



# **Anthropometric and metabolic correlates of sympathetic nervous system activation in women with polycystic ovary syndrome**

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## EXECUTIVE SUMMARY

**Background:** Polycystic ovary syndrome (PCOS) is associated with increased metabolic risk and hypertension, which may relate to enhanced sympathetic nervous system (SNS) activation. The cerebral pathways involved in this process are not known.

**Aims:** (1) To compare blood pressure and SNS activation in response to isometric forearm contraction (IFC) between PCOS and control groups. (2) To identify and compare the neuronal signatures of this response. (3) To investigate metabolic and anthropometric correlates of SNS activation.

**Methods:** 20 PCOS (age 29.8 yrs, BMI 26.1 kg/ m<sup>2</sup>) and 20 matched controls (age 29.7 yrs, BMI 26.1 kg/ m<sup>2</sup>; p=NS) were studied. Out-of-scanner tests: measurement of mean blood pressure (MAP) and heart rate (HR) responses to 30% IFC for 180 seconds; baseline and post-task catecholamines, and microneurography (MSNA) in a subgroup of 8 PCOS and 8 controls. In-scanner: Blood oxygen level dependent (BOLD) fMRI using an identical block paradigm design for IFC, BOLD signal correlating to MAP responses (threshold  $Z > 2.3$ , corrected cluster threshold  $p = 0.05$ ).

**Results:** IFC elicited an increase in HR and MAP in PCOS and controls but these did not differ between groups ( $p = 0.16$ [HR] and  $p = 0.06$ [MAP]). Adrenaline increased significantly post-IFC in PCOS (0.68 to 1.23ng/mL  $p < 0.001$ ) but not in controls (0.77 to 0.99ng/mL  $p = 0.14$ ). MSNA burst frequency increased by 68% in the PCOS group compared to 11.9% in controls ( $p = 0.002$ ). Brain activation indexed by the BOLD signal in response to IFC was significantly greater in the PCOS group compared to the control group in the right orbitofrontal cortex ( $p < 0.0001$ ), left angular gyrus and lateral occipital cortex ( $p = 0.04$ ). When the BOLD signal was separately corrected for insulin sensitivity, BOLD signal in the right orbitofrontal cortex was no longer significant.

**Conclusions:** PCOS is associated with enhanced SNS activation and increased regional brain activation in response to IFC. The right orbitofrontal cortex BOLD signal change in the PCOS group is associated with insulin sensitivity.

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## **PUBLICATIONS**

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## **ORAL PRESENTATIONS**

Young women with polycystic ovary syndrome display enhanced sympathetic neural activation associated with altered regional cerebral activity: an fMRI study. WT Edwards Medal Presentation, Cardiff Medical Society meeting, November 2015.

Young women with polycystic ovary syndrome display enhanced sympathetic neural activation associated with altered regional cerebral activity: an fMRI study. Donnell D. Etwiler International Scholar Presentation, International Diabetes Center, Minneapolis, USA. April 2016.

## **POSTER PRESENTATION**

AJ Lansdown, EAH Warnert, RG Wise, DA Rees. Enhanced orbitofrontal cortex activation following sympathetic neural stimulation in young women with polycystic ovary syndrome: an fMRI study. Society for Endocrinology BES meeting, November 2015, Edinburgh, UK.

## ABBREVIATION LIST

AHI	Apnoea-hypopnoea index
AI	Augmentation index
aPWV	Aortic pulse wave velocity
BF	Burst frequency
BI	Burst incidence
BMI	Body mass index
BOLD	Blood oxygen level dependent
CPAP	Continuous positive airways pressure
DBP	Diastolic blood pressure
ELISA	Enzyme linked immunosorbent assay
FEAT	fMRI Expert Analysis Tool
FSH	Follicle stimulating hormone
fMRI	functional magnetic resonance imaging
GLM	General linear model
HOMA-IR	Homeostatic Model Assessment-insulin resistance
HR	Heart rate
HRR	Heart rate recovery
HRV	Heart rate variability
IFC	Isometric forearm contraction
LH	Luteinising hormone
MAP	Mean arterial pressure
MSNA	Muscle sympathetic nerve activity
OGTT	Oral glucose tolerance test
OSA	Obstructive sleep apnoea
PCOS	Polycystic ovary syndrome
SBP	Systolic blood pressure
SNS	Sympathetic nervous system
WHR	Waist-hip ratio

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# **CHAPTER 1**

## **INTRODUCTION**

## **1.1 PCOS INTRODUCTION**

### **1.1.1 Overview**

Polycystic ovary syndrome (PCOS) is characterised by oligo/anovulation, hyperandrogenism and altered ovarian morphology, encompassing a broad range of phenotypes depending on the definition of the syndrome. PCOS is associated with increased metabolic risk and hypertension as well as its reproductive consequences [Erhmann 1995, Dunaif 1997] and there is the suggestion that it is the hyperandrogenic phenotype which presents with a worse cardiometabolic profile [Daan 2014]. There is evidence that activity of sympathetic nervous system (SNS) is enhanced in metabolic disease, including obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and obstructive sleep apnoea (OSA) [Alvarez 2002, Thorp 2015, Sabath 2015, Drager 2015]. Since these conditions are prevalent in PCOS, it has been both hypothesised and demonstrated that sympathetic overactivity is present in PCOS and may be related to its pathogenesis and natural history [Sverisdottir 2008, Lambert 2015]. In addition higher centres have been implicated relating to increased sympathetic outflow but these have never been studied in patients with PCOS [Kramer 2014, Critchley 2000(a)]. This study therefore aimed to compare blood pressure and SNS activation responses between PCOS and control groups, to identify any higher

centres involved in these responses and to investigate metabolic and anthropometric correlates of the SNS activation.

### **1.1.2 Historical aspects**

Hippocrates (460 BC- 377 BC) noted that: “But those women whose menstruation is less than three days or is meagre, are robust, with a healthy complexion and a masculine appearance; yet they are not concerned about bearing children nor do they become pregnant.” [Hansen AE 1975]. Likewise Soranus of Ephesus (c. 98-138 AD) made note that: “[s]ometimes it is also natural not to menstruate at all...It is natural too in persons whose bodies are of a masculine type...we observe that the majority of those not menstruating are rather robust, like mannish and sterile women” [Temkin 1991]. PCOS does not seem to be a newly recognised entity.

In more recent centuries, the renaissance surgeon and obstetrician, Ambroise Pare (1510-1590 AD) observed: “Many women, when their flowers or tearmes be stopped, degenerate after a manner into a certaine manly nature, whence they are called Viragines, that is to say stout, or manly women; therefore their voice is loude and bigge, like unto a mans, and they become bearded.” [Paré A, 1634].

At the beginning of the 20<sup>th</sup> century, Bullock and Sequeira described the association between masculinisation and endocrine pathology linking the adrenals and gonads [Bullock 1905]. This had followed reports over the centuries that virilisation was associated with specific organs.

It was only in 1935, however, that the syndrome of ovarian dysmorphology, hyperandrogenism and menstrual irregularity was first fully described by American gynaecologists Irving F Stein, Sr and Michael L Leventhal. In their landmark paper, they noted of the clinical characteristics of seven women: 'The breasts were usually normal. In some patients, there was a distinct tendency toward masculinizing changes. A typical rhomboid hairy escutcheon, hair on the face, arms and legs and coarse skin was noted. No voice changes were observed. The external genitalia were normal, but in some, the labia minora was markedly hypertrophied. Libido is apparently not affected by the changes noted in the ovaries.' Surgical exploration of these women often revealed ovaries that were enlarged to two to four times their original size and full of tiny fluid filled cysts. Some of the ovaries were flat and they called them "oyster ovaries" due to their shape and greyish colour. A description of ovaries with sago-like grains or a polycystic appearance was put forth in the earliest descriptions. [Stein and Leventhal 1935]. A number of years after this first description, the eponymous syndrome was named Stein-Leventhal Syndrome.

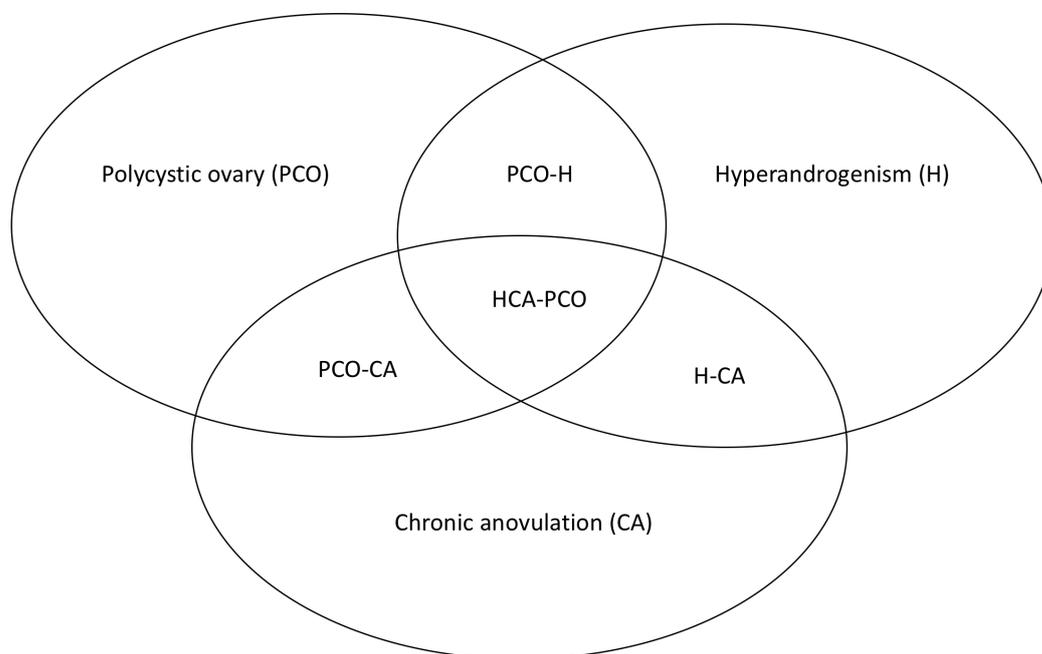
### **1.1.3 Definitions**

In more recent years, the term polycystic ovary syndrome has replaced this and several diagnostic criteria have been reported to aid in its diagnosis. Both the National Institute of Health (NIH) 1990 consensus and Androgen Excess and Polycystic Ovary Syndrome Society (AEPCOS) 2009 task force report identify the importance of androgen excess, with the need for both hyperandrogenism and ovarian dysfunction /oligo-anovulation to make a diagnosis of PCOS [Zawadzki 1992, Azziz R 2009]. The Rotterdam criteria, however, encompasses a wider PCOS phenotype, with only two out of three of hyperandrogenism, oligo-anovulation and polycystic ovaries on ultrasound scan required to define the syndrome [Rotterdam Group 2004]. Although these three different criteria have been established, the Task Force for the Endocrine Society in 2013 recommended using the Rotterdam criteria for the diagnosis of PCOS in their guidance [Legro RS 2013].

### **1.1.4 Clinical manifestations**

Clinical manifestations of PCOS are therefore diverse, with a broad spectrum of clinical presentations possible. Using the current Rotterdam criteria, four different phenotypes are possible (Figure 1.1): (1) hyperandrogenism (clinical or biochemical)

and chronic anovulation; (2) hyperandrogenism and polycystic ovaries on ultrasound but with ovulatory cycles; (3) chronic anovulation and polycystic ovaries without hyperandrogenism; (4) hyperandrogenism, chronic anovulation and polycystic ovaries. The prevalence of PCOS is therefore variable depending on the criteria applied, ranging from 6-25% in the pre-menopausal population [Livadas 2013, Conway G 2014].



**Figure 1.1**

**The different phenotypes in PCOS**

### **1.1.5            Cardiometabolic risk by PCOS definition**

How PCOS is defined is important since it is becoming more apparent that the different definitions in use have differences with respect to cardiometabolic risk. There is increasing evidence that insulin resistance and features of the metabolic syndrome are more prevalent in the classic, hyperandrogenic PCOS phenotype, as defined by the NIH criteria [Moran 2015, Fauser BC 2012, Ehrmann D 2005]. In contrast, the prevalence of metabolic syndrome in the milder phenotype (oligo-anovulatory patients with polycystic ovaries on ultrasound scan but without hyperandrogenism) are closer to control subjects than to the other phenotypes [Gambineri 2009]. The PCOS phenotype could therefore be a predictor of cardiovascular and metabolic risk but longterm studies are needed to show this.

## **1.2                LONG-TERM SEQUELAE**

### **1.2.1            Infertility and cancer risk**

Infertility affects 40% of women with PCOS, which is the commonest cause of anovulatory infertility [Teede 2010]. Approximately 90%–95% of anovulatory women presenting to infertility clinics have PCOS, accounting for a major cause of infertility

that requires treatment for ovulation induction or assisted reproductive techniques [Balen 2002]. In addition, spontaneous abortion occurs more frequently in PCOS with incidences ranging from 42%–73% [Gluek 2001, Jakubowicz DJ 2002]. Even following successful conception, pregnancy in PCOS carries complications, with a 3-4 fold increased risk of pregnancy-induced hypertension and pre-eclampsia, a 3-fold increased risk of gestational diabetes and a 2-fold higher chance of premature delivery. It is thought that features of hyperandrogenism, insulin resistance and obesity may contribute to these adverse outcomes [Palomba 2015]. More recently a retrospective observational study by Rees *et al* of over 9000 women with PCOS confirmed reduced fertility rates, increased miscarriage, pre-eclampsia, gestational diabetes and premature delivery in PCOS compared with controls. Of PCOS births, significantly more (27.7%) were by Caesarean section compared with 23.7% of controls. Infants born to mothers with PCOS also had an increased risk of neonatal jaundice and respiratory complications [Rees DA 2016].

A recent systematic review and meta-analysis of observational studies assessed the risk of gynaecological cancers in women with PCOS compared to controls. There was an increased risk of endometrial cancer, but the risk of breast and ovarian cancer was not increased in women of all ages. It did, however, find that when women with PCOS below age 54 alone were considered, the risk of endometrial cancer increased further,

and the risk became significantly increased for ovarian cancer [Barry 2014]. Conversely, Morgan *et al* found no increase in gynaecological cancer in those with PCOS versus matched controls in their retrospective observational study [Morgan CL 2012].

### **1.2.2 Cardiometabolic consequences**

PCOS is strongly associated with obesity and metabolic consequences. Studies performed to date make it difficult to establish the direction of association between PCOS and obesity, [Pasquali 2006], but it appears that abdominal visceral adiposity worsens the metabolic and reproductive features of PCOS [Lim 2013]. Conversely, it is also possible that androgen excess favours abdominal adiposity from early ages, facilitating insulin resistance [Welt 2013]. Certainly there is evidence of impaired insulin secretion and insulin action [Ehrmann 1995, Dunaiff 1997] leading to an increased risk of metabolic syndrome, glucose dysregulation and T2DM. Longitudinal studies demonstrate that there is a risk intrinsic to PCOS in developing T2DM, that this risk increases steadily with BMI and is particularly high for BMI over 30kg/m<sup>2</sup> [Moran 2010, Morgan 2012, Pasquali 2013].

Obstructive sleep apnoea (OSA) is an established risk factor for cardiovascular disease and is associated with a greater risk of insulin resistance and T2DM. Compared with age- and weight-matched controls, women with PCOS have an increased risk of OSA, which occurs in 44-70% of obese patients with the syndrome [Fogel 2001, Vgontzas 2001, Gopal 2002]. OSA correlates significantly with insulin resistance and glucose intolerance in patients with PCOS [Tasali 2008].

In addition, women with PCOS have an increased prevalence of subclinical markers of premature cardiovascular disease. Many studies have suggested that measures of cardiovascular risk are increased in young or middle-aged women with PCOS, including endothelial dysfunction [Paradisi et al., 2001], carotid intima-media thickness [Talbot et al., 2000], arterial stiffness [Meyer et al., 2005] and myocardial dysfunction [Orio et al., 2004], but it is difficult to establish how much of this risk is due to PCOS *per se* and how much to obesity. Rees *et al*, in a cross-sectional study, comprising 84 young women with PCOS and 95 volunteers, showed central arterial stiffness and diastolic dysfunction were associated with both insulin resistance and abdominal obesity, but PCOS itself did not confer increased risk [Rees E 2014]. The long-term effects of such subclinical changes in PCOS on cardiovascular morbidity and mortality remain uncertain, although evidence to date has failed to demonstrate an increased

incidence of cardiovascular events in pre- or post-menopausal women with PCOS [Wild 2000, Schmidt 2011, Morgan 2012].

Furthermore, PCOS has been associated with dyslipidaemia, with elevated LDL cholesterol and triglycerides, and significantly lower HDL cholesterol compared with healthy volunteers [Kim 2013]. The correlation between PCOS and hypertension remains unclear, with conflicting results from studies [Zimmermann 1992, Holte 1996, Meyer 2005], although a recent large longitudinal Australian study of over 8000 women found that in those with PCOS, the prevalence of hypertension was higher, and that this was independent of BMI [Joham AE 2015]. Table 1.1 outlines a number of key studies investigating blood pressure and hypertension in PCOS. It is clear that a potentially important confounder in the interpretation of these studies is obesity, which is itself a significant risk factor for hypertension. This variable was not consistently controlled for across the studies. However, in those studies which did adjust the analyses for BMI, the association between hypertension and PCOS is still not always clear. It is also apparent that other factors, including ethnicity and the use of medications such as the oral contraceptive pill, are further confounders which may affect blood pressure regulation and the development of hypertension [Lo 2006, Joham 2015, Chang 2016]. Interestingly, Chen *et al* studied the effect of hyperandrogenism on blood pressure and noted that total testosterone was

independently related to systolic and diastolic blood pressure, and hyperandrogenism in women with PCOS associated with hypertension ( $p=0.029$ ) [Chen 2007]. This strengthens the emerging concept that it is the hyperandrogenic phenotype of PCOS that is more associated with metabolic dysfunction, and also raises the question of what role androgens may play in blood pressure regulation in PCOS.

In addition, PCOS has been associated with the increased risk of developing non-alcoholic fatty liver disease (NAFLD) [Kelley 2014]. A meta-analysis showed NAFLD was diagnosed three to four times more often in PCOS compared to controls, which can progress from simple steatosis to alcoholic steatohepatitis, liver fibrosis, cirrhosis and eventually hepatocellular carcinoma [Ramezani-Binabaj M 2014]. Several cross-sectional and case-control studies have demonstrated that the prevalence of NAFLD is increased in young women with PCOS and that this is independent of obesity and other coexisting metabolic syndrome features [Targher G 2016]. Interestingly, it has been shown that those women with the hyperandrogenic PCOS phenotype have significantly higher liver fat than those with normal androgen phenotype, independent of insulin resistance and obesity, sitting with the known evidence that the hyperandrogenic form of PCOS is associated with increased cardiometabolic problems [Jones H 2010]. Furthermore PCOS women are more likely to have the more severe forms of NAFLD, including advanced fibrosis, and cirrhosis. It is also

**Table 1.1 Key studies investigating blood pressure and hypertension in PCOS**

<b>Author</b>	<b>Year</b>	<b>Number studied</b>	<b>Blood pressure measures</b>	<b>Main findings</b>
Zimmermann S <i>et al</i>	1992	14 PCOS, 18 controls	24-hr ambulatory systolic & diastolic BP Left ventricular mass by echocardiography	No difference in systolic, diastolic BP or in left ventricular mass between groups
Conway GS <i>et al</i>	1992	102 PCOS, 19 controls	Automatic recumbent blood pressure	Obese women with PCOS had higher systolic blood pressure than lean women with PCOS and controls
Holte J <i>et al</i>	1996	17 PCOS, 16 controls	24-hr ambulatory systolic & diastolic BP	PCOS group had higher daytime systolic BP and mean arterial BP compared to controls No difference between groups in daytime diastolic BP or night-time recordings
Wild S <i>et al</i>	2000	319 PCOS, 1060 controls	Data collected from medical records 124 women attended a clinical examination	Higher levels of hypertension in PCOS group (p=0.04) Adjustment for BMI, OR 3.2 (1.7-6.0)
Elting MW <i>et al</i>	2001	346 PCOS	Telephone questionnaire asking for presence of hypertension	Hypertension present in 9% Hypertension also occurred significantly more in the younger (35–44 years) PCOS group compared with Dutch female population but the group was significantly more obese
Vrbíková J <i>et al</i>	2003	50 PCOS, 335 controls	Mean of two resting blood pressure recordings (Hypertension defined as on antihypertensive treatment or BP $\geq$ 140/90mmHg)	PCOS women had higher blood pressure than controls Arterial hypertension was more prevalent in PCOS compared to controls
Meyer C <i>et al</i>	2005	100 PCOS, 20 controls	24-hr ambulatory BP monitoring	No difference in total systolic & diastolic BP between groups
Lo JC <i>et al</i>	2006	11,035 PCOS, 55,175 controls	Resting blood pressure measured on 2 outpatient visits (Hypertension defined as systolic BP $\geq$ 140mmHg and/or diastolic BP $\geq$ 90mmHg)	PCOS associated with hypertension (OR 1.41, 1.31-1.51) In PCOS, blacks were more likely and Hispanics less likely to have hypertension
Chen MJ <i>et al</i>	2007	151 PCOS	Mean of 2 readings after resting for >30 minutes	Free androgen index (FAI) and total testosterone independently related to systolic and diastolic blood pressure Higher FAI in women with PCOS increased the risk of elevated blood pressure (SBP $\geq$ 130 mm Hg and/or DBP $\geq$ 85 mm Hg) (p=0.029)
Joham AE <i>et al</i>	2015	8612 subjects (5.8% PCOS)	Women asked if they had been treated for hypertension (outside pregnancy) in the last 3 years	Hypertension reported by 5.5% with PCOS compared to 2.0% in controls (p<0.001) Higher prevalence of hypertension in PCOS in those taking the oral contraceptive pill (OCP)
Chang AY <i>et al</i>	2016	117 PCOS, 204 controls	Resting BP measured and asked if on antihypertensive medication (Hypertension defined average systolic BP $\geq$ 140mmHg, diastolic BP $\geq$ 90mmHg)	PCOS increased the risk of hypertension across racial /ethnic groups. Black women with PCOS had significantly higher systolic blood pressure than white women with PCOS

hypothesised that NAFLD can also exacerbate hepatic and systemic insulin resistance and may therefore play a role in the pathophysiology of PCOS [Targher G 2016].

### **1.2.3 Pro-inflammatory state and cardiometabolic risk**

There is increasing evidence that the long-term cardiometabolic consequences in PCOS are linked to a low-grade chronic inflammatory state. As inflammation underpins insulin resistance in obesity and type 2 diabetes, it is thought to also be the cause of insulin resistance when present in PCOS, which in turn may be related to excess abdominal adiposity. Independent of excess adiposity and insulin resistance, hyperandrogenism activates circulating mononuclear cells in the fasting state to increase their sensitivity to nutrients, and is a potential mechanism for initiating inflammation in PCOS. This may in turn promote atherogenesis [González F 2015]. It is thought that the triad of hyperinsulinemia, hyperandrogenism, and low-grade inflammation may therefore act together in the pathophysiology of PCOS. Dysregulation of the sympathetic nervous system may also act as an important component, in addition to these, all contributing to the pathophysiology of PCOS [Shorakae S 2015].

