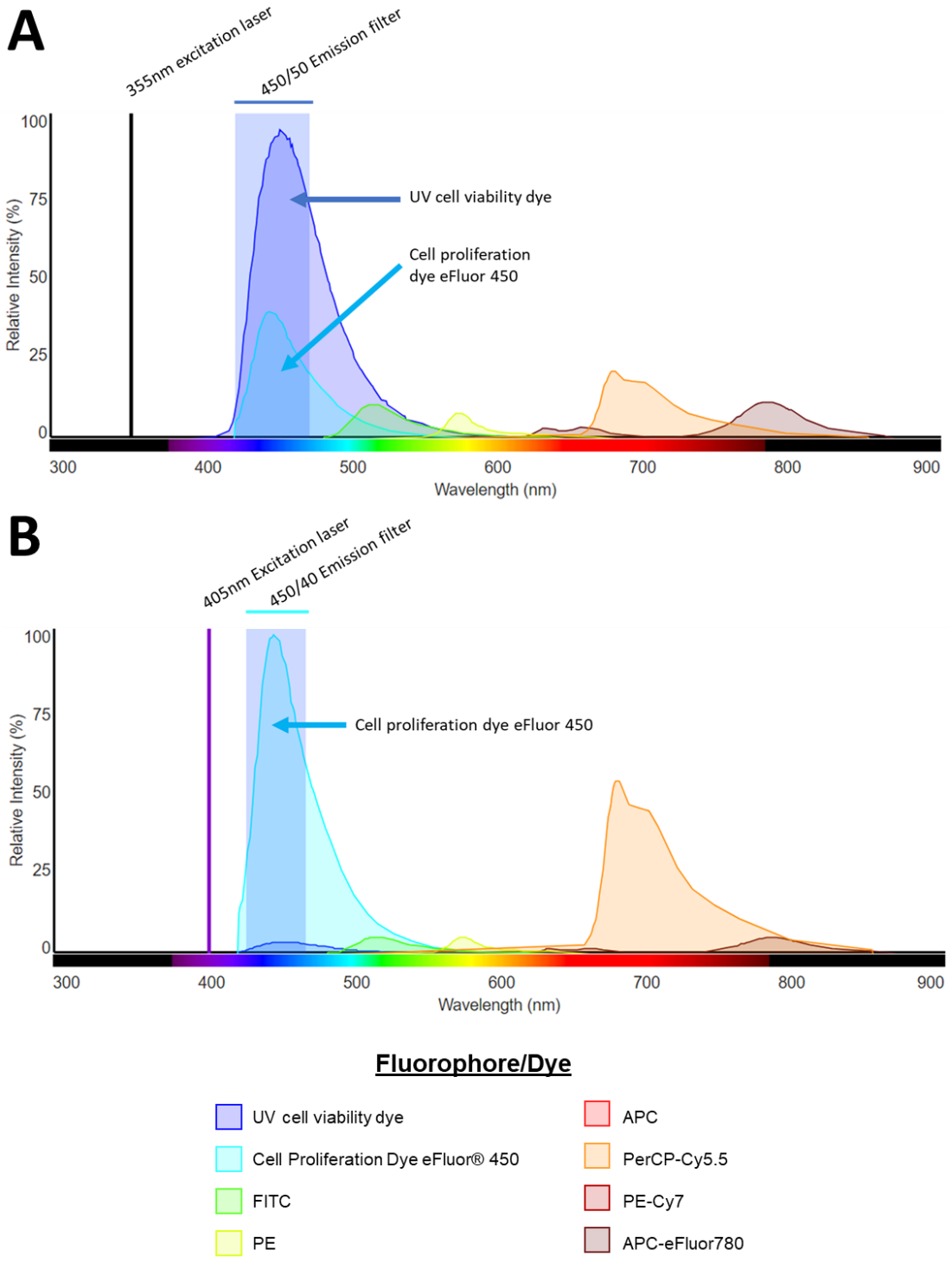
The PSS Scale is reprinted with permission of the American Sociological Association, from Cohen, S., Kamarck, T., and Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behavior, 24*, 386-396.

Cohen, S. and Williamson, G. Perceived Stress in a Probability Sample of the United States. Spacapan, S. and Oskamp, S. (Eds.) *The Social Psychology of Health*. Newbury Park, CA: Sage, 1988.