## **1.3 HYPERTENSION**

### **1.3.1 Pathogenesis**

Hypertension is viewed as a multifactorial disease which arises due to an interaction between several genetic and environmental factors, including psychological stressors, obesity and increased sodium intake [Korner 1982]. Individually each factor is likely to account for a small proportion of variation in blood pressure but together they have an additive effect [Carretero 2000]. Blood pressure has been shown to increase with ageing, which may in part be due to a loss of homeostatic control of blood pressure regulation [Miall and Chinn 1973, Korner 2007].

The sympathetic nervous system had initially been considered to be involved in the pathogenesis of essential hypertension but the observation that sympathetic afferent denervation in dogs did not alter long-term control of mean arterial pressure subsequently led to the conclusion that neuronal influences were not important in longer-term blood pressure control [Cowley *et al* 1973]. However, it subsequently became recognised that the efferent arm of the SNS can function independently of the baroreceptor reflex and therefore its role may be more significant than first thought [Thresher 2007].

### **1.3.2 Sympathetic Nervous System (SNS)**

The autonomic nervous system comprises of the sympathetic and parasympathetic nervous systems. The sympathetic nervous system has a role in regulating the 'fight or flight' response under conditions of stress, and also regulates various tissue functions including chronotropic and inotropic control of the heart, vascular tone, pupillary diameter and urinary output (Figure 1.2). It is the neurotransmitter noradrenaline into the synaptic cleft and subsequent activation of alpha and beta adrenergic receptors that mediates these actions. In the normal physiological state, a fine balance exists between parasympathetic and sympathetic activity to maintain homeostasis.

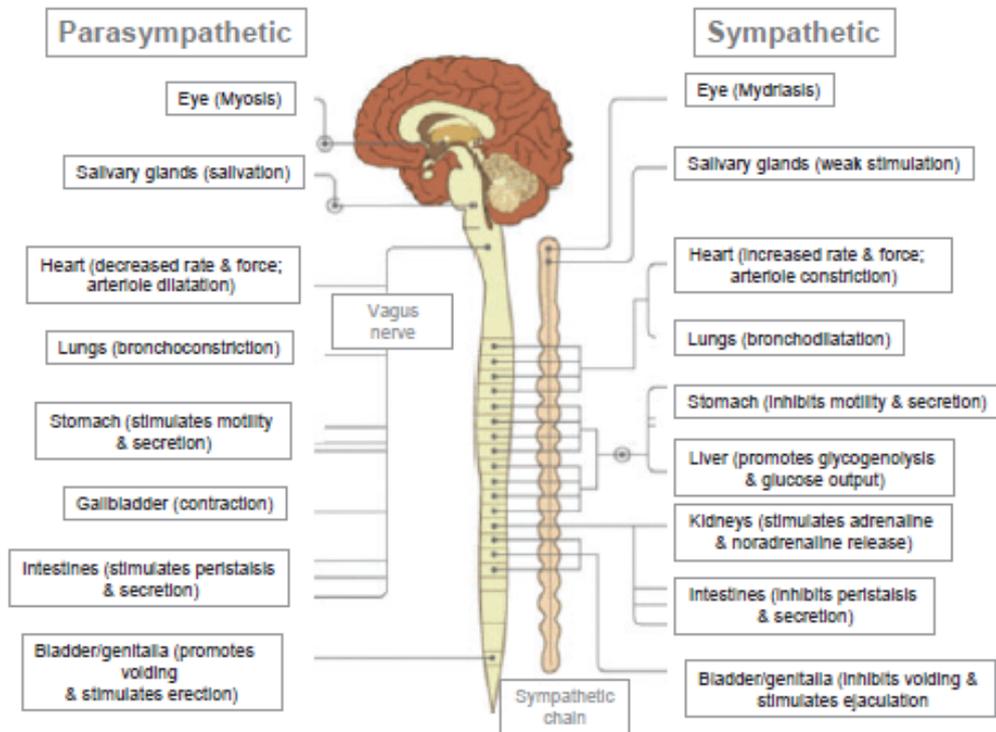


Figure 1.2

## The autonomic nervous system and its target organs

[Adapted from Lansdown A *et al* 2012]

### 1.3.3 Sympathetic Nervous System in Hypertension

In hypertensives, both systemic and regional noradrenaline turnover is increased [Goldstein 1983, Esler *et al* 1989], as well as increased cardiac, renal and muscular sympathetic drive [Julius and Majahalme 2000]. Thus primary hypertension has now become viewed as a hyperadrenergic state [Grassi and Mancia 2004]. It seems that

increased sympathetic activity may play a causative role in the natural history of the disease, with increased plasma noradrenaline levels preceding the onset of hypertension in adults [Julius 1991]. Furthermore, sympathetic activity is related to the severity of hypertension [Grassi 2007], the risk of cardiovascular disease [Minami 1999] and is a predictor of future systolic blood pressure [Flaa 2008a].

The sympathetic nervous system influences short- and, probably long-term control of blood pressure. Blood pressure varies over a 24-hour period, with around 25% of this variation attributed to the baroreceptor arch, suggesting that central SNS efferent activity is responsible for a significant proportion of blood pressure variability by affecting cardiac output and peripheral vascular resistance [Grassi *et al* 2010].

#### **1.3.4 Central drivers of SNS activation in hypertension**

The brainstem appears to be involved in the regulation of blood pressure. In animal models, selective lesion and direct stimulation experiments have identified three medullary regions that form a cardiovascular regulatory centre [Colombari 2001]. In addition, electrical stimulation of the rostral ventrolateral medulla (RVLM) in humans produced tachycardia and a pressor response [Ozer *et al* 2005]. More recently, Coulson *et al* used blood oxygen level-dependent (BOLD) functional magnetic

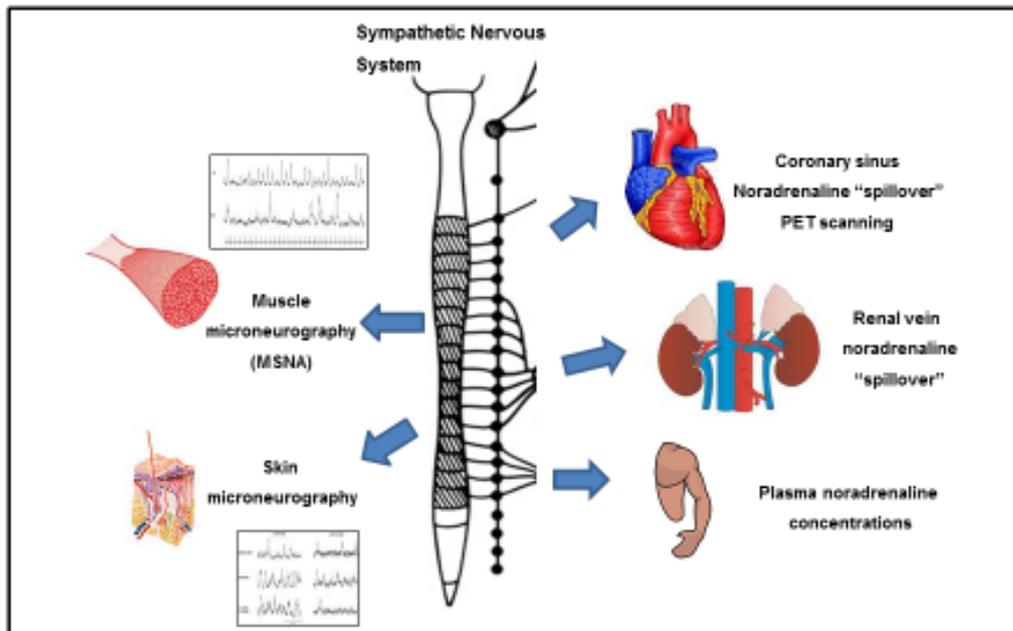
resonance imaging (fMRI) in 12 healthy young adults to identify the neural response to blood pressure changes following isometric forearm contraction (IFC), a recognised stimulator of the sympathetic nervous system. Mean Arterial Pressure (MAP) increased from  $85 \pm 6$  mm Hg to  $108 \pm 15$  mm Hg ( $P=0.0001$ ). The brainstem BOLD signal change associated with this rise was localised to the ventrolateral medulla, suggesting a possible role for this neural centre in MAP control [Coulson JM 2015].

Other studies have suggested a role of the brainstem and other areas of the cerebral cortex in blood pressure control in humans. In a positron emission tomography (PET) study, muscle sympathetic nerve activity (MSNA) during baroreflex unloading was associated with increased activation in the left orbitofrontal cortex and the rostral ventrolateral medulla, suggesting a role of these regions in the baroreflex-mediated control of sympathetic activity [Kramer 2014]. Critchley *et al* also used PET imaging to correlate the rise in MAP from mental stressor tasks and physical exercise to areas of localised increased cerebral blood flow. They found that these areas included the dominant anterior cingulate, postcentral gyrus, cerebellum, insular cortex and orbitofrontal cortex [Critchley *et al* 2000(a)]. Higher centres are therefore emerging as playing a key role in sympathetic drive and blood pressure control in humans.

In humans, there have been historical challenges in studying these higher centres in sympathetic activation and blood pressure changes, chiefly due to technical difficulties in accessing such brain regions, and also a lack of robust functional imaging tools available. However, with the emergence of various modalities during recent years, such as the aforementioned fMRI and PET, identification of such regions has vastly improved and developed. As these tools continue to improve, such as the availability of higher strength MRI scanners with enhanced signal and spatial resolution, so such regions can be more easily and accurately defined.

#### **1.4 MEASUREMENT OF SNS ACTIVITY**

Various methods exist in order to measure the regional sympathetic activity of target organs, including heart rate and blood pressure variability [Axelrod 1981], microneurography [Hagbarth 1968, Sundlof 1977] and estimation of noradrenaline spillover by isotope dilution [Esler 1984a, Esler 1984b, Esler 2003] (Figure 1.3).



**Figure 1.3**

**Methods for measuring SNS activity [Adapted from Lansdown A *et al* 2012]**

The beat-to-beat variation in heart rate (heart rate variability, HRV) is an indirect method of measuring autonomic tone, with contributions from both the sympathetic and parasympathetic nervous systems. The high frequency component (HF: 0.15 to 0.4Hz) reflects vagal control and both the low frequency component (LF: 0.04 to 0.15Hz) and the LF:HF ratio reflect sympathetic activity, which can both be measured with power spectrum analysis. While both measures are influenced by autonomic mechanisms, their specificity as markers of sympathetic activation alone is limited [Hirsch 2003, Grassi 2008].

A more direct measure of sympathetic nerve activity is microneurography, which was first described by Hagbarth and Vallbo in 1968 [Hagbarth 1968]. Fine electrodes are used to record 'bursts' of activity from multiple or single afferent and efferent nerve axons in the skin and muscle. The easily accessible common peroneal nerve is typically chosen for this method and measures the efferent postganglionic muscle sympathetic nerve activity (MSNA) [Vallbo 1979, Macefield 1994]. MSNA has been shown to correlate well with sensory, somatomotor and autonomic effector (e.g. blood pressure, heart rate) responses [Vallbo 1979]. Microneurography can provide instantaneous data on sympathetic neural output but it is invasive so can only be used to measure activity in superficial nerves.

Measurement of regional plasma noradrenaline spillover can provide information on sympathetic activity in individual organs, such as the heart and kidneys. Following nerve depolarisation, any remaining noradrenaline in the synapse, the "spillover", is washed out into the plasma and the plasma concentration is therefore directly related to the rate of sympathetic neuronal discharge [Brown 1957]. By infusing tritiated noradrenaline, outward flow of endogenous noradrenaline can be measured by isotope dilution [Esler 1984a, Esler 1984b]. Plasma noradrenaline concentrations can also be measured from subjects without the use of radiolabelled noradrenaline but this

offers limited sensitivity, is not reproducible and is unable to distinguish between increased central catecholamine production and decreased clearance [Grassi 1999].

These methods should be used in combination to measure sympathetic tone as none is considered 'gold-standard'.

## **1.5 EXPERIMENTAL STIMULI TO ACTIVATE THE SNS**

The sympathetic nervous system (SNS) can be activated using different experimental stimuli, broadly divided into non-exercise and exercise SNS stimuli. Non-exercise stimuli include the cold pressor test, mental stress, apnoea and drugs, while exercise stimuli include isometric forearm contraction (IFC) and orthostatic exercise.

The cold pressor test is one such non-exercise stimulus that can evoke a pressor response and increased generalised sympathetic tone. It involves immersing the subject's hand in ice water for 2 minutes with the subject avoiding isometric contraction, the Valsalva manoeuvre or held expiration during that period. The painful cold stress results in sympathetic discharge of noradrenaline with arteriolar constriction, increased heart rate, and increased cardiac contractility. These responses combine to increase blood pressure - a pressor response. The cold pressor

test is a useful method of stimulating generalised sympathetic tone but not in assessing the SNS response to exercise and can be less practical in certain circumstances, such as its use in an MRI environment [Victor 1987]. Emotional or mental stress can also evoke a pressor response, and mental arithmetic or displaying of emotionally charged images have been used to cause sympathoexcitation [Macefield 2013, Fonkoue 2015]. Apnoea can also result in large increases in MSNA and the use of end-expiratory breath-holds, for example, have been used in experiments to generate increased sympathetic outflow [Kimmerly 2013]. Mental stress and breath-holding exercises both have the advantage of being easily used in any environment but reproducibility and duration of task can be variable. Alpha-adrenergic agonists (such as phenylephrine and clonidine) have been used in study settings to induce sympathoexcitation, vasoconstriction and a pressor response [Limberg 2014(a)], although their use is more technically challenging and requires close supervision.

An exercise-SNS stimulus that is well-validated is Isometric forearm contraction (IFC). IFC has been shown to generate a haemodynamic response, characterised by a rise in arterial blood pressure, heart rate and SNS activation. It represents a complex interaction between central command, parasympathetic and sympathetic influences, arterial and cardiopulmonary baroreflex control, and the exercise pressor reflex

[Kaufman MP 2002, McCloskey 1972]. It has been widely used in studies and is easily reproducible, although depending on the task strength and duration, may be uncomfortable for the subject. Orthostatic stress is another potent adrenergic stressor method where heart rate and blood pressure are regulated by the baroreceptor reflex in an effort to maintain systemic blood pressure. Both passive upright tilt and active standing have been used experimentally to induce sympathoexcitation [Cao 2014]. A significant disadvantage is that there are limitations of where it can be used. For example, such an approach would be impractical in the MRI environment when studying higher centres involved in sympathoexcitation.

A number of stimuli can therefore be used, each with different strengths and weaknesses.

## **1.6 SYMPATHETIC NERVOUS SYSTEM IN METABOLIC DISEASE**

### **1.6.1 Sympathetic activity in obesity**

Abdominal visceral obesity is most associated with increased sympathetic activity, with resting MSNA firing greater than 50% more compared to those with subcutaneous obesity [Alvarez 2002, Grassi 2004, Sivenius 2003]. It has been proposed that chronic

sympathetic activity in obesity is a physiological response to stimulate thermogenesis and stabilise body weight during periods of overeating [Landsberg 2001].

In addition, leptin may play an important role in sympathetic activity and obesity. Leptin has been shown to be elevated in obesity and can act directly on skeletal muscle to impair glucose transporter-4 translocation, resulting in hyperinsulinemia and activation of the sympathetic nervous system [Maffei 1995]. Leptin can also act centrally in the hypothalamus and brainstem to increase sympathetic activity and may also act at the level of the nucleus tractus solitarius to affect neurons involved in baroreflex sensitivity [da Silva 2013, Ciriello J 2013]. In obesity, it has been suggested that leptin-expressing neurons in the ventromedial hypothalamic nucleus become resistant to the hyperleptinaemia found in obesity, reducing its appetite-reducing effects but enabling sympathetic activation [Myers 2008, Thorp 2015].

### **1.6.2 Insulin and sympathetic activity**

The effect of insulin upon the sympathetic nervous system has been demonstrated in various ways. Animal studies have shown that intracerebroventricular injection of insulin increases sympathetic activation acting via the arcuate nucleus [Rahmouni 2004, Cassaglilia 2011, Thorp 2015]. In humans, it has been shown that systemic

infusion of insulin results in increased MSNA outflow [Vollenwieder 1993, Young 2010] but this effect may be blunted in obesity and the metabolic syndrome [Vollenweider 1994, Straznicky 2009]. It was Landsberg who hypothesised that central insulin resistance may therefore contribute to sympathetic activation by increasing insulin-mediated glucose metabolism in hypothalamic neurons and causing suppression of the inhibitory pathway between the hypothalamus and sympathetic centres in the brainstem [Landsberg 1986].

Conversely, it has been suggested that increased sympathetic activity may cause insulin resistance [Thorp 2015, Moreira 2015]. Observations from a perfused human forearm model which showed that increased sympathetic excitation causes acute insulin resistance [Jamerson 1993] and it has been shown that noradrenaline responses to a cold pressor test independently predicted insulin resistance after 18 years [Flaa 2008]. In patients with type 2 diabetes, MSNA has been shown to be significantly elevated at rest compared to those with prediabetes [Straznicky 2012].

### **1.6.3 SNS and NAFLD**

The circadian pattern has been shown to be key in liver lipid accumulation, with the subsequent development of steatosis, and this has been shown to be regulated by the

autonomic nervous system and the feeding-fasting cycle. It has been suggested that an imbalance in sympathetic and parasympathetic tone between organs might therefore be a pathophysiological driver that leads to the development of NAFLD [Sabath E 2015].

#### **1.6.4 SNS in arterial stiffness**

The sympathetic nervous system may also play a role in arterial stiffness and subclinical cardiovascular disease.

In animal models, recombinant leptin was administered to mice every other day for 1 week, and mesenteric arteriole myography revealed that the treatment caused significant impairment of endothelium-dependent vasorelaxation. Leptin treatment also augmented the blood pressure response to angiotensin II, effects which neutralised following sympathetic denervation to the mesenteric vasculature. These findings suggested that the effects of leptin are mediated by sympathetic nervous system activation and may contribute to vascular stiffness and hypertension in obesity [Wang J 2013].

In human subjects, investigation has shown an independent relationship between carotid-femoral pulse wave velocity (PWV), a measure of arterial stiffness, and resting MSNA that is independent of age, BMI, waist circumference, waist-to-hip ratio, heart rate, pulse pressure or blood pressure [Swierblewska E 2010]. A further study has shown sex differences in the relationship between MSNA and aortic wave reflections, with a positive correlation between sympathetic activity and augmentation index in young men but a strongly inverse relationship in young women [Casey DP 2011]. This correlation is lost in older women, with postmenopausal women with higher MSNA having higher aortic pressures and indices of aortic wave reflection, which may relate to their increased risk of cardiovascular disease [Hart EC 2013]. In addition, short-term sympathetic activation (via a cold pressor test) decreases muscular artery compliance in normotensive young humans [Boutouyrie P 1994]. Whether sympathetic activity plays a major role in modulating the elastic properties of central arteries or the tone of peripheral muscular arteries remains unknown.

Therefore the SNS appears to play a role in arterial stiffness, but it is not known yet whether this correlates with clinically significant disease.

### **1.6.5 SNS in obstructive sleep apnoea**

It has been suggested that the hypoxic stress associated with OSA can cause a range of pathological systemic events including sympathetic activation and systemic inflammation, which in turn may be contributors in the development of cardiovascular disease and metabolic dysfunction [Drager LF 2015]. Indeed, OSA has been shown to be associated with increased MSNA and this is independent of body weight [Grassi 2005b]. Cardiac sympathetic drive is also increased in subjects with OSA, even when established cardiovascular disease is not present [Narkiewicz 1998]. Interestingly, a systematic review of randomised studies using continuous positive airways pressure (CPAP) intervention versus sham CPAP have consistently shown a reduction in sympathetic activation and this occurs rapidly in response to the therapy [Jullian-Desayes I 2015, Hedner 1995]

## **1.7 THE SYMPATHETIC NERVOUS SYSTEM IN PCOS**

As PCOS is associated with obesity, insulin resistance, NAFLD, arterial stiffness and obstructive sleep apnoea, it has been supposed that the syndrome itself may be associated with heightened sympathetic nerve activity. Both animal models and

human studies have examined this correlation, and to determine how it may be related to the pathogenesis and metabolic disturbances found in PCOS.

### **1.7.1 Ovarian sympathetic tone**

Bernuci *et al* showed that rats exposed to single or repeated cold stress over 4 weeks resulted in increased expression of ovarian norepinephrine terminals, derranged follicular development and PCO morphology, suggesting a role of the sympathetic nervous system in its development [Bernuci 2013]. Similarly, a further study performing unilateral or bilateral vagotomy in PCO rats restored ovulation in both ovaries [Linares 2013]. A study in which nerve growth factor-producing cells were injected into the ovaries of rodents showed subsequent development of other features of PCOS, including increased visceral versus subcutaneous fat, glucose intolerance, hyperinsulinaemia, as well as increased sympathetic outflow [Wilson JL 2014].

In women with PCOS, a higher density of ovarian catecholaminergic fibres were found compared with controls [Heider 2001] and a human further study showed ovarian nerve growth factor production was enhanced in women with PCOS [Dissen 2009].

These studies may imply a role of sympathetic activation in the pathogenesis and natural history of PCOS.

### **1.7.2 Generalised sympathetic tone**

A number of studies have shown a generalised increase in sympathetic nerve activity in women with PCOS, as outlined in table 1.2.

One early study compared plasma and urinary catecholamine levels in 14 PCOS adolescent patients with age and BMI-matched controls [Garcia-Rudaz 1998]. Serum free plasma adrenaline and noradrenaline levels at rest were similar in patients and controls. However, patients with PCOS had significantly higher urinary excretion of normetanephrine and lower levels of dihydroxyphenylglycol (DHPG), which led to the notion that there may be an alteration of noradrenaline reuptake into the presynaptic nerve or deamination to DHPG in PCOS.

Furthermore, several studies have compared heart rate variability (HRV) and heart rate recovery (HRR) post-exercise in subjects with and without PCOS [Yildirim 2006, Tekin 2008, Giallauria 2008, Di Domenico 2012, Saranya K 2014].

<b>Study</b>	<b>Subjects</b>	<b>Measure of SNS activity</b>	<b>Results</b>
Garcia-Rudaz C <i>et al</i> (1998)	14 PCOS, 9 controls (adolescents)	Serum free adrenaline and noradrenaline and urinary catecholamines at rest	Plasma free adrenaline and noradrenaline similar in both groups PCOS patients had a higher urinary excretion of normetanephrine than controls
Yildirim A <i>et al</i> (2006)	30 PCOS, 30 controls	Heart Rate Variability (HRV) analysis at rest	Low-frequency /High-frequency ratio significantly higher in PCOS, indicating increased SNS activity
Tekin G <i>et al</i> (2008)	26 PCOS, 24 controls (age, BMI and physical activity matched)	Heart rate Recovery (HRR) post-exercise tolerance test, and systolic blood pressure (SBP) changes	PCOS HRR significantly lower at 1 minute compared to controls Exaggerated SBP response to exercise
Giallauria F <i>et al</i> (2008)	75 PCOS, 75 controls (age and BMI matched)	HRR post-exercise test	PCOS showed a significantly reduced HRR compared to controls
Sverrisdóttir YB <i>et al</i> (2008)	20 PCOS, 18 controls (age and BMI matched)	MSNA at rest	MSNA increased in PCOS women compared with controls MSNA in PCOS positively correlated to testosterone and cholesterol
Stener-Victorin E <i>et al</i> (2009)	20 PCOS	MSNA at rest pre- and post- 16 weeks low-frequency electroacupuncture (EA) or exercise or no intervention	Low-frequency EA and physical exercise significantly decreased MSNA burst frequency compared with untreated group
Tasali E <i>et al</i> (2011)	19 PCOS	24-h plasma noradrenaline levels pre- and post 8 weeks of CPAP	Only 9 women were compliant with CPAP and completed the study 25% reduction in mean 24-hr plasma noradrenaline levels Magnitude of decrease strongly associated with hours of CPAP use
Schlaich MP <i>et al</i> (2011)	2 PCOS (obese, hypertensive)	MSNA and noradrenaline spillover at baseline and 3 months post-renal denervation	2.5- 3-fold increase in MSNA and norepinephrine spillover compared with normotensive controls (previously reported) Decrease in MSNA and norepinephrine spillover post-procedure
Jedel E <i>et al</i> (2011)	72 PCOS	MSNA at rest in Montgomery Åsberg Depression Rating Scale (MADR-S) score <11 versus ≥11	42 patients recorded. MSNA not statistically significantly different between two groups
Di Domenico K (2012)	30 classic anovulatory PCOS, 16 ovulatory PCOS, 23 controls (age matched)	HRV at rest and after a mental stress test	During mental stress, classic PCOS showed lower HRV response compared with control group. PCOS testosterone levels inversely associated with low frequency component, low frequency/high frequency ratio
Saranya K (2014)	31 PCOS, 30 controls (age-matched)	HRV at rest and HRV, heart rate and blood pressure response to standing, deep breathing and isometric hand grip	Low-frequency/High frequency HRV was increased in PCOS. Heart rate and blood pressure changes were greater in response to standing and handgrip in PCOS, less in response to deep breathing in PCOS
Lambert EA (2015)	19 PCOS, 21 control (age and BMI matched)	MSNA and HRV at rest	Women with PCOS had elevated multiunit MSNA compared with controls. HRV did not differ between groups

**Table 1.2 Studies investigating SNS activity in PCOS**

One initial study compared resting HRV in 30 PCOS patients and 30 healthy controls, showing that PCOS patients had higher low frequency (LF) component, lower high frequency (HF) component and higher LF:HF ratio compared with controls [Yidirir 2006]. This suggested an increased sympathetic and decreased parasympathetic component of HRV in the PCOS group. Di Domenico *et al* assessed HRV in response to mental stress in different phenotypes of PCOS, showing that women with PCOS appear to have impaired autonomic modulation in response to sympathetic stimulation. Patients with classic anovulatory PCOS showed lower HRV response when compared to the control group, and this remained even after adjustment for age and BMI. When patients with classic and ovulatory PCOS were considered together, total testosterone levels were inversely associated with the LF component, LF:HF ratio, and the difference between HF response at rest and after the stress test, suggesting that androgens may account for the blunted autonomic response in this group [Di Domenico 2012]. Saranya K *et al* compared HRV at rest and HRV, heart rate and blood pressure responses to standing, deep breathing and isometric handgrip in 31 women with PCOS and 30 age-matched controls. They found that LF:HF ratio was increased in PCOS, that heart rate and blood pressure changes were greater in response to standing and handgrip in PCOS, and less in response to deep breathing, concluding that PCOS patients have altered autonomic modulation in the form of

increased sympathetic and decreased parasympathetic reactivity and HRV [Saranya 2014].

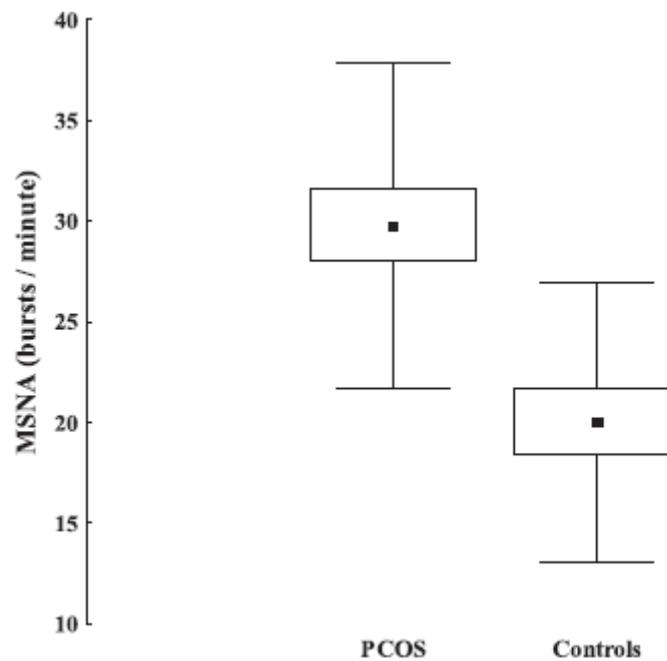
Two other groups studied HRR post-exercise in patients with PCOS and controls [Tekin 2008, Giallauria 2008]. One study compared 26 women with PCOS and 24 matched controls undergoing a modified Bruce protocol exercise tolerance test, with measurements of both HRR and recovery [Tekin 2008]. The PCOS group had an exaggerated systolic blood pressure response to exercise and a delayed recovery systolic blood pressure, implying sympathetic stimulation that persisted into recovery. The reduction in HRR suggested lower vagal parasympathetic activity in women with PCOS. Giallauria *et al* compared HRR after a cardiopulmonary exercise test in 75 overweight women with PCOS with 75 age/BMI-matched healthy volunteers [Giallauria 2008]. Similarly the PCOS group showed a reduction in HRR compared to healthy controls, and in addition this correlated inversely with BMI and insulin sensitivity but not with testosterone.

Only two studies have assessed sympathetic nerve activity in PCOS in comparison to matched controls by the use of MSNA. The first of these examined a group of 20 women with PCOS matched by age and BMI to 18 healthy controls with a normal BMI. It was found that both resting burst frequency and burst incidence (both markers of

sympathetic activity) were increased in the PCOS group as compared with controls. Figure 1.4 shows the resting burst frequency for both groups. MSNA correlated positively with total and free testosterone, and but less with cholesterol levels in the PCOS group. No correlation was found between MSNA and insulin resistance or central obesity. Given the relationship between MSNA and testosterone, it was suggested that the relationship between MSNA and testosterone may indicate that the degree of sympathoexcitation relates to the severity of the syndrome (or the degree of hyperandrogenism). [Sverrisdottir 2008]. This, in turn, may lead to a hypothesis that the classic hyperandrogenic PCOS phenotype, which is more associated with metabolic dysfunction, as defined by the NIH criteria [Moran 2015, Fauser BC 2012, Ehrmann D 2005] could have the sympathetic nervous system as a key driver of these consequences. It may strengthen evidence that it is PCOS, rather than visceral adiposity and obesity, that itself may be associated with increased SNS activation.

More recently, Lambert *et al* examined multiunit and single-unit muscle SNS activity by microneurography and HRV in 19 overweight/obese women with PCOS compared with 21 control overweight/obese women presenting a similar metabolic profile. Women with PCOS had elevated multiunit muscle SNS activity ( $41 \pm 2$  vs  $33 \pm 3$  bursts per 100 heartbeats,  $P < 0.05$ ). In contrast to Sverrisdóttir's study, there was no

relationship between MSNA and testosterone, countering the hypothesis that hyperandrogenaemia is related to elevated sympathetic tone. There was a strong



**Figure 1.4**

**Muscle sympathetic nerve activity (MSNA) expressed as burst frequency (burst/min) in women with polycystic ovary syndrome (PCOS) and in healthy matched controls (p=0.0003). [Sverisdottir 2008]**

relationship between MSNA and plasma glucose and insulin concentration as well as with HOMA-IR, which remained after controlling for the presence of PCOS. This suggested that while insulin resistance is linked to sympathetic tone in

overweight/obese women, in women with PCOS sympathetic activation may not be adequately explained by obesity or insulin resistance alone. [Lambert 2015]. This sits against the hypothesis, therefore, that the more hyperandrogenic PCOS phenotype (with greater metabolic dysfunction) may be related to increased sympathetic tone.

## **1.8 INTERVENTIONS TO REDUCE SNS ACTIVITY IN PCOS**

Some evidence exists to suggest that interventions may reduce the SNS activity in PCOS, which in turn may have an effect on the cardiometabolic consequences.

### **1.8.1 Lifestyle measures**

In obese subjects with the metabolic syndrome, Straznicky *et al* showed that MSNA and whole body noradrenaline spillover rate were reduced during active weight loss [Straznicky 2011]. The noradrenaline spillover was maintained during the maintenance phase of the study but MSNA increased, suggesting regional changes may occur in SNS activity in response to weight loss [Straznicky 2011]. Interestingly, the addition of moderate intensity aerobic exercise training to a weight loss programme in subjects with the metabolic syndrome did not result in additional beneficial reductions in sympathetic activity beyond weight loss alone [Straznicky 2010].

In contrast, however, an interventional study followed on from the HRR in cardiopulmonary exercise testing work by Giallauria *et al.* 124 subjects with PCOS were assigned to a 3-month exercise programme versus no intervention. The group with intervention showed significant improvements in HRR post-exercise compared to no significant changes in the control group [Giallauria 2008b], suggesting a vital role of exercise training in reducing sympathetic tone in PCOS.

### **1.8.2 Pharmacological**

Insulin sensitisers have been used to try and modulate sympathetic activity, given that insulin resistance may be important in its overactivity. Metformin given intravenously to hypertensive rats led to reductions in heart rate, blood pressure and efferent renal sympathetic nerve activity and these were thought to be mediated by a direct action in the central nervous system [Petersen 1996]. However, metformin did not affect MSNA, renal and total body noradrenaline spillover, blood pressure or insulin sensitivity in a double blinded, randomised cross over study conducted in 6 obese insulin resistant hypertensive men [Gudbjornsdottir 1994]. As thiazolidinediones also improve insulin sensitivity, one study randomised 30 patients with type 2 diabetes to either pioglitazone or no pioglitazone 4 weeks after a myocardial infarction. In the

pioglitazone group MSNA decreased significantly and arterial baroreceptor sensitivity increased significantly, with there being a significant relationship between change in MSAN and insulin sensitivity (HOMA-IR) [Yokoe 2012].

Centrally acting imidazoline receptor-1 agonists, such as moxonidine, have been studied with regards to their effects on the SNS and insulin resistance, given their sympatholytic effects. In a multicentre study, 98 postmenopausal hypertensive women were randomised to either atenolol or moxonidine. Both agents led to a significant reduction in diastolic blood pressure but only in the moxonidine group was there a significant improvement in insulin sensitivity ( $p=0.019$ ) [Kaaja 2007]. This may suggest a role of such sympatholytic agents in improving metabolic profile through inhibition of the SNS.

### **1.8.3 Electroacupuncture**

A number of studies have shown that electroacupuncture (EA) can improve insulin resistance and metabolic dysfunction, and this may be through the mechanism of altered inflammation and /or sympathetic nervous system activation [Martinez 2016]. Furthermore, specific EA studies in PCOS have shown an improvement in symptoms which is thought to be due to both spinal and central mechanisms [Stener-Victorin

2013]. Specifically there may be a role of neuropeptides mechanistically in the altered sympathetic drive as low-frequency EA has been shown to cause the release of serotonin, dopamine, endogenous opioids and oxytocin in the central nervous system [Andersson S 1995].

One study randomly assigned 20 overweight patients with PCOS to either low-frequency EA, physical exercise or no intervention. They found that both low-frequency EA and exercise significantly decreased MSNA burst frequency compared to the untreated group. These changes were accompanied by improvements in body composition in both treated groups, but not in blood pressure, insulin sensitivity or lipid profile [Stener-Victorin 2009].

A subsequent larger randomised trial of 74 women with PCOS showed that low-frequency EA and physical exercise improved hyperandrogenism and menstrual frequency without affecting BMI compared with untreated controls, with the EA group showing greater improvements in menstrual regularity and testosterone levels than the exercise group [Jedel 2011(b)]. No measures of sympathetic activity were undertaken in this study but the findings, taken with the results of their earlier trial, may

suggest that these benefits are mediated at least in part by a reduction in sympathetic activity.

#### **1.8.4 Continuous positive airways pressure (CPAP)**

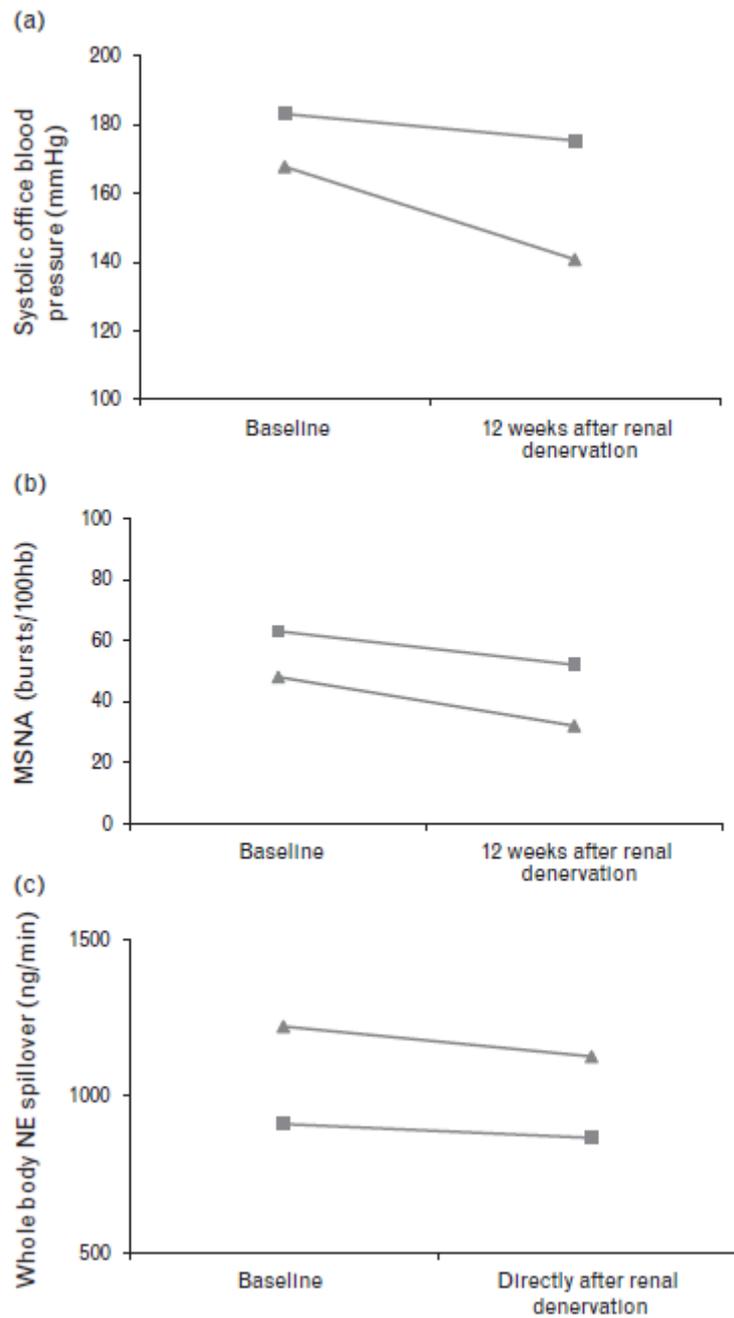
As discussed, continuous positive airways pressure (CPAP) intervention CPAP has consistently shown a rapid reduction in sympathetic activation in obstructive sleep apnoea [Jullian-Desayes I 2015].

Given the prevalence of obstructive sleep apnoea in PCOS, one study has specifically looked at the effect of CPAP on SNS activation in this group. The study used both HRV and 24 hour plasma catecholamine profiles as measures of SNS activity, in addition to measuring insulin sensitivity and blood pressure in 9 obese women with PCOS. Measurements were made at baseline and following 8 weeks of nightly CPAP [Tasali 2011]. It was found that daytime and night-time noradrenaline fell significantly and this strongly correlated with increasing hours of CPAP use. There was also a reduction in LF and an increase in HF components of HRV, suggesting reduced SNS activity. The use of CPAP also improved insulin sensitivity and reduced diastolic blood pressure. Although the study was small and only examined short-term outcomes, it supports the use of CPAP as an intervention to reduce SNS activity in PCOS.

### 1.8.5 Renal denervation

Percutaneous catheter-based radiofrequency ablation of the renal sympathetic nerves has been used as a method of reducing SNS activation in recent years. Particularly, the Symplicity-1 and Symplicity-2 trials targeted patients with resistant hypertension and demonstrated that denervation of the renal nerve resulted in sustained improvements blood pressure, reduced renal and total body noradrenaline spillover [Schlaich 2009, Krum 2009]. Symplicity-3, however, failed to demonstrate an improvement in ambulatory blood pressure in either the 24-hour or day and night periods [Bakris GL 2014]. Although questions have since arisen regarding screening at entry into the trial, frequent medication changes during the study and the ablation technique used, enthusiasm for renal denervation as a treatment for resistant hypertension has abated [Epstein M 2015]. Based on these initial observations, Maranon *et al* examined the effects of renal denervation in hyperandrogenaemic female rats and found that it resulted in a reduction in mean arterial pressure as compared to controls [Maranon 2015]. Taking this approach one-step further, Schlaich *et al* examined the effects of renal denervation on SNS activation, blood pressure and insulin resistance in two obese PCOS subjects with hypertension [Schlaich 2011]. Both patients had elevated MSNA and total body noradrenaline spillover at baseline, compared with normotensive controls. Following renal denervation, MSNA,

noradrenaline spillover, blood pressure and insulin resistance were reduced in both patients (Figure 1.5). These findings support the role of increased sympathetic tone in the pathogenesis of PCOS and may suggest a place for this intervention in improving cardiometabolic outcomes.



**Figure 1.5**

**Changes in systolic blood pressure (a) and MSNA (b) in the two patients at 12 weeks after bilateral renal denervation and (c) whole body norepinephrine spillover at baseline and directly after renal denervation. NE, norepinephrine**

[Adapted from Schlaich *et al* 2011]

## 1.9 STUDY HYPOTHESIS AND AIMS

Previous studies have shown that PCOS is associated with sympathoexcitation but the central regulatory pathways are unknown. Advanced MRI has the potential for the first time to illuminate some of the cerebral control pathways implicated in this activation, as has been discussed, and to establish if there are central differences in neural responses to sympathoexcitation between subjects with PCOS and controls, which may underly susceptibility to hypertension.

I hypothesise that increased SNS activation in women with PCOS is associated with increased BOLD fMRI activity within the brainstem and higher brain structures in response to SNS-mediated pressor responses to IFC. Potential mediators of this activation may include central obesity, androgens and insulin resistance. This novel approach of using functional neuroimaging will improve our understanding of the contribution of adiposity and insulin resistance to the neuronal control of blood pressure and may inform subsequent clinical trials which seek to target insulin sensitivity (e.g. metformin), OSA (CPAP), and weight reduction (e.g. lifestyle or pharmacological intervention), leading to improved vascular outcomes for patients with PCOS and other metabolic disorders associated with altered insulin sensitivity.

The aims of this thesis are:

1. To determine whether blood pressure and SNS responses to IFC are increased in women with PCOS compared with age- and BMI-matched controls.

2. To compare BOLD fMRI signal activity in the brainstem and higher brain structures associated with increased SNS activity in women with PCOS compared with age- and BMI-matched controls.

3. To investigate the metabolic and anthropometric correlates of SNS activity in young women with PCOS and age- and BMI-matched controls.

# **CHAPTER 2**

# **MATERIALS AND METHODS**

## **2.1 STUDY APPROVAL**

The study was approved by Cardiff University (Study Sponsor), Cardiff and Vale University Health Board, and the South East Wales Research and Ethics Committee in October 2012 (REC reference: 12/WA/0239). A copy of the REC approval can be found in Appendix 1, the Patient information sheet and consent form in Appendix 2 and Volunteer information sheet and consent form in Appendix 3.

## **2.2 SUBJECTS**

### **2.2.1 Patients**

Patients were recruited from the Endocrine and Gynaecology clinics at the University Hospital of Wales, Cardiff, the Endocrine clinic at Morriston Hospital, Swansea, Morlais Medical Practice, Merthyr Tydfil, and via a PCOS database held at the Centre for Endocrine and Diabetes Sciences, Institute for Molecular and Experimental Medicine, Cardiff University School of Medicine. Recruitment took place between May 2013 and June 2014. All patients gave written, informed consent.

The diagnosis of PCOS was based on the consensus Rotterdam criteria i.e. at least 2 out of 3 of the following [Rotterdam 2004]:

- Androgen excess (clinical symptoms of hyperandrogenism and/or elevated testosterone/androstenedione),
- Ovulatory dysfunction (fewer than 6 menstrual cycles per year)
- Polycystic ovaries on ultrasound scan (one or both ovaries with 12 or more follicles measuring between 2 and 9mm and /or an ovarian volume > 10cm<sup>3</sup>)

Androgen-secreting neoplasms, Cushing's syndrome, Congenital adrenal hyperplasia, hyperprolactinaemia and thyroid disease were excluded by biochemical testing, including 17-hydroxyprogesterone (17-OHP), prolactin and TFT measurements. Patients were aged between 18 and 45 years and were not included in the study if they were pregnant (pregnancy test was performed at initial visit), breastfeeding, or had a history of current or previous use (within 3 months) of glucocorticoids, lipid lowering agents, anti-hypertensives, anti-diabetics or anti-obesity drugs. Patients with a history of hypertension or diabetes were also excluded. Patients with any contraindication to an MRI scan were also excluded, including pacemakers, implanted hearing aids, neurostimulators, intracranial metal clips and metallic bodies in the eye.

### **2.2.2 Healthy volunteers**

The control group consisted of healthy volunteer females with regular menstrual cycles (menses every 27-32 days) matched for age ( $\pm$  2yrs) and BMI ( $\pm$  2Kg/m<sup>2</sup>). Their healthy state was determined by history, physical examination and hormonal evaluation (testosterone, androstenedione, thyroid function, prolactin). Healthy volunteers were excluded if they were pregnant (test performed at first visit). Control subjects with signs of hirsutism or with a personal history of diabetes or hypertension, or a family history of PCOS were excluded. Those with any contraindication to MRI were also excluded. Healthy volunteers were recruited by advertisement among staff and students at the University Hospital of Wales, Cardiff University and in the local press. Recruitment took place between June 2013 and May 2014. All healthy volunteers gave written, informed consent.

### **2.3 ANTHROPOMETRIC MEASUREMENTS**

Anthropometric assessment took place at the Clinical Research Facility, University Hospital of Wales. Assessment included measurement of height, weight, waist circumference and hip circumference. Height was measured using a Seca 242 measuring rod [Seca, Birmingham, UK] and weight with Seca sit on scales [Seca,

Birmingham, UK]. Waist circumference was measured at minimal respiration and reported to the nearest 0.5cm by positioning a tape measure parallel to the floor and immediately above the iliac crest. Hip circumference was measured in a similar manner around the widest portion of the buttocks.

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. The Waist-Hip Ratio (WHR) was determined by dividing the waist circumference (cm) by the hip circumference (cm).

## **2.4 INSULIN SENSITIVITY MEASUREMENTS**

Studies were conducted between 0900 and 1200 hours in a quiet physiological lab maintained at 20°C in the Clinical Research Facility, University Hospital of Wales. All subjects attended for blood sampling in a fasted state from midnight. An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm.

A standard 75g oral glucose tolerance test (OGTT) was performed using Polycal Liquid© [Nutricia Ltd, Wiltshire, UK] and insulin sensitivity was assessed by means of insulin and glucose responses. Glucose and insulin were measured by drawing blood from the cannula at 0, 30, 60, 90 and 120 minutes.