8.5. Figures

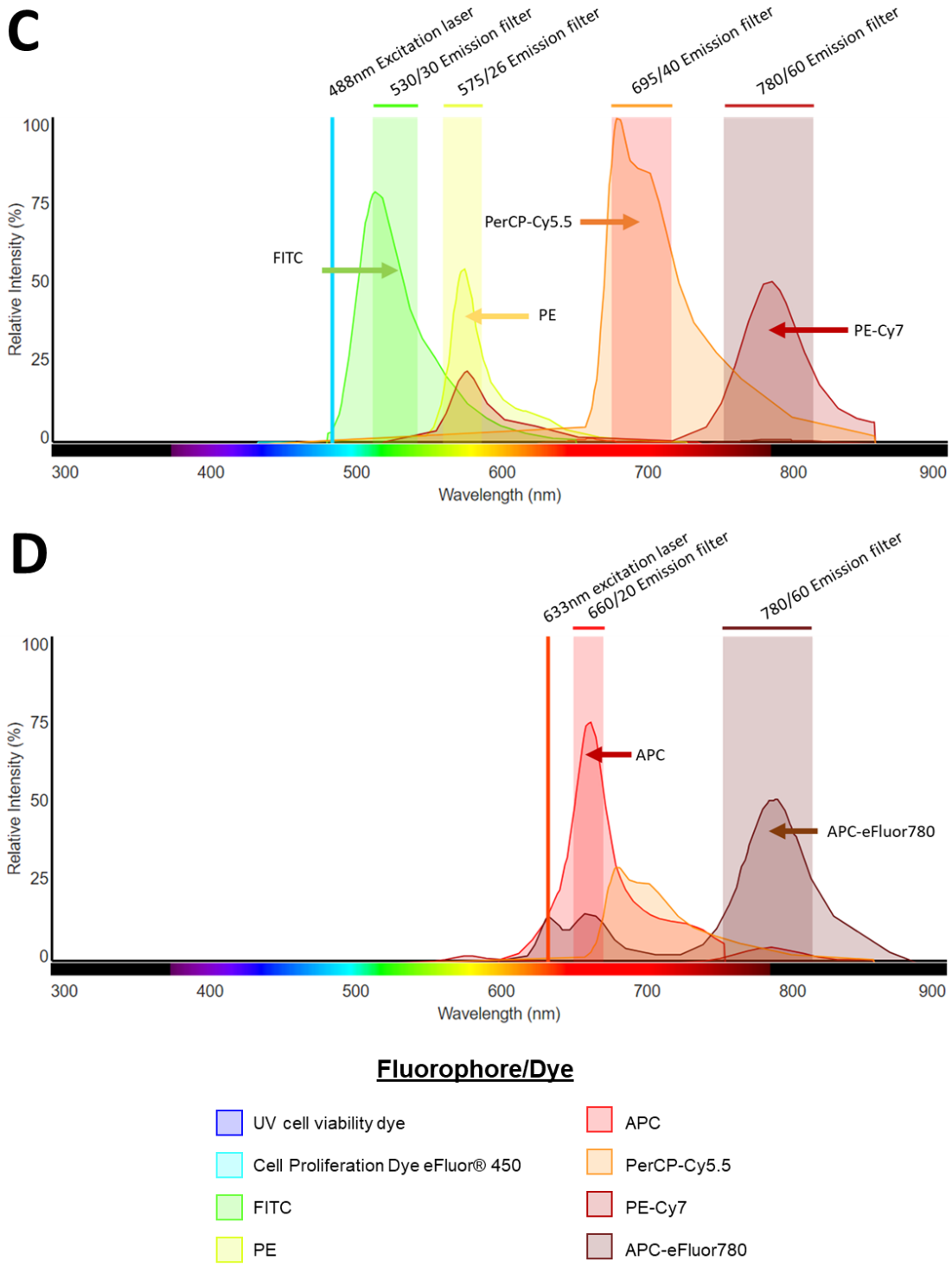


Appendix Figure 1 Example of automated HRV analysis by Kubios HRV
HRV measurements for patient 014 prior to VNS implantation



Appendix Figure 2 Fluorescence emission of fluorophores and dyes selected for flow cytometry

Fluorophore and dye combinations were selected using the Fluorescence SpectraViewer available online (https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html). The LSRII flow cytometer has 4 excitation lasers at (A) 355nm, (B) 405nm, (C) 488nm, and (D) 633nm with a selection of emission filters for each laser. Combinations used were chosen to have a relatively high intensity of emission, peak of emission passing through emission filter, and the least amount of overlapping fluorescence emission for each excitation laser.