Glucose was measured using the Aeroset chemistry system [Abbott Diagnostics, Berkshire, UK]. Insulin samples were centrifuged within 1 hour and aliquots stored at -80 °C until measurement, at a maximum of 6 months post-sampling, using an immunometric assay specific for human insulin [Invitron, Monmouth, UK].

The area under the curve (AUC) for insulin and glucose was calculated from the values obtained during the OGTT using the trapezoid method. The homeostatic model assessment (HOMA) method was also used to estimate insulin resistance (HOMA-IR) [Matthews et al., 1985]. In addition, the Matsuda Index was calculated using the glucose and insulin values from the OGTT and the equation  $(10,000/\text{square root of } [\text{fasting glucose} \times \text{fasting insulin}] \times [\text{mean glucose} \times \text{mean insulin during OGTT}])$  [Matsuda *et al*, 1999].

## **2.5 BIOCHEMICAL MEASUREMENTS**

Fasting basal samples were taken pre-oral glucose tolerance test, as outlined above, for biochemical analysis. Serum was prepared by centrifugation of blood at 4000 rpm for 8 minutes. Serum total cholesterol, high density lipoprotein cholesterol (HDL) and triglycerides were assayed using an Aeroset automated analyser [Abbott Diagnostics, Berkshire, UK]; LDL cholesterol (LDL) was calculated using Friedewald's formula.

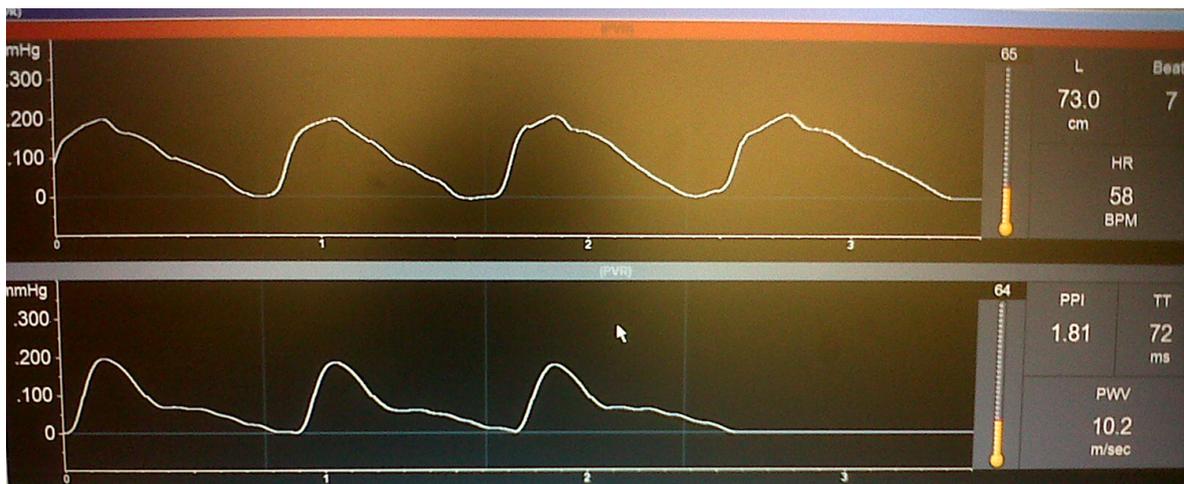
Total testosterone was measured by liquid chromatography-tandem mass spectrometry [Quattro™ Premier XE triple quadrupole tandem mass spectrometer, Waters Ltd, Watford, UK]. Androstenedione was measured by tandem mass spectrometry using an in-house method. Thyroid function tests (TFTs) were assayed using the Abbott Architect platform [Abbott Laboratories, Illinois]. HbA1c measurements were determined using a high-performance liquid chromatography (HPLC) assay [TOSOH HLC-723G8, Tosoh Corporation, Tokyo, Japan]. The intra- and inter-assay coefficients of variation were all <9%.

## **2.6 PULSE WAVE VELOCITY ASSESSMENT**

Measurements were made with the patient resting supine between 0900 and 1200 hours in a quiet physiological lab maintained at 20°C in the Clinical Research Facility, University Hospital of Wales.

The Vicorder system [Skidmore Medical, Bristol, UK] measures simultaneous pressure waveforms by a volume displacement technique, using blood pressure cuffs placed around the sites of interest. It has the advantage over applanation tonometry in that it is operator-independent, does not require the subject to undress and has been shown to be a validated and repeatable tool for assessment of pulse wave

velocity and measurement of central systolic blood pressure [Hickson SS 2009, Puccini G 2013]. Measurements were obtained using the Vicorder device by placing a 100 mm wide blood pressure cuff around the upper thigh to measure the femoral pulse and a 30 mm partial cuff around the neck at the level of the carotid artery. The cuffs were each inflated to 60 mm Hg, and high-quality waveforms were recorded simultaneously for 3 s with the subject in the supine position, using a volume displacement method. Path length was defined as the distance from the suprasternal notch to the top of the thigh cuff as indicated by the manufacturer. Figure 2.1 shows a screenshot from the Vicorder device.



**Figure 2.1**

**Screenshot of Vicorder device showing recording of pulse wave velocity**

## **2.7 ISOMETRIC FOREARM CONTRACTION**

### **2.7.1 Rationale**

Isometric forearm contraction (IFC) has been shown to generate a haemodynamic response, characterised by a rise in arterial blood pressure, heart rate and SNS activation. It is thought the increase in SNS activation is driven by (i) stimulation of mechanoreceptors during muscle stretch (ii) chemoreceptor stimulation from accumulation of metabolites in the exercising muscle (also sensitising the mechanoreceptors) (iii) central command, although to a lesser degree. Group III and IV afferent fibres, both sensitive to mechanical and chemical stimulation, are thought to be involved in the exercise pressor reflex [Kaufman MP 2002, McCloskey 1972]. The stimulation of these muscle afferents results in increased MSNA outflow, with subsequent vasoconstriction. Increased heart rate and cardiac output also contribute to the blood pressure rise. Thus there may be an interaction of the mechanoreflex and metaboreflex in the central nervous system, enhancing the MSNA response [Cui J, 2007].

The degree of maximum effort during IFC is also thought to be relevant in the metabo-mechanoreflex. In lean healthy individuals, the increase in MSNA during exercise until

10% of maximal voluntary contraction is mediated by central command/mechanoreceptors located in the skeletal muscle, whereas the increase in sympathetic nerve activity during 30% IFC is mediated by central command/mechanoreceptors and metaboreceptors [Seals 1988, Rowell 1992].

Coulson and colleagues recently confirmed a pressor response to IFC in healthy male and female volunteers and described the influence of the SNS on this response by the use of selective  $\alpha$ - (phentolamine) and  $\beta$ -adrenoceptor (esmolol) antagonists [Coulson 2015]. Results from Coulson's study showed no difference in pain perception between a 40% of maximum isometric grip (pain score  $1.5 \pm 0.7$ ) or a <5% isometric grip (pain score  $1.5 \pm 0.8$ ).

A range of IFC strengths and durations have been used in studies. During sustained handgrip at 30% maximum, heart rate and blood pressure increase during the first two minutes of the exercise, with MSNA increasing during the second minute [Mark AL, 1985]. The approach which I adopted for my protocol is therefore based upon those studies performing IFC at 30% maximum grip for 3 minutes which demonstrated a significant haemodynamic response to 30% IFC and also evoked MSNA activation [Negrao 2001, Ichinose M 2006].

## 2.7.2 Protocol

Maximum grip strength was determined by asking the volunteer to squeeze an electronic hand dynamometer (90kg capacity range) [Zhongshan Camry Electronic Co. Ltd, China] with their dominant hand to maximum effort on three separate attempts, with a 60 second period of rest between each squeeze, as previously recommended [Hamilton 1994, Innes 1999] (Figure 2.2). The mean maximum grip strength was determined and 30% IFC calculated by:

$$30\% \text{ IFC} = 0.3 \times \frac{S1 + S2 + S3}{3}$$

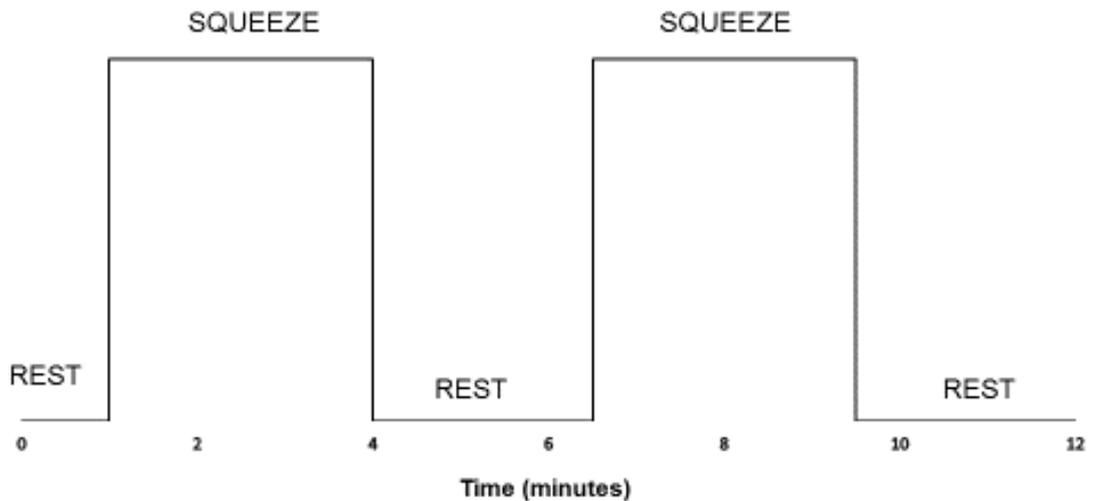
(S1, S2, S3 represent three initial maximum squeeze attempts (measured in kg)).



**Figure 2.2**

**Electronic hand dynamometer used for IFC**

Subjects were then left to rest in a supine position for 5 minutes before the experiment began. The protocol followed a block design 12 minutes in total, comprising 1 minute rest, 3 minutes squeeze, 2.5 minutes rest, 3 minutes squeeze and 2.5 minutes rest (Figure 2.3). The subjects were instructed as to the rest and squeeze periods, and targeted to sustain 30% IFC during the squeeze periods.



**Figure 2.3**

**IFC paradigm**

**2.7.3 Catecholamine measurement**

Blood was drawn from the intravenous cannula in the non-dominant arm of the subject in a supine position after a 10 minute rest period (pre-IFC catecholamines). Following 3 minutes of IFC at 30% maximum handgrip strength, further blood was immediately drawn for post-IFC catecholamines. Samples were centrifuged at 2000rpm at 4°C within 10 minutes of collection and aliquots stored at -80°C until analysis. Measurements took place within 6 months of sampling using an Epinephrine ELISA

Kit [Abnova, Taoyuan County, Taiwan] and Norepinephrine ELISA Kit [Abnova, Taoyuan County, Taiwan]. The intra- and inter-assay coefficients of variation were <15.4% for the epinephrine kit and <16.1% for the norepinephrine kit.

#### **2.7.4 Blood pressure and heart rate measurement**

Resting blood pressure (mmHg) and heart rate (beats/min) were measured at time zero of the IFC paradigm (after a 10 minute rest period in the supine position) using an Omron HEM-907 blood pressure monitoring device [Omron Healthcare UK Ltd, Milton Keynes, UK]. The blood pressure cuff was inflated on the non-dominant arm for each subject. Measurements were then made of blood pressure and heart rate every 30 seconds throughout the 12 minute protocol. Mean arterial blood pressure (MAP) was calculated using the formula:

$$\text{MAP} = \frac{(2 \times \text{DBP}) + \text{SBP}}{3}$$

3

DBP = diastolic blood pressure, SBP = systolic blood pressure.

## **2.8 MRI**

### **2.8.1 Introduction**

MRI is a non-invasive, non-ionising radiation modality that enables imaging of the anatomical structure of the central nervous system. The technique of functional magnetic resonance imaging (fMRI) has been developed to allow mapping of local physiological or metabolic changes from altered brain electrical activity. fMRI provides excellent spatial resolution (the ability to distinguish different locations within an image, a few mm) and temporal resolution (the rate at which images are acquired, approximately 3 seconds). For this reason fMRI represents an important technique in experimental neuroscience.

### **2.8.2 Physics of MRI**

Atomic nuclei that have an odd number of protons possess angular momentum. Nuclear magnetic resonance (NMR) allows the measurement of electromagnetic induction from the magnetic moments of the nuclei that have undergone polarisation in a magnetic field [Bloch 1946].

Since hydrogen atoms have a magnetic moment due to the intrinsic momentum of their nuclei, tissues that contain varying densities of water molecules (containing hydrogen) can be distinguished. When placed in a strong external magnet field, a small proportion of hydrogen nuclei orientate parallel to the magnet field. When an alternating radiofrequency (RF) pulse is applied in a different plane to the external magnetic field, the nuclei become aligned to the RF field. When the RF pulse stops, this causes the nuclei to return to their original orientation ('relaxation') and re-radiation of RF energy is detected as the MRI signal.

Relaxation measured in different planes allows modification of the contrast between different tissue densities. T1 relaxation is the recovery of longitudinal magnetisation whereas T2 relaxation is the decay of transverse magnetisation. A T2 contrast image, which is sensitive to the presence of deoxygenated blood, can be generated by measuring the transverse magnetic decay that results from magnetic field variations in the tissue within the applied static magnetic field.

### **2.8.3 Physiology of BOLD fMRI**

In 1936, Pauling and Coryell discovered that the haemoglobin molecule has magnetic properties depending on whether it is oxygen bound or not. Oxygenated haemoglobin

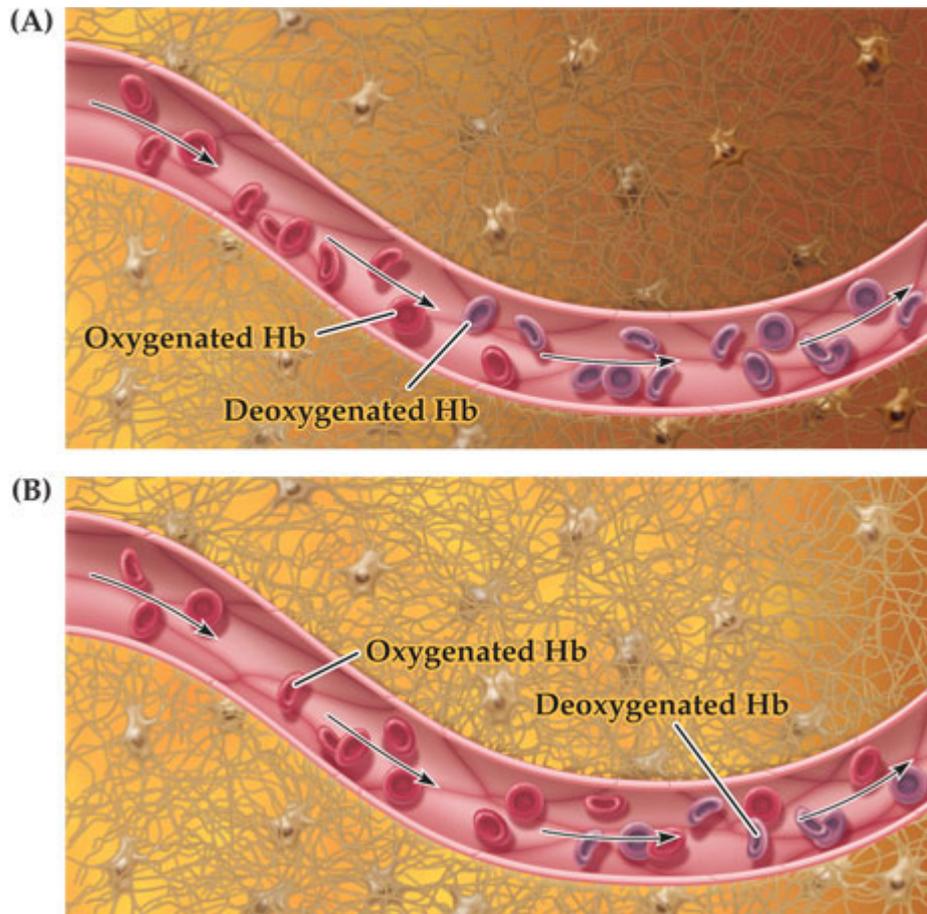
is diamagnetic, with no unpaired electrons and zero magnetic moment, whereas deoxyhaemoglobin has unpaired electrons with a significant magnetic moment and is thus paramagnetic [Pauling and Coryell 1936].

Since paramagnetic substances distort the surrounding magnetic field, nearby protons experience different field strengths and therefore change the orientation of their rotational axis at different frequencies, resulting in more rapid decay of transverse magnetisation. Therefore MR pulse sequences show more signal where high amounts of oxygenated haemoglobin are present compared to where blood is highly deoxygenated. This finding was verified in experiments in the 1980s when it was found that the decay of transverse magnetisation depended on the proportion of oxygenated haemoglobin in a test tube of blood. Deoxygenation increases the volume magnetic susceptibility within the erythrocytes and creates local field gradients around these cells and in the blood vessels containing them [Thulborn 1982]. Deoxyhaemoglobin has therefore since been utilised as a natural contrast agent during MRI and the modality is therefore known as Blood Oxygenation Level Dependent (BOLD) fMRI [Ogawa 1990].

Under normal physiological conditions, a proportion (about 40%) of oxygenated haemoglobin is converted to deoxygenated haemoglobin in the capillary bed (Figure

2.4A). During neuronal activation in the brain (Figure 2.4B), there is an overcompensatory increase in bloodflow, with more oxygenated haemoglobin supplied than is required by those areas of the brain with metabolically active neurons. Consequently this results in a decrease in the amount of deoxygenated haemoglobin and an increased, brighter MR signal [Roy and Sherrington 1890, Huettel SA 2009].

The matching of local cerebral blood flow to neuronal activity is not exact which affects the temporal resolution of BOLD fMRI. Following neuronal activation, in some areas of the brain such as the visual and primary motor cortex, there is an initial decrease in oxygenated haemoglobin (oxy-Hb), followed by a delivery of oxy-Hb in excess of demand for approximately 6 seconds. This has been interpreted as reflecting local deoxygenation of blood in the capillary bed preceding the onset of activation-associated hyperaemia [Buxton 1998]. This “initial dip” may provide a more accurate measure of the localisation of activation. A brief, post-stimulus, undershoot is then seen before returning to baseline.



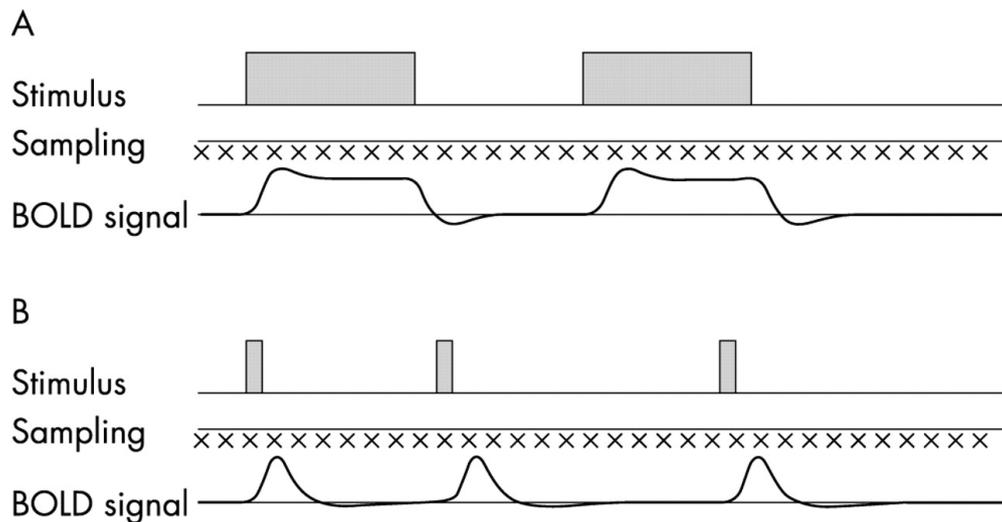
**Figure 2.4**

**Overview of BOLD signal generation: (A) Under normal conditions oxygenated haemoglobin is converted to deoxygenated haemoglobin at a constant rate within the capillary bed. (B) When neurons become active the vascular system supplies more oxygenated haemoglobin than needed by the neurons, through an overcompensatory increase in blood flow. This results in a decrease in the amount of deoxygenated haemoglobin, leading to a brighter MR image. [from Huettel et al, 2009]**

#### **2.8.4 BOLD experimental design**

BOLD fMRI can be used to detect relative signal intensity changes in response to a change in regional neural stimulation in the brain. The means of achieving this is to compare brain responses during different states of the same imaging session by introducing a stimulus. The 'block' design is a relatively common method used in which there are relatively long alternating periods (at least 30 seconds) between a stimulus and rest period (Figure 2.5A) [Friston KJ 1999]. In some cases, a stimulus cannot be maintained for long periods and thus an 'event' related design can be utilised while discrete stimuli are repeated throughout the experiment while data are acquired (Figure 2.5B) [Buckner 1998]. Event related fMRI requires longer acquisition times compared with the block paradigm in order to achieve a sufficient signal-to-noise ratio.

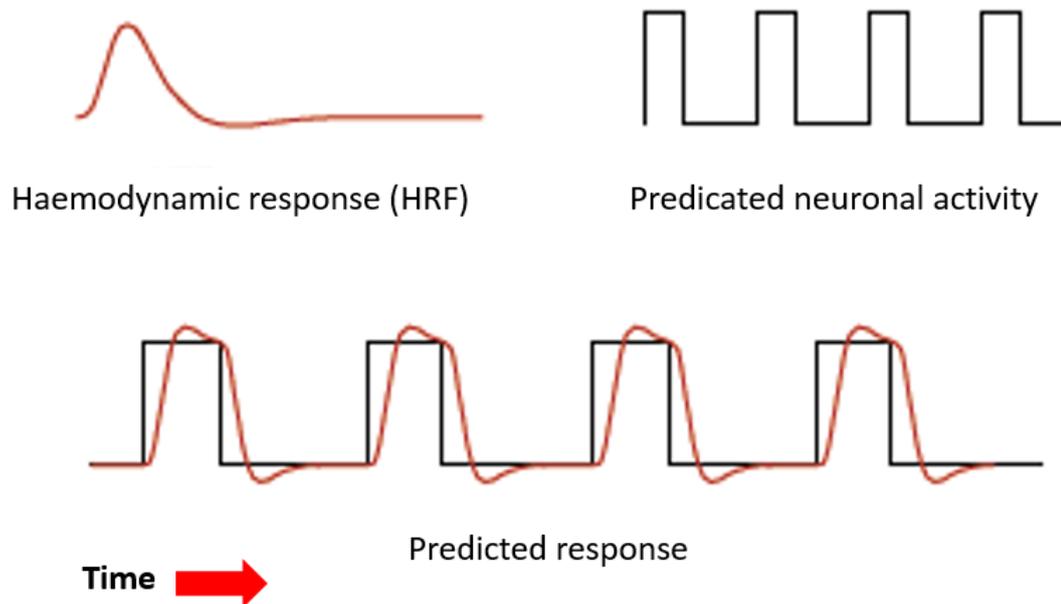
As the neural haemodynamic response to a stimulus is delayed and prolonged it requires modelling of the predicted haemodynamic response to a given stimulus (Figure 2.6).