Appendix Figure 2 (cont'd) Fluorescence emission of fluorophores and dyes selected for flow cytometry

Fluorophore and dye combinations were selected using the Fluorescence SpectraViewer available online (https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html). The LSRII flow cytometer has 4 excitation lasers at (A) 355nm, (B) 405nm, (C) 488nm, and (D) 633nm with a selection of emission filters for each laser. Combinations used were chosen to have a relatively high intensity of emission, peak of emission passing through emission filter, and the least amount of overlapping fluorescence emission for each excitation laser.

8.6. Tables

Appendix Table 1 Summary of HRV parameters

	Measure	Units	Description
Time-Domain	RR	[ms]	The mean of RR intervals
	STD RR (SDNN)	[ms]	Standard deviation of RR intervals
	HR	[1/min]	The mean of heart rate
	STD HR	[1/min]	Standard deviation of instantaneous heart rate values
	RMSSD	[ms]	Square root of the mean squared differences between successive RR intervals
	NN50		Number of successive RR interval pairs that differ more than 50 ms
	pNN50	[%]	NN50 divided by the total number of RR intervals
	HRV Triangular index		The integral of the RR interval histogram divided by the height of the histogram
	TINN	[ms]	Baseline width of the RR interval histogram
Frequency-Domain	Peak frequency	[Hz]	VLF, LF, and HF band peak frequencies
	Absolute power	[ms^2]	Absolute powers of VLF, LF, and HF bands
	Relative power	[%]	Relative powers of VLF, LF, and HF bands VLF [%] = VLF [ms^2] / total power [ms^2] × 100% LF [%] = LF [ms^2] / total power [ms^2] × 100% HF [%] = HF [ms^2] / total power [ms^2] × 100%
	Normalised power	[n.u.]	Powers of LF and HF bands in normalized units LF [n.u.] = LF [ms^2] / (total power [ms^2] – VLF [ms^2]) HF [n.u.] = HF [ms^2] / (total power [ms^2] – VLF [ms^2])
	LF/HF		Ratio between LF and HF band powers
Non-linear	SD1, SD2	[ms]	The standard deviation of the Poincaré plot perpendicular to (SD1) and along (SD2) the line-of-identity
	ApEn		Approximate entropy
	SampEn		Sample entropy
	D_2		Correlation dimension
	DFA		**Detrended fluctuation analysis:**
	α1		Short term fluctuation slope
	α2		Long term fluctuation slope
	RPA		**Recurrence plot analysis:**
	Lmean	[beats]	Mean line length
	Lmax	[beats]	Maximum line length
	REC	[%]	Recurrence rate
	DET	[%]	Determinism
	ShanEn		Shannon entropy

Table taken from Kubios HRV version 2.2 User's guide 2014.

Appendix Table 2 Sample size calculations for questionnaires

Questionnaire	Sample size required for when α=0.05, 1-β=0.8	
	3M – PS	6M – PS
LSSS	32	118
NDDI-E	1030	1067
GAD-7	63	182
FAS	45	78
PSS	29	20

Appendix Table 3 Sample size calculations for measures of HRV

Measure of HRV	Sample size required when α=0.05, 1-β=0.8	
	3M – PS	6M – PS
Mean (ms)	33	18
SDNN (ms)	187	19.62
Mean HR (bpm)	475	20
Stdev HR (bpm)	41	1023
RMSSD (ms)	154	1049
NN50	284	474
pNN50 (%)	299	441
HRV Triangular Index	86	6923
TINN (ms)	109	10391
LF Peak Frequency (Hz)	22	53
LF Absolute Power (ms^2)	307	40
LF Relative Power (%)	69	13
LF Normalised Power (n.u.)	36	1526
HF Peak Frequency (Hz)	45	28
HF Absolute Power (ms^2)	1313	150
HF Relative Power (%)	4659	6
HF Normalised Power (n.u.)	45	112
LF/HF Ratio	409	275
SD1 (ms)	1228	582
SD2 (ms)	399	52384
Approx Entropy	40	44
Sample Entropy	3423	153
D2	355	1002
DFA α1	1125	406
DFA α2	288	665
Lmean (beats)	192	1209
Lmax (beats)	14342	17
REC (%)	454	375
DET (%)	168	53
Shannon Entropy	117	118
MSE min	24	41
MSE max	86	2247
CSI	31159	22
CVI	433	93

Appendix Table 4 Full HRV results

	One-way ANOVA p value (n=7)	3M – PS (n=11)			6M – PS (n=8)		
		Mean diff.	SD of diff.	T test p value	Mean diff.	SD of diff.	T test p value
Mean (ms)	0.2626	-34.07	76.53	0.1706	-50.12	80.53	0.1217
SDNN (ms)	0.6438	+2.54	13.91	0.5583	+1.164	19.62	0.8715
Mean HR (bpm)	0.211	+1.162	10.17	0.7127	+3.885	6.628	0.1413
STD HR (bpm)	0.4489	+0.5203	1.31	0.2171	+0.1164	1.496	0.8321
RMSSD (ms)	0.7335	-2.238	11.11	0.519	-1.214	15.8	0.8341
NN50	0.7172	-2.727	18.42	0.6339	+2.875	25.13	0.7557
pNN50 (%)	0.7291	-1.464	10.15	0.6426	+1.557	13.12	0.7468
HRV Triangular Index	0.3912	-1.022	3.759	0.3882	-0.0931	3.115	0.935
TINN (ms)	0.6443	+17.73	73.67	0.4433	+1.875	76.86	0.9469
LF Peak Frequency (Hz)	0.3571	-5.014	8.973	0.0936	-1.686	4.83	0.3563
LF Absolute Power (ms^2)	0.4509	-51.36	360.6	0.6468	-130.5	324	0.2921
LF Relative Power (%)	0.235	+6.625	21.85	0.3383	-8.021	10.8	0.0738
LF Normalised Power (n.u.)	0.7876	+13.57	31.92	0.1891	+0.7669	12.04	0.8622
HF Peak Frequency (Hz)	0.2545	-0.1955	0.5189	0.2398	-0.0477	0.09748	0.2088
HF Absolute Power (ms^2)	0.8325	-16.13	234.9	0.8244	-60.39	295.5	0.5814
HF Relative Power (%)	0.3939	-0.4536	12.45	0.9062	-6.381	5.149	0.0099
HF Normalised Power (n.u.)	0.7021	-38.88	102.8	0.238	-3.287	13.86	0.5237
LF/HF Ratio	0.6487	+0.2188	1.775	0.6911	+0.1161	0.7713	0.6832
SD1 (ms)	0.8766	-0.4865	6.852	0.8186	-1.157	11.21	0.7787
SD2 (ms)	0.6896	+1.9	15.23	0.6877	+0.2854	26.27	0.9763
Approx Entropy	0.4676	+0.0465	0.1155	0.2117	+0.0489	0.1277	0.3148
Sample Entropy	0.5578	-0.0186	0.438	0.8907	-0.0830	0.4099	0.5849
D2	0.5841	+0.1953	1.475	0.6699	+0.1389	1.767	0.8305
DFA α1	0.8643	+0.0188	0.2529	0.8107	+0.0268	0.2166	0.7367
DFA α2	0.6669	-0.0437	0.2973	0.6366	+0.0339	0.3516	0.7927
Lmean (beats)	0.2241	-0.8388	4.653	0.5632	+0.4657	6.507	0.8453
Lmax (beats)	0.5053	+2	96.32	0.9465	+22.25	35.29	0.1177
REC (%)	0.2406	-1.223	10.46	0.7064	+1.657	12.87	0.7264
DET (%)	0.461	-0.3643	1.886	0.5362	+0.6005	1.722	0.3567
Shannon Entropy	0.2408	-0.0969	0.418	0.4599	+0.0993	0.4299	0.5345
MSE min	0.2154	+0.2184	0.4082	0.1064	+0.1642	0.4134	0.2982
MSE max	0.9019	+0.188	0.6915	0.3883	+0.0363	0.691	0.8862
CSI	0.3974	-0.0022	0.1593	0.9637	-0.0580	0.1054	0.1638
CVI	0.9474	+0.0294	0.2456	0.6996	-0.1459	0.56	0.4852