**Figure 2.5**

**‘Block’ versus ‘event’ related fMRI designs: Schematic representation of a block design functional magnetic resonance imaging (fMRI) paradigm (A) and an event related fMRI paradigm (B). For the block design a relatively long (e.g. 30 second) stimulation period is alternated with a control period. For the event related design a brief stimulus period is used, which can either be periodic or randomised. In both cases volumes of data (indicated by the crosses) are collected continuously, typically with a repeat time of three to five seconds.**

[from Matthews PM 2004]



**Figure 2.6**

**The modelling of the predicted haemodynamic response to a given stimulus.**

**The red haemodynamic response function (HRF) is modelled against the black 'block' periods of stimuli [adapted from Jezzard 2001]**

Various factors can affect the BOLD signal and sufficient resolution of the BOLD signal needs signal intensity that can be detected above background noise. Potential sources of noise include physiological factors such as cerebral blood flow, respiratory patterns and arterial carbon dioxide concentrations, as well as noise within the electronics of the MRI system [Wise *et al* 2007]. Breath-holding can also affect the signal [Murphy

*et al* 2010]. Physiological noise can be removed by regressing from the data signals that correlate with the breathing and cardiac cycles.

### **2.8.5 Scan protocol**

The scan protocol was designed to show how BOLD signal correlates with the IFC (pressor) task, using a block design.

Subjects were fitted with a nasal cannula to measure end tidal CO<sub>2</sub>. Respiration pattern was determined by a strain-gauge band around the chest. Heart rate was collected from a pulse oximeter in the left hand [MedRad, USA]. Physiological data were collected with a computer-based data acquisition and analysis system [CED 1401, Cambridge, UK].

An in-house MRI-compatible handgrip device was positioned in the dominant hand and connected to a pressure transducer. The pressure signal was collected with a computer-based data acquisition and analysis system [CED 1401, Cambridge, UK] and displayed on a screen located inside the scanner via a digital projector.

Participants were asked to perform a maximum voluntary contraction to determine their maximum grip strength on two occasions. A target of 30% of maximum grip strength was set for the experiment and the block paradigm followed as shown in Figure 2.3. Subjects followed visual instructions presented on the screen as to the rest and squeeze periods, with a target bar showing when 30% squeeze had been achieved. Psychology software in Python (PsychoPy) version 1.78 [Peirce 2007] was used to run the visual stimulus. Subjects performed the block paradigm on two occasions with time to rest between the runs.

The experimental protocol included visual stimuli in the form of a flashing check-board (2Hz lasting 15s) that appeared twice at the end of both rest periods.

### **2.8.6 fMRI**

A 3-Tesla whole body MRI scanner [General Electric, US] was used for all scans. The head was held immobile in an eight-channel head coil by foam pads. A continuous series of 232 fMRI image volumes (echo-planar images using BOLD contrast, scan time = 12m) were collected for each run. Voxel size was  $1.5 \times 1.5 \times 3 \text{mm}^3$ , matrix  $128 \times 128 \times 40$  and Field-of-view (FOV)  $192 \times 192 \text{mm}^2$  in plane. The slice thickness was 2.2mm and slice gap 0.8mm. Each volume covered the entire brain and brainstem (TR

= 3.1s, TE = 25ms). Slices were tilted 10°-15° from the axial to the coronal plane to reduce signal loss due to dephasing in the brainstem resulting from through-slice susceptibility-induced gradients [Weiskopf 2006]. Parameters used were adapted to image the brainstem, including slice orientation and using a slightly shorter echo time. Structural images were collected using a T1-weighted sequence order to facilitate visualisation. Each T1 scan was registered to the MNI152, an average T1 brain image constructed from 152 normal subjects at the Montreal Neurological Institute (MNI), Montreal, QC, Canada, using FLIRT software and linear registration [Jenkinson 2001 & 2003].

### **2.8.7 Image analysis**

During initial image analysis, a number of pre-processing steps are performed, including reduction of head movement artifact, spatial smoothing, and temporal smoothing by filtering out temporal variations that were slower than the experimental paradigm [Turner *et al* 1998].

The Brain Extraction Tool (BET), available in FEAT, was used to remove surrounding tissue around the brain, as the experiments within this study were only concerned with analysis of brain structures.

During scanning, head movement by a subject can interfere with assigning the correct time series to the correct voxel. McFLIRT, a motion correction algorithm, was used to ensure that the fMRI time series was representative of the same volume of brain tissue [Jenkinson M 2002].

High pass filtering allows the removal of scanner drift. To prevent the removal of variations related to the experimental paradigm, a filter cut-off of 330 seconds was used.

A lack of uniformity in the magnetic field can result in signal distortion and dropout. This is most noticeable at the tissue-air interfaces such as the air-filled sinuses. Acquiring separate field maps therefore allows for the correction of any distortion at each voxel [Jenkinson 2003].

Physiological noise correction was applied retrospectively to the imaging data using in-house software developed in the Cardiff University Brain Research Imaging Centre [Harvey *et al.* 2008].

Analysis of the scans was by FEAT (fMRI Expert Analysis Tool, version 6.00) software (available on-line at [www.fmrib.ox.ac.uk/fsl](http://www.fmrib.ox.ac.uk/fsl)). FMRI images were un-warped, motion corrected, spatially smoothed and spatially normalised to the MNI template. A high-pass filter of 330 seconds was used. To generate contrast images, task-related BOLD activation was estimated with a design matrix specifying a general linear model (GLM) that included a waveform based on each person's IFC during the scan protocol. The visual stimulus shown in the scan session was also included in this analysis.

BOLD signal changes for blood pressure condition were modelled with a waveform derived from the blood pressure recordings made out of scanner during the 12-minute paradigm.

Z statistic images were thresholded using clusters determined by  $z > 2.3$  and a cluster significance threshold of  $P = 0.05$  [Worsley 2001]. Significant BOLD signal intensity changes were colour coded and rendered onto an individual's T1-weighted anatomic image set. The resulting statistical parametric maps were used in higher level analysis to determine differences between PCOS and control groups.

### **2.8.8 Statistical analysis**

Statistical analysis is required to determine which voxels are activated by a stimulus. The Oxford Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) expert analysis tool (FEAT) software was used for both the individual and group (lower and higher) level analysis to correlate the change in BOLD signal with the modelled neural haemodynamic response to IFC. This software uses univariate analysis to analyse the time series of each voxel independently through general linear modeling (GLM).

### **2.8.9 General Linear Modelling**

GLM analyses the extent to which the timing of the measured BOLD signal correlates with the model of the haemodynamic response function (HRF) to a stimulus (Figure 2.6). The stimulus function is convolved with the HRF to allow for the temporal delay and blurring in the HRF. A specific stimulus model is called an explanatory variable (EV). When the model fitting is performed, parameters are estimated that describe the amplitude of the signal component in the data that is represented in each EV. The parameter estimates a conversion to T-scores and Z statistics.

The equation for GLM is given by the equation:

$$y = X\beta + e$$

Where  $y$  = the observed fMRI time course in a specific voxel

$$X = EV$$

$\beta$  = parameter estimate (intensity of the signal response for a voxel)

$e$  = error in the model

### **2.8.10 Multiple comparison problems**

Running the statistical analysis separately for each voxel creates a massive multiple comparisons problem. To correct for multiple comparisons, Gaussian Random Field (GRF) is used as a statistical map based on the notion that neighbouring voxels are not activated independently from each other but are more likely to activate together in clusters. Application of this GRF correction method, however, requires that the fMRI data are spatially smoothed which substantially reduces its high spatial resolution.

### **2.8.11 Registration**

Image alignment to a standard space brain template is essential in fMRI studies with multiple participants to allow comparisons of spatial locations. FEAT contains the software FLIRT (FMRIB's Linear Image Registration Tool) that allows the linear transformation of imaging data [Jenkinson *et al.* 2001 & 2002]. This registration from functional space to standard space is a two-step process, using a mid-point reference of a structural T1 image – the functional low resolution image is aligned to a T1 high-resolution image, which is then registered to a standard space template.

### **2.8.12 Group analysis**

FEAT is able to compare the BOLD response from several subjects during higher level analysis. This also improves the signal-to-noise ratio and allows easier identification true BOLD activation areas by comparing the intra- and inter-subject variability.

As the paradigm was run twice, an intermediate level FEAT analysis was run for each subject by combining their two lower-level FEAT outputs, to produce an average for each subject. These were then used in the higher-level Feat analysis that could be used in the group analyses.

## **2.9 MUSCLE SYMPATHETIC NERVE ACTIVITY (MSNA)**

A pilot sub-study was undertaken in PCOS subjects and healthy volunteers who agreed to undergo microneurography in order to record a more direct measure of sympathetic activity.

Microneurography studies were conducted between 0830 and 1530 hours in a quiet physiological lab maintained at 20°C in the Clinical Research Facility, University Hospital of Wales. These measurements were undertaken by Dr Yrsa Sverrisdóttir, Oxford University, who has many years' experience in undertaking microneurography.

Direct recordings of multiunit efferent postganglionic muscle sympathetic nerve activity (MSNA) were obtained with a tungsten microelectrode with a tip diameter of a few micrometres inserted into a muscle fascicle of the peroneal nerve, posterior to the fibular head. A low-impedance reference electrode was inserted subcutaneously a few centimetres from the fibular head (Figure 2.7).



**Figure 2.7**

### **Positioned microelectrodes for MSNA recordings**

When a muscle nerve fascicle had been identified, small electrode adjustments were made until a site was found in which spontaneous, pulse-synchronous bursts of neural activity could be recorded. Details of the nerve recording technique and criteria for MSNA have been reported previously [Vallibo 1979]. Bursts identified by inspection of the mean voltage neurogram were expressed as burst frequency (number of pulse synchronous sympathetic bursts per minute) [bursts/ min (BF)] and burst incidence

(number of pulse synchronic sympathetic bursts per 100 heart beats) [bursts/100 heartbeats (BI)]. During the microneurographic recording, heart rate was monitored via ECG chest electrodes (Figure 2.8).

The nerve recordings were assigned a code and analysed blinded. MSNA data were analysed a) over 300 seconds and b) over the last 60 sec before the 30% IFC, which were the values used to compare to the last 60 seconds of the IFC. As well as burst frequency and burst incidence, Total MSNA activity was measured to take into account both the frequency and size of a sympathetic burst (the product of burst per minute and mean burst amplitude), expressed in arbitrary units. The total MSNA during the last 60 seconds of a rest period was used as a baseline to see the percentage change in MSNA during the last 60 seconds of the 30% IFC.



**Figure 2.8**

**MSNA recordings on a subject while IFC being performed.**

## **2.10 SLEEP STUDIES**

Home sleep studies were undertaken from 2300-0700h, in subjects who agreed to this. The Embletta™ X100 PDS (Portable Device System) [ResMed (UK) Ltd, Abingdon, Oxfordshire] was used for limited channel sleep recordings with airflow monitored by a nasal cannula, chest and abdominal wall motion by belts, and oxygen saturations and heart rate using pulse oximeter. Snoring was derived from the nasal

cannula. Sleep recordings were stored and analysed by standard software, RemLogic-E [Embla, Stowood Scientific Instruments, Oxford, UK] and a sleep report produced for each subject. The apnoea-hypopnoea index (AHI) was calculated as the total number of obstructive respiratory events per hour of sleep, with OSA diagnosed if/when AHI is equal to or greater than 5 events per hour. The severity was then subdivided into mild, moderate or severe depending on the AHI score, according to standard criteria.

## **2.11 SAMPLE SIZE AND STATISTICAL ANALYSIS**

Given its central role in my investigation, I focused my statistical considerations on powering the BOLD fMRI study to examine brain responses to the isometric forearm contraction task. Power calculations are hard to perform for all comparisons in the study given that such work is novel so I focused on detecting basic, task-related changes. My estimates are based on the study at Cardiff University Brain Research Imaging Centre performed in which brain activity changes were detected in the brainstem in a pilot cohort of 12 volunteers [Coulson 2015]. I focused on the brainstem as a primary region of interest for the present study which indexes the brain's response to the task and which is likely to related to SNS output. Coulson *et al* detected a mean IFC (40% of maximum grip) related BOLD signal change in the brainstem (medulla) of

0.3 (arb. units) with a standard deviation of 0.2 (arb. units). This was in healthy volunteers. In the present study I aim to compare a cohort of patients with matched controls. Assuming a similar variance of task-related signal changes in the proposed cohorts, to detect a 50% greater increase in the medullary BOLD signal in response to IFC in patients with  $p < 0.05$  as the required statistical threshold to reject the null hypothesis and 70% detection power I would need to scan 20 volunteers in the patient group and 20 in the matched control group. I would also expect this cohort size to be sufficient to yield significant differences in microneurography measures.

Statistical analysis for the data was performed using SPSS version 20.0 [IBM, New York]. Independent-Samples t Test was used to compare the statistical difference between the mean of the PCOS and control groups, and  $p < 0.05$  was considered statistically significant.

# **CHAPTER 3**

**CLINICAL,**

**CARDIOMETABOLIC AND**

**SYMPATHETIC NEURAL**

**CHARACTERISTICS OF**

**THE STUDY POPULATION**

Polycystic ovary syndrome (PCOS) is an established metabolic disorder associated with insulin resistance, dyslipidaemia and hypertension, as has been outlined in chapter 1 [Dunaif 1997, Talbott 1995, Kim 2013, Joham 2015]. Furthermore, the increasing evidence that insulin resistance, obesity, hypertension and PCOS *per se* are all associated with increased sympathetic tone has been discussed [Masuo 2003, Alvarez 2002, Grassi and Mancia 2004, Sverrisdottir 2008].

The aims of this chapter are therefore to report: (1) the metabolic and anthropometric correlates of SNS activity in my study population of young women with PCOS and the age- and BMI-matched controls, and (2) to determine whether blood pressure and SNS responses to IFC are increased in PCOS compared with controls.

To this end, the clinical, cardiometabolic and sympathetic neural characteristics of my study population will be presented and discussed. The baseline anthropometric and metabolic features measured (as detailed in chapter 2, sections 2.3-2.5) will be reported alongside the results of arterial stiffness and sleep studies (chapter 2, sections 2.6 and 2.10). Furthermore, the SNS response to IFC in the PCOS and control groups are reported (as described in chapter 2, sections 2.7 and 2.9) and the potential mediators that correlate with these responses.

### 3.1 BASELINE ANTHROPOMETRIC AND METABOLIC CHARACTERISTICS

A total of 40 subjects were recruited, 20 with PCOS and 20 healthy volunteers (as described in Chapter 2.2), matched for age and BMI. The baseline anthropometric and metabolic characteristics of the two groups are summarised in Table 3.1.

Mean age for the PCOS group was 29.8yrs and in controls 29.7yrs ( $p=0.92$ ), with no significant difference in BMI between groups (mean 26.1 Kg/m<sup>2</sup> both groups),  $p=0.97$ . The waist-hip ratio (WHR) was calculated for both groups and was greater in the PCOS group (0.88) compared to controls (0.84),  $p=0.04$ . However, neither waist (mean 85.9cm PCOS, 85.1cm,  $p=0.86$ ) nor hip circumference (mean 97.2cm PCOS, 101.4cm controls,  $p=0.24$ ) was different between groups. Although serum testosterone was higher in the PCOS group (1.41nmol/L versus 0.84nmol/L), the difference between groups did not reach statistical significance ( $p=0.09$ ). Total cholesterol was 5.2mmol/L in the PCOS group and 4.8mmol/L in controls ( $p=0.12$ ) with serum triglycerides significantly higher in PCOS subjects as compared to controls (1.34mmol/L, 0.90mmol/L),  $p=0.02$ . Baseline heart rate (HR) and mean arterial pressure (MAP) were measured in both groups, with mean HR 71 beats/min in both

groups ( $p=0.94$ ), and mean MAP 81.6mmHg in the PCOS group, and 83.8mmHg in the control group ( $p=0.54$ ).

**Table 3.1**

**Baseline characteristics of the study population. Results are expressed as mean  $\pm$  SD.  $P<0.05$  is considered statistically significant**

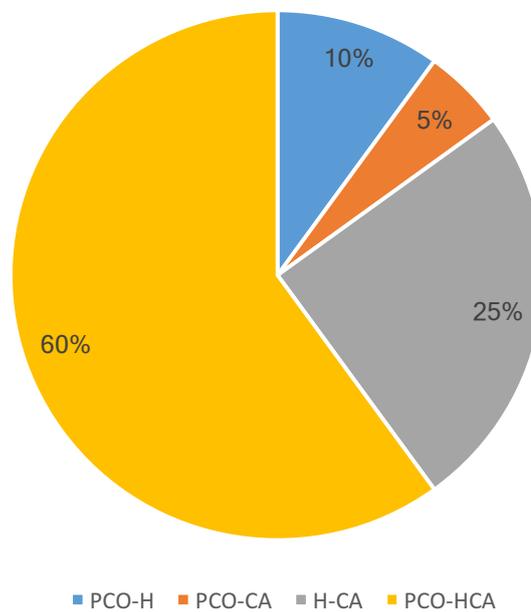
	<b>PCOS (n=20)</b>	<b>Control (n=20)</b>	<b>P value</b>
Age (yrs)	29.80 $\pm$ 4.78	29.65 $\pm$ 4.96	0.92
BMI (Kg/m <sup>2</sup> )	26.05 $\pm$ 4.90	26.11 $\pm$ 4.83	0.97
WHR	0.88 $\pm$ 0.07	0.84 $\pm$ 0.04	<b>0.04</b>
Waist circumference (cm)	85.9 $\pm$ 13.7	85.1 $\pm$ 11.1	0.86
Hip circumference (cm)	97.2 $\pm$ 10.4	101.4 $\pm$ 11.8	0.24
Testosterone (nmol/L)	1.41 $\pm$ 0.77	1.03 $\pm$ 0.53	0.09
Androstenedione (nmol/L)	4.51 $\pm$ 2.99	3.64 $\pm$ 1.28	0.25
HbA1c (mmol/mol)	34.15 $\pm$ 2.76	34.21 $\pm$ 2.64	0.95
Total cholesterol (mmol/L)	5.22 $\pm$ 1.05	4.79 $\pm$ 0.55	0.12
Triglycerides (mmol/L)	1.34 $\pm$ 0.68	0.90 $\pm$ 0.36	<b>0.02</b>
Resting HR (beats/min)	71.05 $\pm$ 8.59	71.26 $\pm$ 7.65	0.94
Resting SBP (mmHg)	114.53 $\pm$ 9.33	117.58 $\pm$ 12.62	0.40
Resting DBP (mmHg)	65.16 $\pm$ 13.33	65.47 $\pm$ 14.31	0.94
Resting MAP (mmHg)	81.63 $\pm$ 11.26	83.84 $\pm$ 10.54	0.54

### 3.2 PCOS CHARACTERISTICS AND PHENOTYPES

Of the 20 patients with PCOS recruited, Figure 3.1 outlines the proportion of each with hyperandrogenism (clinical or biochemical), ovulatory dysfunction and polycystic ovaries on ultrasound scan.

**Figure 3.1**

**Proportion of PCOS subjects with various phenotypes. PCO = polycystic ovaries on ultrasound scan, H = clinical or biochemical hyperandrogenism, CA = chronic anovulation**



Out of the 20 patients, those with biochemical evidence of hyperandrogenism (defined by serum testosterone  $>1.7\text{nmol/L}$ ) were 5/20 (25%).

11/20 (55%) had a normal BMI and 5/20 (25%) were overweight and 4/20 (20%) obese. The range of BMI was 20-38  $\text{Kg/m}^2$ .

Only 2/20 (10%) patients were both biochemically hyperandrogenic and overweight/obese.

2/20 patients had been taking metformin therapy when recruited but had a 3-month washout period before any data was collected. In addition, 4 patients were on the oral contraceptive pill.

### **3.3 INSULIN SENSITIVITY MEASUREMENTS**

39 study participants (20 PCOS, 19 controls) underwent an oral glucose tolerance test, as outlined in chapter 2, with 0, 30, 60, 90 and 120 minute glucose and insulin measured. HOMA-IR, Matsuda index, insulin AUC and glucose AUC were calculated for both groups, as described in chapter 2. Results are shown in Table 3.2.

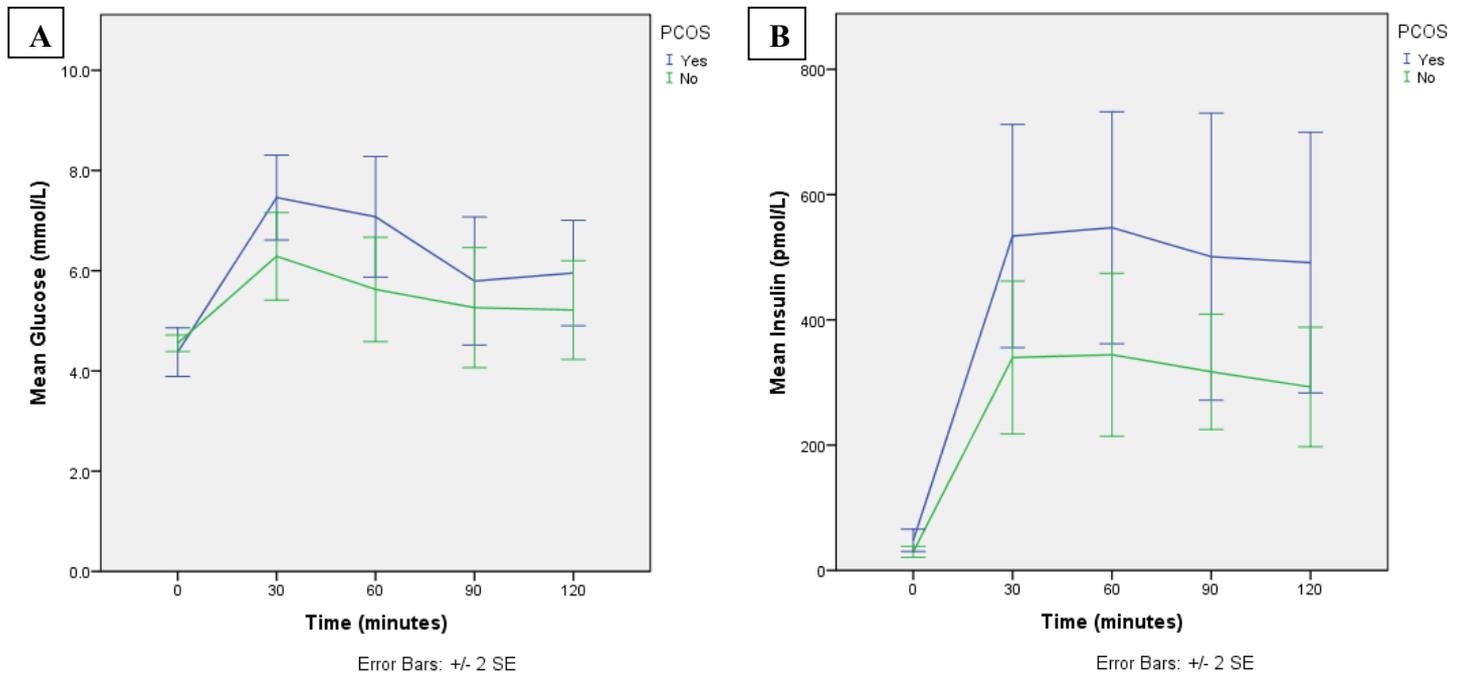
**Table 3.2****Insulin sensitivity indices for PCOS and control groups.**

**Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>PCOS (n=20)</b>	<b>Control (n=19)</b>	<b>P value</b>
Fasting plasma glucose (mmol/L)	4.38 $\pm$ 1.08	4.55 $\pm$ 0.35	0.50
2-hour glucose (mmol/L)	5.96 $\pm$ 2.35	5.22 $\pm$ 2.15	0.31
Glucose AUC (mmol min/L)	764.85 $\pm$ 239.02	661.89 $\pm$	0.17
Fasting insulin (pmol/L)	47.98 $\pm$ 39.64	29.89 $\pm$ 19.71	0.09
Insulin AUC (pmol min/L)	55519.50 $\pm$ 41547.67	35320.26 $\pm$ 21008.31	0.07
HOMA-IR	1.41 $\pm$ 1.10	0.88 $\pm$ 0.65	0.08
Matsuda index	9.11 $\pm$ 6.52	13.39 $\pm$ 9.55	0.12

As anticipated, all indices of insulin resistance were higher in the PCOS group although none of these reached statistical significance. The HOMA-IR was 1.41 compared to 0.88 in controls, Matsuda index 9.11 compared to 13.39 in controls, insulin AUC 55520 pmol min/L compared to 35320 pmol min/L in controls and fasting insulin 47.98 pmol/L versus 29.89 pmol/L in controls. In addition, 2-hour glucose was greater in the PCOS group compared with controls (6.0 mmol/L, 5.2mmol/L), as was

the glucose AUC (765 mmol min/L in the PCOS group compared to 662 mmol min/L in controls), but these were not significantly different.