Note: P values for ANOVA and paired T test was Tukey-corrected

Appendix Table 5 Full cytokine array results

Cytokine	Mean change relative to PS	SD of mean change	Paired T test p value	Cytokine	Mean change relative to PS	SD of mean change	Paired T test p value
ENA-78	-35.03%	42%	0.1139	**VEGF**	-33.27%	49%	0.1132
G-CSF	-39.80%	42%	0.1390	**PDGF BB**	-27.66%	65%	0.1646
GM-CSF	-41.00%	42%	0.2604	**Leptin**	-61.64%	85%	0.1581
GRO	-42.65%	45%	0.1887	**BDNF**	-56.01%	35%	0.0322
GRO-alpha	+8.36%	82%	0.4709	**BLC**	-28.41%	59%	0.1572
I-309	-29.93%	49%	0.1455	**CCL23**	-38.05%	49%	0.0673
IL-1 alpha	-48.32%	34%	0.0160	**Eotaxin 1**	-0.18%	80%	0.3512
IL-1 beta	-46.78%	32%	0.0054	**Eotaxin 2**	-39.57%	50%	0.1258
IL-1	-14.09%	63%	0.2555	**Eotaxin 3**	-19.76%	48%	0.3947
IL-3	-31.61%	57%	0.1099	**FGF 4**	-20.86%	61%	0.1820
IL-4	-15.59%	58%	0.2871	**FGF 6**	-25.44%	54%	0.1629
IL-5	-22.01%	53%	0.4420	**FGF 7**	-46.37%	33%	0.0380
IL-6	-2.87%	81%	0.3957	**FGF 9**	-50.43%	34%	0.0293
IL-7	-32.61%	42%	0.1193	**Flt-3 Ligand**	-27.45%	59%	0.1340
IL-8	-51.70%	41%	0.1875	**Fractalkine**	-47.25%	50%	0.2526
IL-10	-40.93%	55%	0.1985	**GCP-2**	-20.42%	58%	0.1549
IL-12 p40/70	-22.31%	58%	0.2105	**GDNF**	-0.66%	115%	0.2002
IL-13	-35.23%	50%	0.1484	**HGF**	-19.69%	82%	0.1609
IL-15	-22.24%	69%	0.1233	**IGFBP1**	-30.54%	64%	0.1816
IFN gamma	-36.26%	39%	0.0784	**IGFBP2**	-17.22%	61%	0.3207
MCP-1	-30.42%	60%	0.1148	**IGFBP3**	-25.52%	65%	0.1133
MCP-2	-8.93%	69%	0.2348	**IGFBP4**	-10.60%	69%	0.2301
MCP-3	-39.23%	40%	0.0731	**IL-16**	-41.09%	46%	0.0672
M-CSF	-34.86%	54%	0.1087	**IP-10**	-12.39%	68%	0.2141
MDC	-38.52%	40%	0.0491	**LIF**	-34.92%	57%	0.1067
MIG	-35.02%	41%	0.0726	**LIGHT**	-24.81%	67%	0.2281
MIP-1 beta	-38.92%	49%	0.2388	**MCP-4**	+9.57%	94%	0.3410
MIP-1 delta	-20.47%	65%	0.2213	**MIF**	-17.24%	69%	0.2270
RANTES	-88.02%	67%	0.0621	**MIP-3 alpha**	-9.46%	71%	0.3061
SCF	-32.41%	48%	0.1152	**NAP-2**	-8.44%	103%	0.2761
SDF-1	-42.62%	41%	0.0758	**NT-3**	-31.17%	49%	0.1758
TARC	-48.17%	39%	0.0707	**NT-4**	-27.92%	63%	0.1794
TGF-beta 1	-55.94%	41%	0.0785	**OPN**	-8.79%	76%	0.3189
TNF alpha	-55.71%	39%	0.0504	**OPG**	-22.45%	65%	0.2227
TNF beta	-41.47%	44%	0.0645	**PARC**	-16.06%	71%	0.1829
EGF	-18.81%	90%	0.1204	**PLGF**	-42.28%	55%	0.0892
IGF-1	-20.12%	50%	0.2976	**TGF-beta 2**	-40.60%	55%	0.0804
ANG	-9.02%	64%	0.2807	**TGF-beta 3**	-18.61%	63%	0.2374
OSM	-35.40%	53%	0.1764	**TIMP-1**	-1.03%	102%	0.2679
THPO	-39.79%	42%	0.0936	**TIMP-2**	-13.98%	97%	0.1878

Note: P values for paired T test were Bonferroni corrected.