**Figure 3.2**

**Line graphs showing the mean glucose (A) and insulin (B) responses for both PCOS (blue) and controls (green) during the oral glucose tolerance test.**

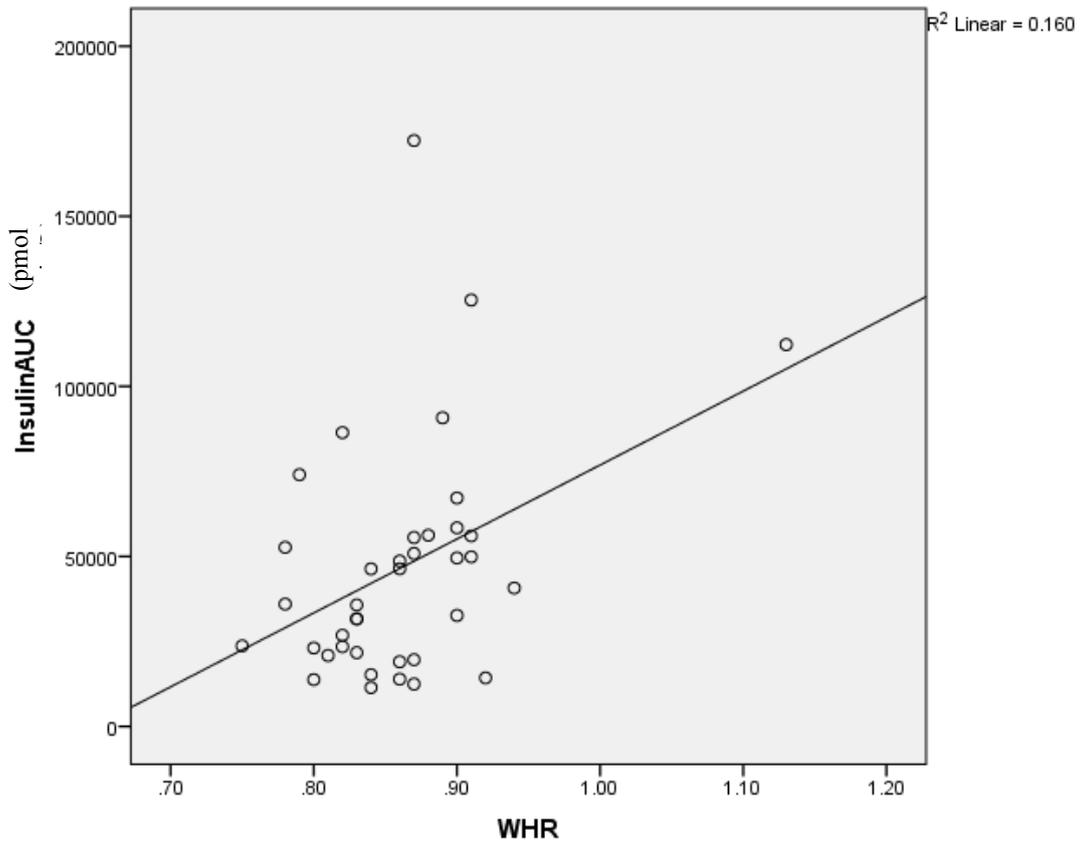
**Values represent the mean values and error bars represent  $\pm 2$  standard errors of the mean**

Figure 3.2 shows the glucose and insulin responses for the PCOS and control groups throughout the OGTT.

As anticipated, when all subjects were grouped together, there was a positive correlation between WHR and insulin resistance (Figure 3.3), with a Pearson correlation of 0.40 between WHR and insulin AUC ( $p=0.01$ ). There was also a positive correlation between BMI and insulin AUC across all subjects,  $r = 0.47$  ( $p=0.002$ ). In the PCOS group alone, there was a positive Pearson correlation between BMI and insulin AUC,  $r = 0.45$ ,  $p=0.04$ , but not a statistically significant correlation between WHR and insulin AUC,  $r = 0.37$ ,  $p=0.11$ , likely due to the comparatively small sample size. Pearson's correlation between BMI and insulin AUC for controls was 0.67 ( $p=0.002$ ), but it was not significant between WHR and insulin AUC in this group either,  $r = 0.20$ ,  $p=0.41$ .

**Figure 3.3**

**Relationship between WHR and insulin AUC (pmol min/L) for all subjects (n = 39),  $r = 0.40$ ,  $p = 0.01$**



### **3.4 ARTERIAL STIFFNESS**

Arterial stiffness was assessed using the Vicorder device, as described in chapter 2. Table 3.3 shows the augmentation pressure, augmentation index and aortic pulse wave velocity (aPWV) for the PCOS and control groups. For all three measurements, the mean values were greater in the PCOS group compared to controls, but no

statistically significant differences between the groups were found. However, the study was not powered to reveal differences in vascular function.

**Table 3.3**

**Arterial measurements for PCOS and control groups**

**Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>PCOS</b>	<b>Control</b>	<b>P value</b>
Augmentation pressure (mmHg)	6.94 $\pm$ 3.90	5.78 $\pm$ 2.90	0.32
Augmentation index (%)	13.63 $\pm$ 6.16	12.17 $\pm$ 6.00	0.47
Aortic Pulse Wave Velocity (m/s)	6.19 $\pm$ 0.63	5.83 $\pm$ 0.73	0.13

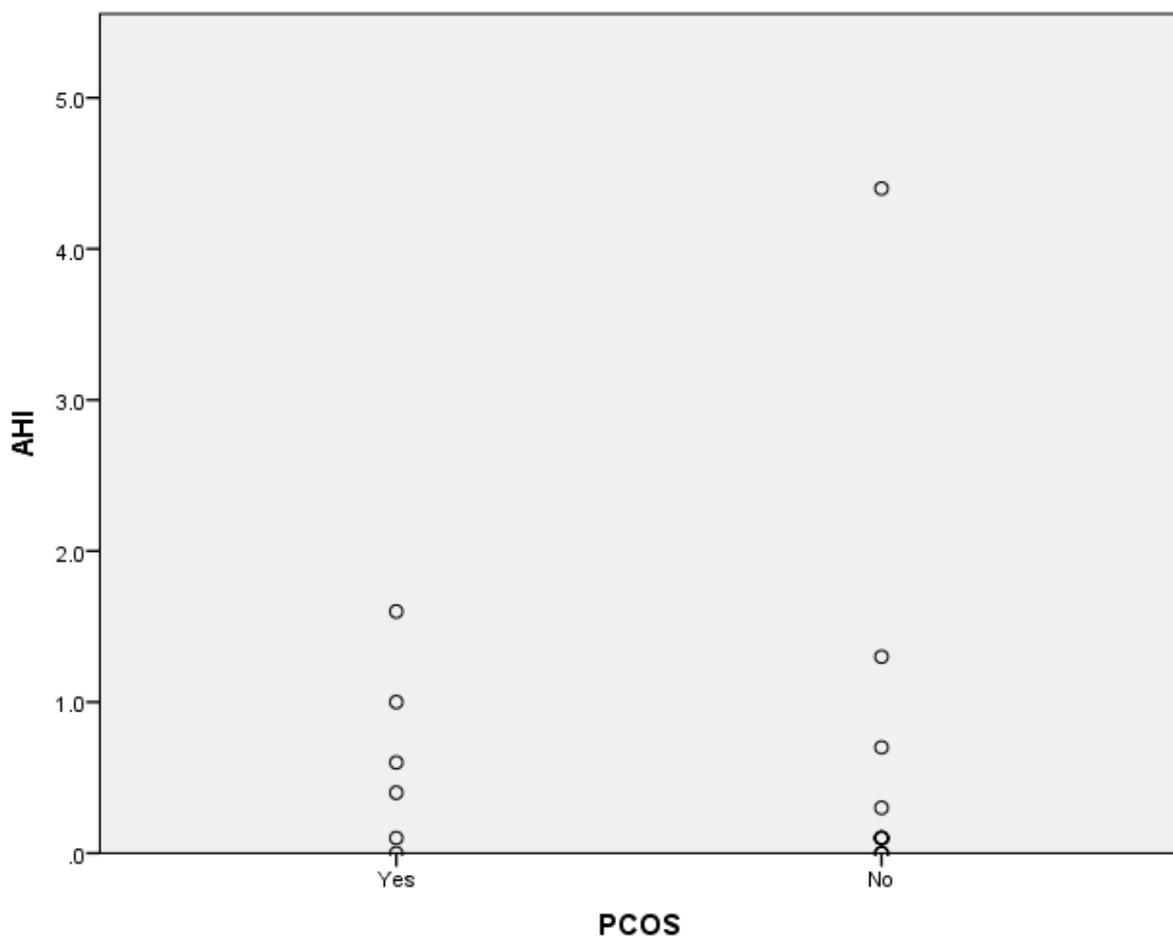
**3.5 SLEEP STUDIES**

A subgroup of 19 subjects (6 PCOS and 13 controls) underwent home sleep studies between 23.00h-07.00h, as outlined in section 2.10. The remaining subjects did not wish to undertake these. The apnoea-hypopnoea index (AHI) was calculated for both groups. The mean AHI for the PCOS group was 0.62  $\pm$  0.60 /hr, compared to 0.56  $\pm$  1.21 /hr in the control group (p=0.92). Figure 3.4 shows the difference in AHI between PCOS and controls. No subjects in either group met the criteria for OSA, as defined

by  $\geq 5$  events /hr. I conducted exploratory analyses to study the association of apnoea-hypopnoea index with anthropometric and indices in the combined group, and each of PCOS and controls separately. I felt justified in combining data from the two groups because no differences were noted in AHI between PCOS and controls. There was a positive correlation,  $r = 0.53$ , of WHR with AHI across all subjects ( $p = 0.02$ ). Similarly this was noted between BMI and AHI,  $r = 0.54$  ( $p = 0.02$ ).

**Figure 3.4**

**AHI differences between PCOS (n=6) and control groups (n=13)**



When analysed by PCOS and control groups separately, there was no significant correlation between WHR and AHI in the PCOS group,  $r = 0.45$ ,  $p = 0.37$ , but a strongly positive correlation between BMI and AHI,  $r = 0.83$ ,  $p = 0.04$ . For controls, there was a statistically significant positive correlation between AHI and WHR,  $r = 0.66$ ,  $p = 0.01$ , but not between AHI and BMI,  $r = 0.51$ ,  $p = 0.07$ .

### **3.6 SNS RESPONSE TO IFC**

#### **3.6.1 Catecholamines**

The plasma catecholamine response to IFC was assessed in 39 subjects (20 PCOS, 19 controls) before and following 3 minutes of 30% sustained IFC, as outlined in section 2.7.3. The mean resting baseline plasma adrenaline concentration in the PCOS group was  $0.68 \pm 0.53$ ng/mL and  $0.77 \pm 0.59$ ng/mL in controls ( $p=0.64$ ). Similarly, the baseline noradrenaline concentration was not statistically different between groups ( $p=0.22$ ),  $18.11 \pm 11.18$ ng/mL in PCOS and  $22.99 \pm 13.33$ ng/mL in controls.

Following IFC, for all subjects ( $n=39$ ), there was a significant rise in plasma adrenaline from  $0.72 \pm 0.55$  ng/mL to  $1.11 \pm 0.67$  ng/mL ( $p<0.001$ ) but no change in noradrenaline

concentrations (baseline  $20.48 \pm 12.36$  ng/mL to post-IFC  $18.82 \pm 11.15$  ng/mL ( $p=0.14$ )). When analysed for each group separately, there was a significant rise in adrenaline concentrations following IFC from  $0.68 \pm 0.53$  ng/mL to  $1.23 \pm 0.71$  ng/mL in the PCOS group ( $p<0.001$ ) but no change in noradrenaline concentrations from  $18.11 \pm 11.18$  ng/mL to  $16.77 \pm 10.01$  ng/mL ( $p=0.38$ ) (Figure 3.5). For the controls, adrenaline levels did not significantly change following IFC ( $0.77 \pm 0.59$  ng/mL to  $0.99 \pm 0.61$  ng/mL ( $p=0.14$ )) nor did noradrenaline concentrations ( $22.99 \pm 13.33$  ng/mL to  $20.99 \pm 12.12$  ng/mL ( $p=0.25$ )).

When expressed as percentage change from the resting state, the proportional rise in adrenaline concentrations following IFC was greater in the PCOS group compared to controls (313.8% vs 105.9%) but this was not significantly different ( $p = 0.32$ ).

Figure 3.5

Change in adrenaline concentrations (ng/mL) pre- and post-IFC in PCOS group

( $p < 0.001$ ) and control group ( $p = 0.38$ ). Adrpre = plasma adrenaline

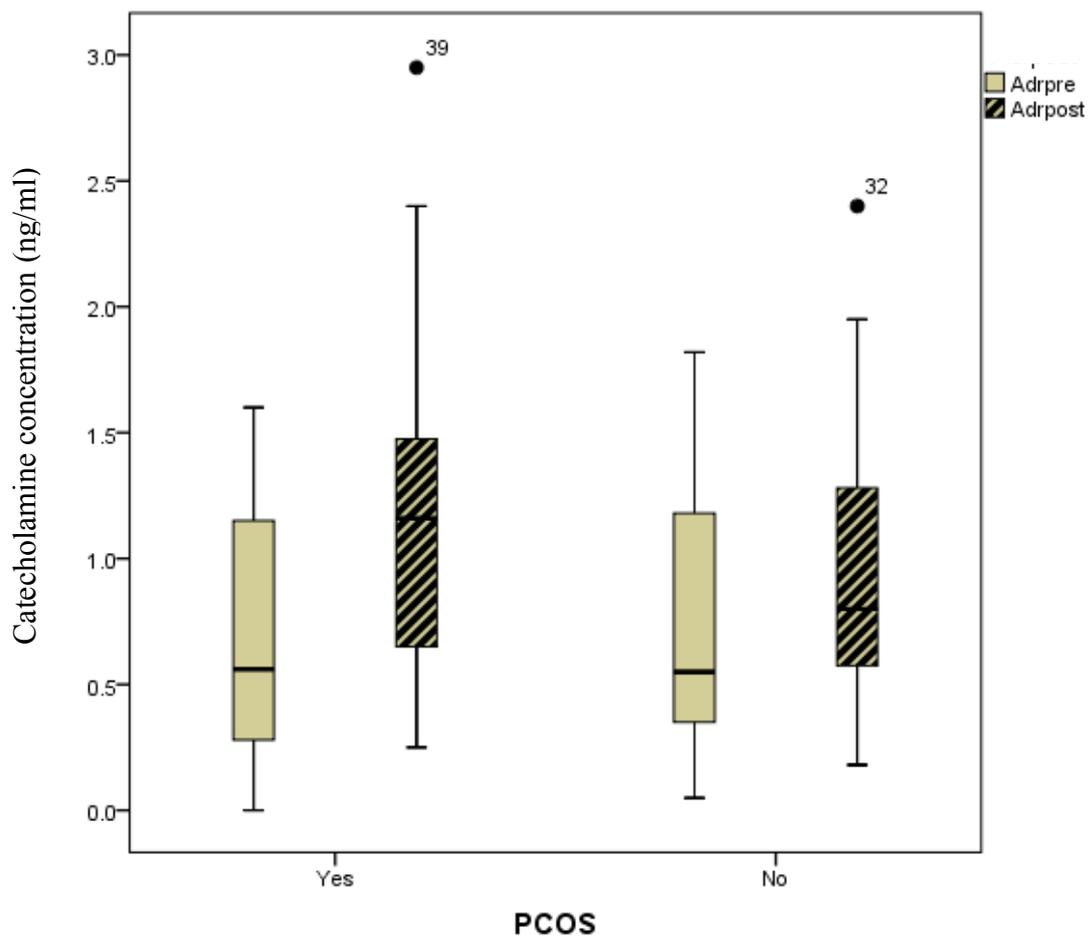
concentration pre-IFC; Adrpost = plasma adrenaline concentration post-IFC.

The solid black line within each box represents the median concentration, the

ends of the boxes the upper and lower quartiles, and the extremes of the

whiskers the minimum and maximum concentrations. Black dots show the

outlier values.



### 3.6.2 Pressor response

38 subjects (19 PCOS, 19 controls) had measurements of heart rate and blood pressure in the resting state and following 3 minutes of sustained 30% IFC, as described in the methods section 2.7.4. Table 3.1 shows the baseline resting HR, SBP, DBP and MAP for both groups, with no significant differences between the PCOS and control groups. Following IFC, taking all subjects as one group, as expected there were significant rises in HR, DBP, SBP and MAP, as shown in table 3.4

**Table 3.4**

**Haemodynamic measurements for all subjects (n=38) pre- and post-IFC.**

**Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>Mean <math>\pm</math> SD Pre-IFC</b>	<b>Mean <math>\pm</math> SD Post-IFC</b>	<b>Mean difference (95% CI)</b>	<b>P value</b>
HR (beats/min)	71.2 $\pm$ 8.0	75.9 $\pm$ 8.2	4.7 (3.3-6.2)	<0.001
SBP (mmHg)	116.1 $\pm$ 11.1	126.5 $\pm$ 12.4	10.4 (8.0- 12.8)	<0.001
DBP (mmHg)	65.3 $\pm$ 13.6	74.5 $\pm$ 13.3	9.2 (7.1- 11.4)	<0.001
MAP (mmHg)	82.7 $\pm$ 10.8	91.8 $\pm$ 11.7	9.1 (7.4- 10.8)	<0.001

For the PCOS group, following IFC, there was a mean increase in HR of 5.6 (3.0-8.3) beats/min ( $p<0.001$ ), SBP 12.6 (9.1-16.0) mmHg ( $p<0.001$ ), DBP 9.7 (7.3-12.1) mmHg ( $p<0.001$ ) and MAP 10.7 (8.2-13.3) mmHg. Similarly, for controls, the mean rise in HR was 3.8 (2.4-5.3) beats/min ( $p<0.001$ ), SBP 8.3 (4.9-11.6) mmHg ( $p<0.001$ ), DBP 8.7 (4.9-12.5) mmHg ( $p<0.001$ ) and MAP 7.5 (5.2-9.7) mmHg ( $p<0.001$ ).

The percentage increase in HR, SBP, DBP and MAP for each group was calculated, with the results shown in table 3.5.

**Table 3.5**

**Mean percentage increase ( $\pm$ SD) in HR, DBP, SBP and MAP for PCOS and control groups (%).  $P<0.05$  is considered statistically significant**

	<b>PCOS (n=19)</b>	<b>Controls (n=19)</b>	<b>P value</b>
Percentage HR increase (%)	8.5 $\pm$ 8.4	5.4 $\pm$ 4.4	0.155
Percentage SBP increase (%)	11.0 $\pm$ 5.9	7.4 $\pm$ 6.7	0.090
Percentage DBP increase (%)	15.0 $\pm$ 7.4	11.3 $\pm$ 8.0	0.157
Percentage MAP increase (%)	13.5 $\pm$ 6.5	9.3 $\pm$ 6.8	0.058

The percentage rises in all measures of the pressor response to IFC were greater in the PCOS group compared with controls, but these did not reach statistical significance.

### **3.6.3 MSNA**

A subset of participants underwent microneurography (MSNA) recordings on a separate occasion, as outlined in section 2.9. Resting data were obtained from a total of 16 subjects (8 PCOS, 8 controls). Only 14 of these subjects (7 PCOS, 7 controls) were able to proceed with full MSNA recordings post-IFC due to technical difficulties, including inability to locate the peroneal nerve for recordings (n=1) and a participant who was unable to keep their leg in position (n=1). The baseline characteristics of these two groups are shown in table 3.6. Both groups were matched by age and BMI. The only significant difference between the two groups was that insulin AUC was greater in the PCOS group compared to controls ( $p = 0.03$ ).

**Table 3.6****Baseline characteristics of the subjects undergoing MSNA recordings.****Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>PCOS (n=7)</b>	<b>Controls (n=7)</b>	<b>P value</b>
Age (yrs)	29.57 $\pm$ 6.37	30.14 $\pm$ 6.23	0.87
BMI (Kg/m <sup>2</sup> )	27.29 $\pm$ 4.89	27.14 $\pm$ 6.20	0.96
WHR	0.92 $\pm$ 0.10	0.84 $\pm$ 0.04	0.08
Testosterone (nmol/L)	1.56 $\pm$ 0.82	1.20 $\pm$ 0.32	0.30
Insulin AUC (pmol min/L)	80599.29 $\pm$ 29766.85	46367.14 $\pm$ 23083.73	<b>0.03</b>
HOMA-IR	1.53 $\pm$ 0.92	1.03 $\pm$ 0.98	0.34
Matsuda	5.58 $\pm$ 4.46	11.71 $\pm$ 8.26	0.13
Resting HR (beats/min)	69.83 $\pm$ 9.70	68.67 $\pm$ 7.87	0.82
Resting MAP (mmHg)	83.00 $\pm$ 10.58	85.00 $\pm$ 8.27	0.72

The resting MSNA data for both groups are shown in table 3.7. Resting Burst frequency (BF) and Burst Incidence (BI) was not significantly different between the PCOS group and controls.

As anticipated, IFC induced an increase in MSNA activity in both groups, as shown in table 3.8. However, this only reached statistical significance with respect to BF and BI in the PCOS group. In the controls (n=7), the increase in BF or BI post-IFC was not statistically significant, but the total MSNA rise was (p=0.048).

**Table 3.7**

**Resting MSNA in PCOS and control groups, including burst frequency (BF), burst incidence (BI) and total MSNA activity. Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>PCOS (n=8)</b>	<b>Controls (n=8)</b>	<b>P value</b>
Burst frequency (BF) (bursts/min)	26.5 $\pm$ 4.4	31.6 $\pm$ 8.8	0.16
Burst incidence (BI) (bursts/100 heart beats)	37.0 $\pm$ 9.4	44.1 $\pm$ 11.3	0.19
Total MSNA activity	2.6 $\pm$ 1.3	2.6 $\pm$ 0.7	0.97

**Table 3.8**

**Mean changes in BF, BI and total MSNA following IFC in PCOS (n=7) and controls (n=7). Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

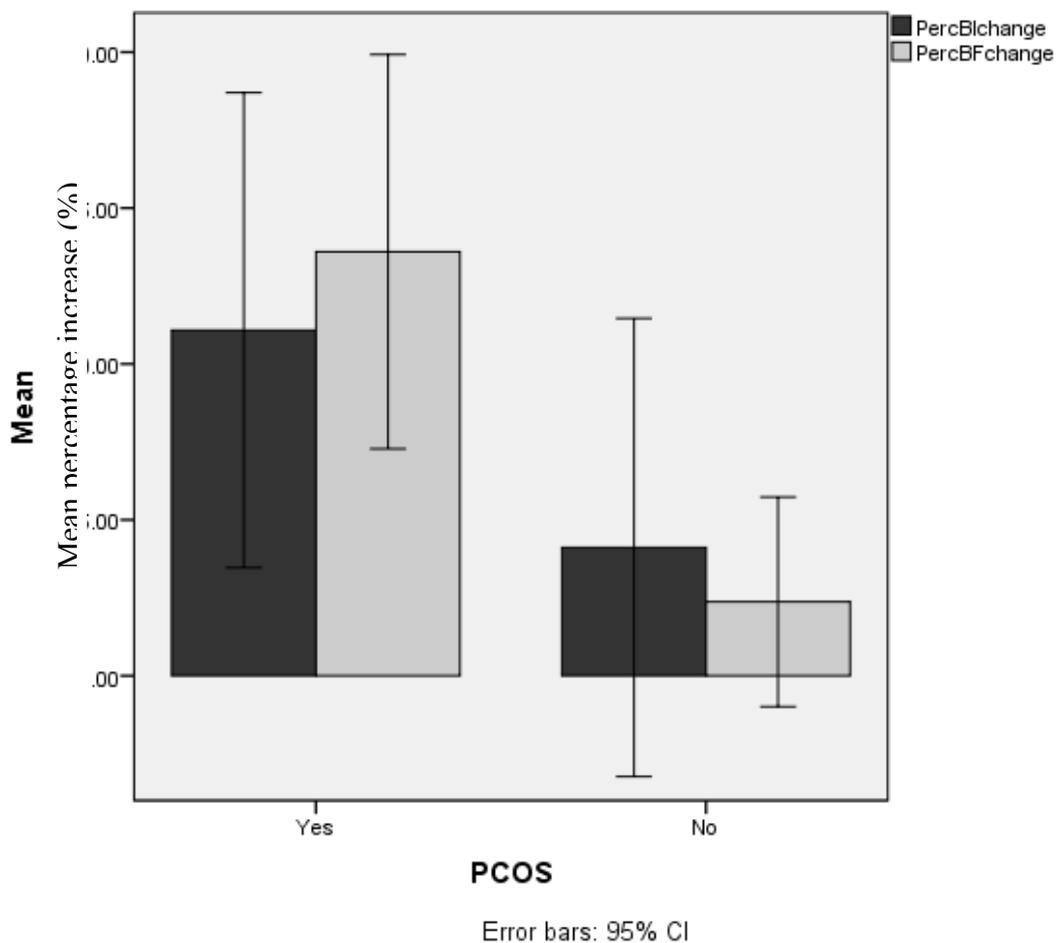
		<b>Pre-IFC</b>	<b>Post-IFC</b>	<b>Difference</b>	<b>P value</b>
<b>BF (bursts/min)</b>	<b>PCOS</b>	25.9 $\pm$ 4.4	42.9 $\pm$ 8.2	17.0 (10.0-24.0)	<b>0.001</b>
	<b>Control</b>	29.6 $\pm$ 7.1	34.9 $\pm$ 4.5	5.3 (2.5-13.1)	0.149
<b>BI (bursts/100 heartbeats)</b>	<b>PCOS</b>	36.3 $\pm$ 9.9	54.4 $\pm$ 12.1	18.1 (8.4-27.9)	<b>0.004</b>
	<b>Control</b>	42.0 $\pm$ 10.3	47.9 $\pm$ 7.1	5.9 (4.1-15.8)	0.199
<b>Total MSNA</b>	<b>PCOS</b>	2.4 $\pm$ 1.3	5.5 $\pm$ 3.1	3.1 (1.4-4.8)	<b>0.004</b>
	<b>Control</b>	2.6 $\pm$ 0.7	4.4 $\pm$ 1.7	1.8 (0.0-3.6)	<b>0.048</b>

The percentage increase in BF, BI and total MSNA from pre- to post-IFC was calculated for each group. Figure 3.6 shows the percentage increase in BF and BI for the PCOS and control groups.

Figure 3.6

Mean percentage increase in BI and BF for PCOS and control groups post-IFC.

Black bars represent the percentage BI change for each group post-IFC and light grey bars represent percentage BF change post-IFC. Error bars represent 95% confidence intervals.



BF increased by 68.0% in the PCOS group compared to 11.9% in controls ( $p=0.002$ ) and BI increased by 55.4% and 20.5% in PCOS and controls respectively ( $p=0.133$ ).

The total MSNA increase (PCOS: 124.1%, controls: 86.4%) was not statistically significantly different between groups ( $p=0.420$ ).

### **3.7 POTENTIAL MEDIATORS OF SNS ACTIVATION**

When exploring potential mediators of SNS activation, as differences were found between PCOS and controls with respect to post-IFC BI and BF, I did not consider it valid to combine all subject data for MSNA, BI and BF and have therefore undertaken subsequent analyses of these separately.

#### **3.7.1 BMI**

Simple correlation showed that for the PCOS group, BMI was associated with resting adrenaline levels and percentage rise in MAP. In the control group, resting MSNA burst incidence (BI) and resting burst frequency (BF) correlated positively with BMI (Table 3.9). There was no significant association between BMI and any other parameters associated with sympathetic activation, although I recognise that the sample size for microneurography measurement was small.

**Table 3.9**

**Correlation between BMI and measures of SNS activity**

<b>Parameter: BMI</b>	<b>Group</b>	<b>Correlation</b>	<b>R<sup>2</sup></b>	<b>P value</b>
Resting MAP (mmHg)	All	0.05	0.002	0.78
	PCOS	0.05	0.002	0.84
	Control	0.06	0.003	0.81
Percentage MAP rise	All	0.41	0.16	<b>0.01</b>
	PCOS	0.66	0.44	<b>0.002</b>
	Control	0.10	0.01	0.69
Resting adrenaline levels (ng/mL)	All	0.21	0.04	0.21
	PCOS	0.47	0.22	<b>0.04</b>
	Control	0.01	0.00	0.95
Percentage adrenaline rise	All	0.20	0.04	0.23
	PCOS	0.27	0.08	0.24
	Control	0.25	0.06	0.31
Resting BI (bursts/100 heartbeats)	PCOS	0.18	0.03	0.67
	Control	0.72	0.51	<b>0.05</b>
Resting BF (bursts/min)	PCOS	0.52	0.27	0.19
	Control	0.83	0.69	<b>0.01</b>
Resting total MSNA	PCOS	0.14	0.02	0.74
	Control	0.24	0.06	0.60
Percentage BI rise	PCOS	0.09	0.01	0.86
	Control	0.24	0.06	0.61
Percentage BF rise	PCOS	0.57	0.32	0.18
	Control	0.64	0.41	0.12
Percentage total MSNA rise	PCOS	0.58	0.33	0.18
	Control	0.39	0.15	0.39

**3.7.2 Waist circumference**

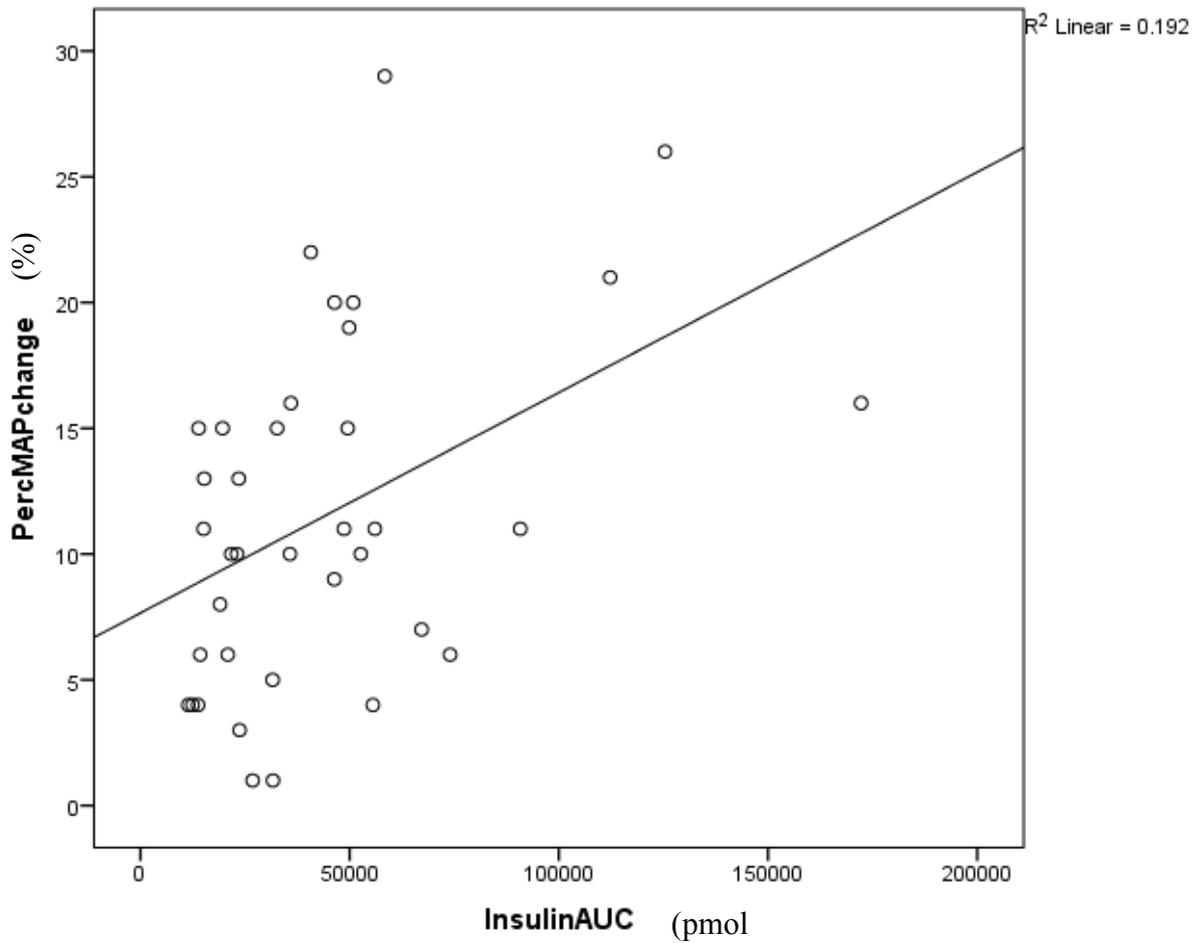
For the PCOS group, WHR correlated significantly with percentage rise in MAP,  $r = 0.46$ ,  $p = 0.045$  and with percentage rise in plasma adrenaline,  $r = 0.79$ ,  $p < 0.001$ .

Similarly, for the control group, WHR correlated with rise in MAP,  $r = 0.53$ ,  $p=0.03$ , and with adrenaline rise,  $r = 0.55$ ,  $p = 0.02$ . For all subjects analysed together, Pearson's correlation between WHR and percentage MAP rise was  $0.51$ ,  $p=0.002$ , and  $0.7$  ( $p<0.001$ ) between WHR and percentage rise in plasma adrenaline. There was no significant correlation between WHR and any MSNA measurement of sympathetic activity for the PCOS group or control group.

### **3.7.3 Insulin resistance**

Although the PCOS MSNA group was more insulin resistant, there were no correlations found between MSNA and HOMA-IR. For the control group, there was a positive correlation between resting MSNA BF and insulin AUC,  $0.804$ ,  $p=0.02$ , but no relationship between MSNA and insulin AUC in the PCOS group. Overall, for all subjects, there was no association between HOMA-IR and blood pressure measurements. However, by group, for the control group, there was a negative correlation between HOMA-IR and resting MAP,  $r = -0.58$ ,  $p=0.01$ , and a strongly positive association ( $r = 0.69$ ,  $p = 0.001$ ) between HOMA-IR and percentage change in MAP. This was not present for the PCOS group. There was an overall positive correlation between insulin AUC and percentage rise in MAP for all subjects ( $r = 0.44$ ,

p=0.01) (Figure 3.7) but this was not present when analysed by group. There were no significant associations between insulin sensitivity and any catecholamine measure.



**Figure 3.7**

**Correlation between insulin AUC (pmol min/L) and percentage rise in MAP for**

**all subjects, p = 0.01**

### **3.7.4 Testosterone**

For the PCOS group, there was no significant correlation between serum testosterone and any MSNA measures. For the controls, there was a strongly positive correlation between testosterone and percentage rise after IFC in burst incidence (BI),  $r = 0.92$ ,  $p=0.004$  and testosterone and total MSNA rise,  $r = 0.81$ ,  $p=0.03$ . There was also a negative correlation in the control group between testosterone and resting MSNA BI ( $r = -0.70$ ,  $p=0.05$ ) and resting MSNA BF ( $r = -0.73$ ,  $p = 0.04$ ).

### **3.7.5 Obstructive sleep apnoea**

For all subjects, a positive correlation was found between the apnoea-hypopnoea index (AHI) and resting MSNA BF,  $r = 0.66$ ,  $p = 0.05$ . There were no significant associations between MSNA and AHI in each of the PCOS and control groups. For the PCOS group, there was a positive correlation between percentage rise in MAP and AHI,  $0.94$ ,  $p=0.02$ , but this was not present for the control group.

### 3.8 INFLUENCE OF PCOS PHENOTYPE

Amongst the PCOS group, although numbers were small (n=20), analysis was performed to see if there was a difference between insulin sensitivity and SNS response to IFC between the 5 hyperandrogenic (H) patients (serum testosterone >1.7nmol/L) and the non-hyperandrogenic (non-H) patients. As only two patients were both hyperandrogenic and overweight-obese, given the very small number, it wasn't thought statistically valid to compare these with the 18 non-hyperandrogenic normal BMI patients.

In the H patients, Matsuda Index was  $16.81 \pm 2.70$  compared to  $7.53 \pm 5.94$  in non-H (p=0.004), insulin AUC  $34623.00 \pm 18344.31$  pmol min/L in H group, compared to  $62485.00 \pm 45151.20$  pmol min/L in the non-H (p=0.07) and HOMA-IR  $0.74 \pm 0.80$  in H group, compared to  $1.63 \pm 1.12$  in the non-H group (p=0.08).

Resting heart rate was  $70.40 \pm 8.20$  beats /min in the H-group, compared to  $71.29 \pm 9.00$  beats /min in the non-H group (p=0.85). Percentage change in heart rate following IFC was  $5.80 \pm 7.98$  % in the H-group and  $9.50 \pm 8.64$  % in the non-H group (p=0.41).

Resting MAP was  $80.60 \pm 13.48$  mmHg in the H-group and  $82.00 \pm 10.91$  mmHg in the non-H group (p=0.84). The percentage change in MAP following IFC was  $11.60 \pm$

4.56 % in the H-group and  $14.14 \pm 7.04$  % in the non-H group ( $p=0.38$ ). The percentage change in noradrenaline post-IFC was  $-20.00 \pm 33.13$  % in the H-group and  $22.53 \pm 98.31$  % in the non-H group ( $p=0.17$ ), and the percentage change in adrenaline post-IFC was  $93.40 \pm 50.41$  % in the H-group and  $3359.73 \pm 11533.75$  % in the non-H group ( $p=0.29$ ).

For the PCOS subjects undergoing full MSNA recordings, 2/7 were biochemically hyperandrogenic (H) and 5 were non-hyperandrogenic (non-H). There was no statistical difference in any of the resting MSNA recordings or post-IFC recordings between the H and non-H groups.

## **3.9 DISCUSSION**

### **3.9.1 Clinical and cardiometabolic findings**

#### **3.9.1.1 Anthropometric findings**

In this study, 20 PCOS subjects were matched by both age and BMI with 20 healthy volunteers although the waist-hip ratio (WHR) was greater in the PCOS group compared to controls. This supports some previous studies which have explored body

fat percentage and distribution in women with PCOS, showing that compared to BMI-matched controls, PCOS women show significantly greater visceral adipose tissue [Cosar E 2008, Karabulut 2012, Godoy-Matos 2009, Arpaci D 2015], although data is not consistent [Barber 2008]. My study would suggest that, at least by WHR measurements, visceral adiposity is greater in PCOS subjects compared to controls, even when matched by BMI.

### **3.9.1.2 Hyperandrogenism**

Total testosterone and, to a lesser extent, androstenedione levels were greater in the PCOS group compared to controls but these levels did not reach statistical significance. Clinical signs of hyperandrogenism were noted for each patient but the use of validated assessment tools, such as the Ferriman-Gallwey scale to evaluate hirsutism, were not employed. This highlights the heterogeneity of PCOS and the wide range of phenotypes as encompassed by current diagnostic criteria. In my study I used the Rotterdam criteria to diagnose PCOS, and according to these criteria, androgen excess may be defined by clinical symptoms of hyperandrogenism and /or elevated testosterone/androstenedione levels [Rotterdam Group 2004]. This could explain why the testosterone and androstenedione levels were not significantly higher in my patient group. In addition, a lack of difference in testosterone levels may have been due to

inadequate statistical power since my sample size calculations were powered for the primary outcome measure (BOLD fMRI signal change in the brainstem).

### **3.9.1.3 Dyslipidaemia**

The findings from my study cohort are in keeping with previous reports, in that the PCOS group had higher serum triglyceride levels compared with controls [Ollila 2015]. The findings from my small group of patients with PCOS concur with this lipid pattern which may be an effect of central adiposity rather than the syndrome *per se*.

### **3.9.1.4 Insulin sensitivity**

In the PCOS group markers of insulin resistance (fasting insulin, insulin AUC, HOMA-IR and Matsuda index) tended to be more than controls, although none of these were statistically significant. Again, this may be a reflection of how PCOS was defined, whereby the Rotterdam criteria (which embrace a less severe metabolic phenotype) were used to establish the diagnosis. A recent cross-sectional multicentre study of over 2000 women with PCOS showed that those with hyperandrogenic PCOS (53% of total) presented with a worse cardiometabolic profile and a higher prevalence of cardiovascular risk factors, such as obesity and overweight, insulin resistance, and

metabolic syndrome, compared with women with non-hyperandrogenic PCOS. [Daan 2014]. In my study, the PCOS patients recruited had relatively mild biochemical hyperandrogenism which may have been one reason why the measures of insulin sensitivity were not significantly different between groups.

Another important reason why no significant difference in insulin sensitivity was noted between groups may be related to the way insulin resistance was measured. Fasting glucose, 2-hour glucose, HOMA-IR, insulin AUC and glucose AUC calculated and these parameters measure whole body insulin sensitivity rather than specifically peripheral insulin sensitivity. The insulin resistance in PCOS has been shown to be more of a defect in insulin signal transduction in peripheral muscular tissue [Courbold 2005] which is more accurately detected using the euglycaemic hyperinsulinaemic clamp technique.

I also noted a strong correlation between WHR and insulin resistance (as defined by insulin AUC) across all subjects, which would suggest that greater visceral adiposity is associated with increased insulin resistance. Similarly, in PCOS, it has been shown that central obesity is associated with higher fasting insulin levels [Lim 2013]. Indeed, the risk of type 2 diabetes steadily increases with BMI and is particularly high for BMI over 30kg/m<sup>2</sup> [Moran 2010, Morgan 2012, Pasquali 2013].

### 3.9.1.5 Arterial Stiffness

Using the Vicorder device, augmentation pressure, augmentation index and aortic pulse wave velocity were not different between the PCOS group and controls. Augmentation Index (AI) and aortic pulse wave velocity (aPWV) are both measures of arterial stiffness and previous studies in young women with PCOS have been discordant. A number of studies have supported increased arterial stiffness in PCOS [Meyer 2005, Soares 2008, Trakakis 2008] while others have shown no difference to controls [Muneyyirci-Delale O 2007, Ketel 2010, Rees E 2014]. Our group have recently shown that central obesity and insulin resistance are independently associated with arterial stiffness, but not PCOS *per se* [Rees 2014].

It is noteworthy that arterial stiffness in this study was similar in both groups at baseline, although it should be remembered that the study was not powered to reveal differences in vascular function and this was not a major outcome measure for the study population.

### **3.9.1.6 Obstructive sleep apnoea**

The prevalence of obstructive sleep apnoea (OSA) may be increased in PCOS compared to controls, and associated with insulin resistance, glucose intolerance and increased sympathetic activation [Fogel 2001, Tasali 2008, Grassi 2005b]. The sleep studies in the 19 subjects showed no difference in apnoea-hypopnoea index (AHI) between groups and no subject had evidence of sleep apnoea (as defined by  $\geq 5$  events /hr). However, these studies were only in a subgroup of the total study population, with small numbers especially in the PCOS group so it is therefore difficult to make any meaningful interpretations. It does confirm, however, that there were no subjects of the 19 who had sleep apnoea, which may have been a confounding factor when measuring sympathetic activation.

It is interesting that there was an association with increasing WHR and AHI across this subgroup. This is as would be expected, with previous studies showing that WHR is the most reliable correlate of OSA in both sexes [Sharma 2004, Lim 2014]. Unfortunately neck circumference was not measured in my study, which correlates well with the risk of sleep apnoea, and this may have been a simple alternative to employing formal sleep study techniques [Kapur VK 2010].

### **3.9.2 SNS Activation in PCOS**

#### **3.9.2.1 Haemodynamic response to IFC**

Baseline blood pressure and heart rate was measured for both PCOS (n=19) and controls (n=19). No difference was found between the groups, and all participants were confirmed to be normotensive. This was expected as any subject with a personal history of hypertension was excluded from the study, with only normotensive participants enrolled.

Following 30% IFC sustained for 3 minutes, a significant increase in HR and MAP was seen for all participants ( $p < 0.001$ ). The percentage increase in HR, SBP, DBP and MAP was greater in the PCOS group compared to controls but this did not reach statistical significance.

These results contrast with Saranya *et al*, who found that the baseline heart rate, diastolic and systolic blood pressures of the PCOS cases were significantly higher than controls, and that there was a significantly greater rise in diastolic blood pressure following IFC in the PCOS group. However, in their study the two groups were not matched by BMI and the PCOS group had a significantly higher BMI compared to

controls. Fasting plasma glucose and testosterone levels were also significantly higher in the PCOS group and might also explain why differences in cardiovascular parameters were seen in their study and not in mine [Saranya 2014].

In addition blood pressure rise is a crude end-point measurement of physiological pathways and does not directly measure sympathoexcitation.

### **3.9.2.2 Catecholamines**

Plasma catecholamines (adrenaline and noradrenaline) were measured at baseline in both groups and no difference was found. Following IFC there was a significant rise in plasma adrenaline concentrations for both groups, but no change in noradrenaline concentrations. The plasma adrenaline level rise following IFC was significant in the PCOS group ( $p < 0.001$ ) but not in the control group ( $p = 0.14$ ). When expressed as a percentage increase in adrenaline levels from baseline to post-IFC, there was no difference between the groups.

There are a number of points that merit consideration here. First, the plasma catecholamine assay is not considered a 'gold-standard' measure of sympathetic activation, hence results should be interpreted with caution. Plasma noradrenaline

concentrations offer limited sensitivity, are not reproducible and are unable to distinguish between increased central catecholamine production and decreased clearance [Grassi 1999]. Also the noradrenaline concentrations in our study were derived from antecubital fossa sampling, reflecting venous drainage from the forearm alone.

Second, it is interesting that there was a significant rise in plasma adrenaline concentrations for the whole group following IFC but not in noradrenaline. A rise in plasma noradrenaline levels, rather than adrenaline levels, may therefore have been expected, but this was not seen. It is difficult to explain why this was so, although one reason may be the ELISA kits used for analysis which were initially stored under suboptimal conditions, which may have affected the accuracy of the results obtained.

Our results do concur, however, with Garcia-Rudaz *et al*, who found that in the resting state, plasma adrenaline and noradrenaline levels were similar in patients with PCOS and controls [Garcia-Rudaz 1998].

### 3.9.2.3 MSNA

#### Resting MSNA

MSNA was recorded in a subgroup of 14 subjects (7 PCOS and 7 controls); baseline burst frequency (BF) and burst incidence (BI) were not different between the two groups. Only two other studies have compared MSNA at rest in PCOS versus controls and both have demonstrated elevated MSNA compared to controls [Sverisdottir 2008, Lambert 2015]. Sverrisdóttir *et al* showed that resting BF and BI was significantly higher in PCOS compared to controls [Sverisdottir 2008] and Lambert *et al* found a significantly higher resting BI in PCOS versus controls [Lambert 2015]. In my study, resting BF was 26.5 bursts/min in PCOS and 31.6 bursts/min in controls ( $p=0.16$ ), and BI 37.0 bursts /100 heartbeats in PCOS and 44.1 bursts /100 heartbeats in controls ( $p=0.19$ ). It is noteworthy that the resting MSNA BF and BI in our study were higher in the control group than in these two previous studies.

There may be several reasons for these observations. First, it may relate to the case and volunteer selection. In Sverrisdóttir's study PCOS women were defined by the Rotterdam criteria, as with our study, but had significant evidence of hyperandrogenism and insulin resistance compared to their control group. In

Lambert's study, the PCOS population was similarly recruited according to the Rotterdam criteria and showed evidence of significant biochemical hyperandrogenism compared to controls, although there was no difference in measures of insulin sensitivity between groups. In my study population, the PCOS group were defined by the Rotterdam criteria but were not strongly hyperandrogenic, although the subgroup that underwent MSNA were significantly more insulin resistant than age and BMI-matched controls.

In contrast to the other two studies mentioned, my observations were made only in a small subgroup, compared with much larger numbers in their studies. It is therefore potentially difficult to draw any firm conclusions from these data. Furthermore, there are a number of other factors that affect resting MSNA in individuals which may have confounded results, including age, gender and BMI [Ng 1993, Scherrer 1994]. In addition, timing of the menstrual cycle is important as has been observed that SNS activity is highest during the mid-luteal phase of the ovulatory cycle, during which oestrogen and progesterone levels are high, and SNS activity falls during the early follicular phase at which time oestrogen and progesterone levels have fallen [Minson 2000]. This was not taken into account when recordings were made.

## Post-exercise MSNA

Our subgroup underwent sustained IFC at 30% for 3 minutes and it was observed that BF and BI increased significantly in the PCOS group but not in controls. Comparing groups, there was a greater percentage increase in BF in the PCOS group of 68.0% from baseline as compared to an 11.9% increase in controls ( $p=0.002$ ). The percentage increase in BI in PCOS group was 55.4% compared to 20.5% in controls but this was not significantly different.

These findings suggest that there was an augmented sympathetic response to static exercise in PCOS compared to controls. To my knowledge, mine is the first study to observe the effect of static exercise on MSNA response in subjects with PCOS.

Previous studies have sought to examine the autonomic neural control of the cardiovascular system in response to exercise in PCOS [Teikin 2008]. Giallauria *et al* compared heart rate recovery [HRR] after a cardiopulmonary exercise test in 75 young overweight women with PCOS with 75 age and BMI-matched healthy volunteers [Giallauria 2008]. Women with PCOS showed reduced HRR compared to healthy controls, which correlated inversely with BMI and insulin sensitivity but not with testosterone.

More specifically, IFC has been used in women with PCOS as a static SNS exercise stimulus but never with the MSNA response measured. In the study by Sarayna *et al* findings similarly suggested an exaggerated sympathetic response in patients with PCOS in response to IFC [Saranya 2014]. Other studies have also used IFC as an SNS stimulus and recorded the MSNA response. From those studies, an augmented blood pressure and MSNA response has also been seen in subjects with type 2 diabetes [Petrofsky 2005, Holwerda 2016] and hypertension [Sausen 2009, Delaney 2010] but not in the metabolic syndrome [Limberg 2014] or obesity [Negrao 2001].

It is interesting that there was a significant rise in BF in response to IFC but not in BI in the PCOS as compared to controls in my study. This may reflect an increase in heart rate in the PCOS group that was greater than the controls, meaning the rise in bursts per 100 heartbeats ('burst incidence') was not as significant in the PCOS group compared to controls.

Unlike some other studies using IFC, we did not have a post-exercise ischaemia (PEI) aspect to our IFC experiment, so it is not possible to infer any mechanisms underpinning the increased MSNA seen in our PCOS group. There is no way of delineating if the mechanoreflex or metaboreflex (or indeed both) were increased in

the PCOS group compared to controls, only to conclude that there was a difference seen post-static exercise.

#### **3.9.2.4 Potential mediators**

Having observed that there was a significant increase in MSNA burst frequency in the PCOS group compared to controls ( $p=0.002$ ), it is of interest to consider any potential mediators contributing to this difference.

In the PCOS group, there was no correlation between the increase in burst frequency and either BMI, WHR, insulin sensitivity measures, testosterone levels or AHI levels. There are only two other published MSNA studies of PCOS subjects versus controls to-date, as I have discussed earlier, neither of which used a SNS-stimulus task but both measured resting activity alone. Sverrisdóttir's study showed a positive correlation of MSNA with total and free testosterone and suggested that the degree of sympathoexcitation relates to the degree of biochemical hyperandrogenism [Sverrisdottir 2008]. In contrast, Lambert's study described an absence of a relationship between MSNA and testosterone, not supporting the hypothesis that hyperandrogenism is related to the elevated sympathetic tone. However, there was a strong relationship between MSNA and insulin concentration as well as with HOMA-

IR, which remained after controlling for the presence of PCOS, which suggested that the degree of sympathetic activation may not be explained by obesity or insulin resistance alone [Lambert 2015]. In my study, the sample size was much smaller and, as discussed, there was no baseline difference between the two groups in terms of testosterone levels but there was a difference in terms of insulin AUC in the MSNA subgroups, although this did not correlate with BF.

When considering the other measures of SNS activation that showed no difference between groups (catecholamines, HR and BP changes), there was a correlation noted between percentage MAP rise and BMI ( $p=0.012$ ) and WHR ( $p=0.002$ ) across all subjects. This contrasts with Negrão's study, which compared the MSNA response to IFC in 40 young normotensive obese women and 15 age-matched normotensive lean women. They found that the MSNA response was blunted in the obese women [Negrao 2001]. Although it is known that increased adiposity correlates with sympathetic overactivity at rest [Alvarez 2002, Grassi 2004, Sivenius 2003], my data suggest that during IFC there is also a strong correlation between the MAP pressor response and the degree of obesity.

Furthermore, when considering all subjects, there was a positive correlation between insulin AUC and percentage rise in MAP ( $r=0.439$ ,  $p=0.007$ ) which agrees with the

work of Holwerda et al. Although they studied patients with established type 2 diabetes and IFC, they noted an augmented pressor response that significantly correlated with fasting glucose, HbA1c and HOMA-IR [Holwerda 2016].

Finally there was a positive correlation for all subjects between the apnoea-hypopnoea index (AHI) and resting MSNA BF,  $r = 0.66$ ,  $p = 0.05$ . Again this finding stands with previous evidence, that OSA may be associated with increased MSNA, independently of body weight [Grassi 2005b]

To summarise, for the difference seen in BF increase post-exercise, no potential mediators were identified to explain this difference. For rise in MAP, there was a positive correlation with BMI, WHR and insulin AUC for all subjects, suggesting they may play a role in the pressor response. Finally, for all subjects, the established correlation between resting MSNA and AHI was noted in my study, strengthening the link between OSA and SNS activation.

### **3.9.3 Influence of PCOS phenotype**

Although evidence has suggested that the classic hyperandrogenic PCOS phenotype has an increased risk of cardiometabolic dysfunction compared to the non-

hyperandrogenic group [Daan 2014, Moran 2015, Fauser BC 2012, Ehrmann D 2005] the current study did not find this. The only significant difference was the Matsuda index was actually significantly higher in the hyperandrogenic group compared with the non-hyperandrogenic group. There were no differences in the haemodynamic or sympathetic changes between these groups. It is difficult to draw any meaningful conclusions from these findings as the study was not powered to look at such differences and only 5 out of 20 subjects had biochemical evidence of hyperandrogenism. This, in turn, relates back to the weakness in how PCOS was defined in the study and the lack of classic phenotypic PCOS patients recruited.

#### **3.9.4 Strengths and limitations**

Strengths of the current study were that both groups were well-matched for age and BMI. Also a wide range of measures used to assess sympathetic tone and activation in the study population. Although no 'gold-standard' is available, a combination of haemodynamic, catecholamine and microneurography techniques were used to capture SNS activation. This study was also the first to study the MSNA response to IFC in women with PCOS.

Several limitations have been highlighted. The current cohort recruited for the PCOS group was based upon the Rotterdam criteria. As has been discussed, this encompasses a broader phenotype, including a less androgenic and less insulin resistant group of patients, as seen in our study population. Therefore, the NIH 1990 or AE-PCOS 2009 criteria for diagnosing PCOS could have been used to better define a more hyperandrogenic, insulin-resistant group of patients for inclusion in the study. In addition, the way body composition and visceral adiposity were measured using WHR and BMI alone was a potential weakness. The value of WHR as a reflection of visceral adiposity has been debated, with some evidence suggesting it is inaccurate [Busetto 1992, Joy 2009]. More objective measures, such as single-slice CT abdomen, MR abdomen, or dual energy x-ray absorptiometry (DEXA) may therefore have potentially been more accurate ways to record visceral adiposity [Kvist 1988, Kullberg 2007, Yoon DY 2008, Kaul 2012]. Alternatively, Bioelectrical impedance analysis (BIA) could have been used as a simple tool to assess body composition [Lee SY 2008]. Another limitation was the way insulin sensitivity was assessed in the study population. We assessed total body insulin sensitivity using a standard 75-g OGTT but the 'gold-standard' test to more accurately assess peripheral insulin resistance, as seen in PCOS, would have been using a euglycaemic hyperinsulinaemic clamp technique to calculate the ISI.

Furthermore, there were confounders, including cigarette smoking, alcohol consumption and menstrual cycle phase not accounted for when measuring sympathetic activity. Also, although the total sample size was reasonable, the group that underwent microneurography was small (n=14) and therefore the findings from this subgroup, although of interest, may lack power. Furthermore, circulating catecholamines were measured but these offer limited sensitivity. In addition the haemodynamic changes were measured using an automatic, non-invasive, oscillatory sphygmomanometer and a device to measure continuous heart rate and blood pressure changes throughout the paradigm could have been used to more accurately monitor the haemodynamic changes.

### **3.10 CONCLUSION**

In conclusion, my study has shown that women with PCOS had increased central adiposity and hypertriglyceridaemia compared to age- and BMI-matched controls. However, I was unable to show a statistically significant increase in insulin resistance or biochemical hyperandrogenism. Vascular function and AHI were also similar in both groups, and no subjects (of those screened) had evidence of OSA.

When studying SNS responses, there was no significant difference in resting or post-excitatory haemodynamic or catecholamine responses in PCOS subjects compared to the control group (with the exception of a post-IFC rise in adrenaline). In the subgroups that underwent microneurography, there was no difference in resting sympathetic tone between groups. However, after IFC there was a significantly greater increase in burst frequency in the PCOS group compared to controls. Of note, the PCOS subgroup that was studied was more insulin resistant than the controls, with a higher insulin AUC ( $p = 0.03$ ).

# **CHAPTER 4**

## **IDENTIFICATION OF HIGHER BRAIN CENTRES ASSOCIATED WITH SNS ACTIVATION**

The higher regional brain centres involved in sympathoexcitation in PCOS are unknown. Imaging studies in humans without PCOS have highlighted higher cortical centres associated with sympathetic activation and blood pressure control, including the dominant anterior cingulate, postcentral gyrus, cerebellum, insular cortex and orbitofrontal cortex [Critchley *et al* 2000, Kramer 2014]. In addition, fMRI work by Coulson *et al* has identified BOLD signal change in the ventrolateral medulla associated with blood pressure rise induced by IFC [Coulson JM 2015].

The main aim of this chapter is to report any BOLD fMRI signal activity in the brainstem and higher brain structures associated with SNS activation in women with PCOS compared with age- and BMI-matched controls.

First the BOLD signal change associated with blood pressure variation will be presented, followed by the changes associated with both IFC motor activation and the visual stimulus. The influence of other factors (testosterone, insulin sensitivity and BMI) will then be reported, followed by a final discussion of the findings.

## **4.1 BASELINE ANTHROPOMETRIC AND METABOLIC CHARACTERISTICS**

40 subjects (20 PCOS, 20 controls) underwent fMRI scanning according to the protocol as outlined in chapter 2, section 2.8.5. All subjects completed the 12-minute block paradigm as shown in Figure 2.3, chapter 2. The analysis for the motor activation (section 4.3) and visual activation (section 4.4) was based upon 40 fMRI scans and the group differences between 20 PCOS fMRI scans and 20 control fMRI scans.

The baseline anthropometric and metabolic characteristics of the 40 subjects recruited (20 PCOS and 20 healthy volunteers) are outlined and described in chapter 3, section 3.1.

## **4.2 HAEMODYNAMIC RESPONSE**

### **4.2.1 Subgroup baseline characteristics**

In a subgroup of 30 subjects (15 PCOS and 15 controls), the 12-minute IFC paradigm (Figure 2.3) was performed out-of-scanner with BP and HR recordings made every 30 seconds. In the remaining 10 subjects, either due to equipment failure, time constraints or subject intolerance, these data were not collected.

The baseline characteristics of the subgroup of 15 PCOS and 15 controls are shown in table 4.

**Table 4.1****Baseline characteristics of the haemodynamic response study population.****Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

	<b>PCOS (n=15)</b>	<b>Control(n=15)</b>	<b>P value</b>
Age (yrs)	30.7 $\pm$ 4.9	29.1 $\pm$ 4.8	0.37
BMI (Kg/m <sup>2</sup> )	24.7 $\pm$ 3.8	25.7 $\pm$ 4.4	0.98
WHR	0.86 $\pm$ 0.05	0.85 $\pm$ 0.04	0.35
Testosterone (nmol/L)	1.5 $\pm$ 0.9	1.1 $\pm$ 0.5	0.11
Androstenedione (nmol/L)	4.8 $\pm$ 3.3	3.7 $\pm$ 1.4	0.24
HOMA-IR	1.2 $\pm$ 0.8	0.9 $\pm$ 0.4	0.21
Matsuda index	9.8 $\pm$ 6.0	11.9 $\pm$ 6.2	0.39
Insulin AUC (pmol min/L)	47627.00 $\pm$ 31127.28	32948.57 $\pm$ 19214.23	0.14
Glucose AUC (mmol min/L)	743.90 $\pm$ 250.44	689.89 $\pm$ 243.27	0.56
Resting HR (beats/min)	71.9 $\pm$ 9.7	71.4 $\pm$ 7.7	0.89
Resting SBP (mmHg)	113.4 $\pm$ 8.9	116.9 $\pm$ 13.7	0.43
Resting DBP (mmHg)	62.6 $\pm$ 13.6	63.7 $\pm$ 14.7	0.85
Resting MAP (mmHg)	79.6 $\pm$ 11.1	82.7 $\pm$ 10.7	0.45

#### 4.2.2 Subgroup pressor and catecholamine responses

The HR, BP and plasma catecholamine responses to IFC for the subset of 15 PCOS and 15 controls are shown in table 4.2. There was no statistically significant difference in the HR, MAP or plasma catecholamine change pre- and post-IFC between the PCOS and control groups.

In the PCOS group, 5/15 had biochemical evidence of hyperandrogenism but there were no differences in their HR, BP or plasma catecholamine responses to IFC.

**Table 4.2**

**Changes in HR, MAP and plasma catecholamines pre- and post-IFC for the PCOS and control groups. Results are expressed as mean  $\pm$  SD. P<0.05 is considered statistically significant**

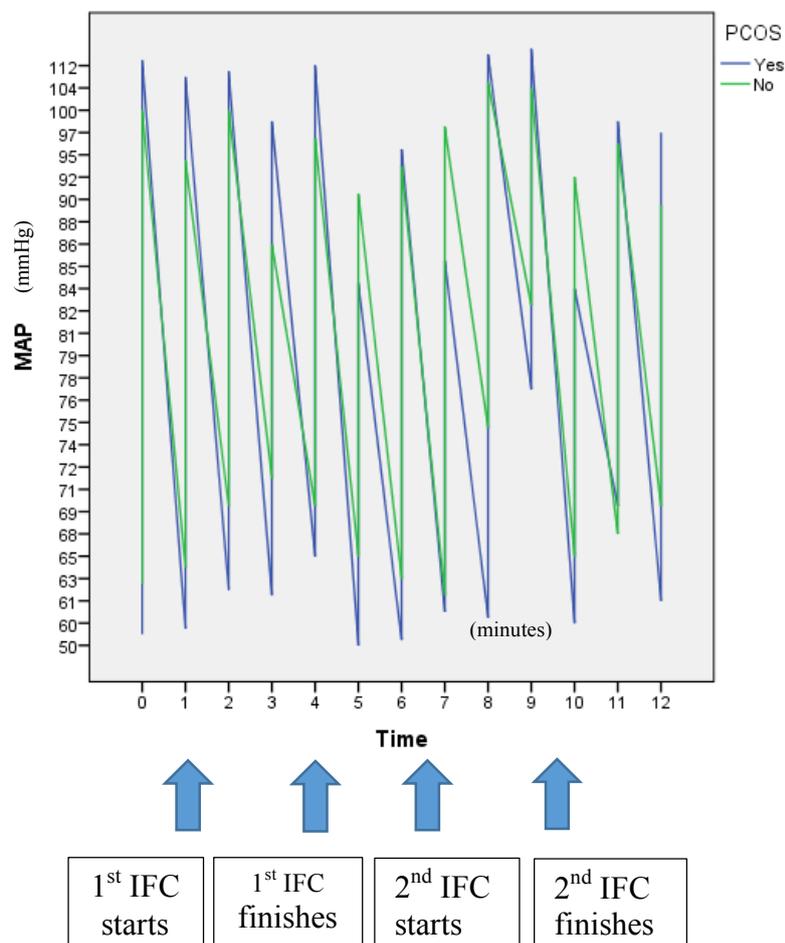
Parameter	Group	Pre-IFC	Post-IFC	P value
HR (beats/min)	PCOS	71.9 $\pm$ 9.7	76.9 $\pm$ 9.0	<0.05
	Control	71.4 $\pm$ 7.7	75.3 $\pm$ 7.5	<0.05
MAP (mmHg)	PCOS	79.6 $\pm$ 11.1	89.3 $\pm$ 13.1	<0.05
	Control	82.7 $\pm$ 10.7	90.8 $\pm$ 9.8	<0.05
Adrenaline (ng/mL)	PCOS	0.8 $\pm$ 0.5	1.4 $\pm$ 0.7	0.001
	Control	0.6 $\pm$ 0.6	0.9 $\pm$ 0.5	0.295
Noradrenaline (ng/mL)	PCOS	21.2 $\pm$ 10.4	19.1 $\pm$ 9.6	0.29
	Control	23.3 $\pm$ 14.1	20.9 $\pm$ 12.7	0.235

### 4.2.3 Blood Pressure Model

Using the blood pressure recordings from every 30 seconds during the paradigm, haemodynamic BP response curves were produced in Matlab (MathWorks, Natick, USA) for the 12-minute period based upon the mean arterial pressure (MAP). An example is shown in Figure 4.1. The text file for each individual subject was used as a blood pressure regressor in their individual Feat analyses using a Double-Gamma HRF convolution.

Figure 4.1

#### Mean blood pressure plots for PCOS and controls during 12-minute paradigm



#### 4.2.4 BOLD response

The change in BOLD signal intensity that fitted the modelled blood pressure responses showed activation in the PCOS group in the right cerebral cortex, right pallidum, right thalamus and right parietal operculum cortex ( $p < 0.0001$ ) and control group in the intracalcarine cortex and lingual gyrus ( $p = 0.003$ ). BOLD signal activation was greater in the PCOS group versus controls in the right orbitofrontal cortex ( $p < 0.0001$ ), left angular gyrus and lateral occipital cortex ( $p = 0.04$ ) (Figure 4.2(a) and (b)). The MNI co-ordinates of the peak responses for each of these are shown in table 4.3.

**Table 4.3**

**MNI co-ordinates of the peak responses for the BOLD activation greater in the PCOS group versus controls**

<b>Cluster region</b>	<b>X (mm)</b>	<b>Y (mm)</b>	<b>Z (mm)</b>	<b>p-value</b>
Right orbitofrontal cortex	16	8	-24	<0.0001
Left angular gyrus /Lateral occipital cortex	-48	-60	20	0.04

Figure 4.2 (a)

BOLD signal activation (modelled for blood pressure) greater in PCOS (n=15) versus controls (n=15) in the right orbitofrontal cortex. The significant region is displayed with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$

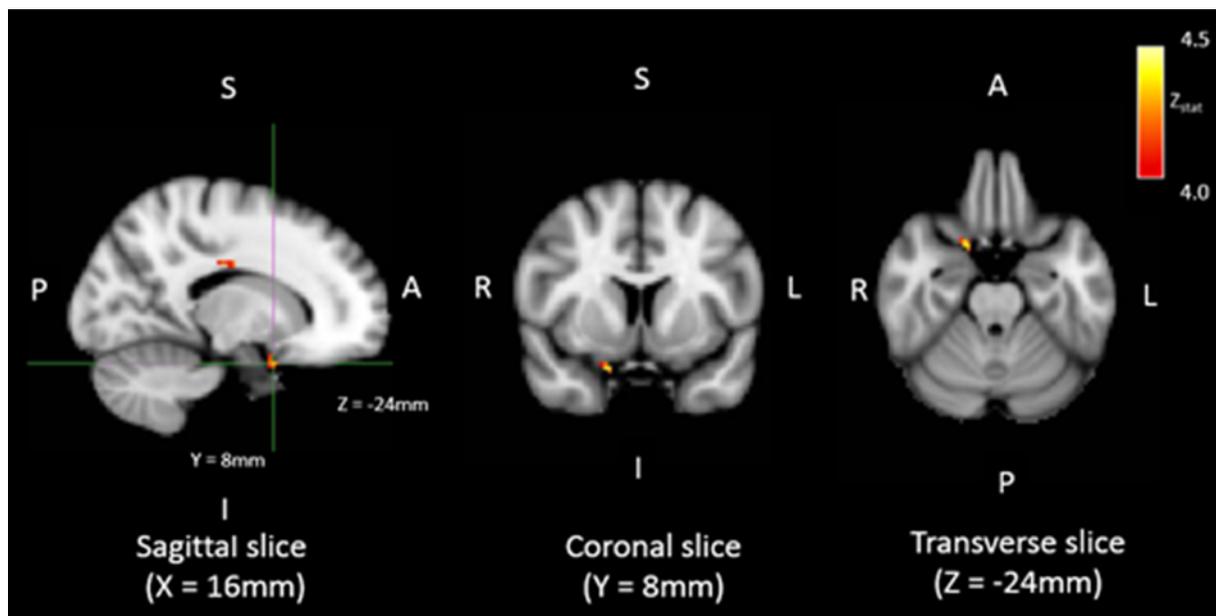
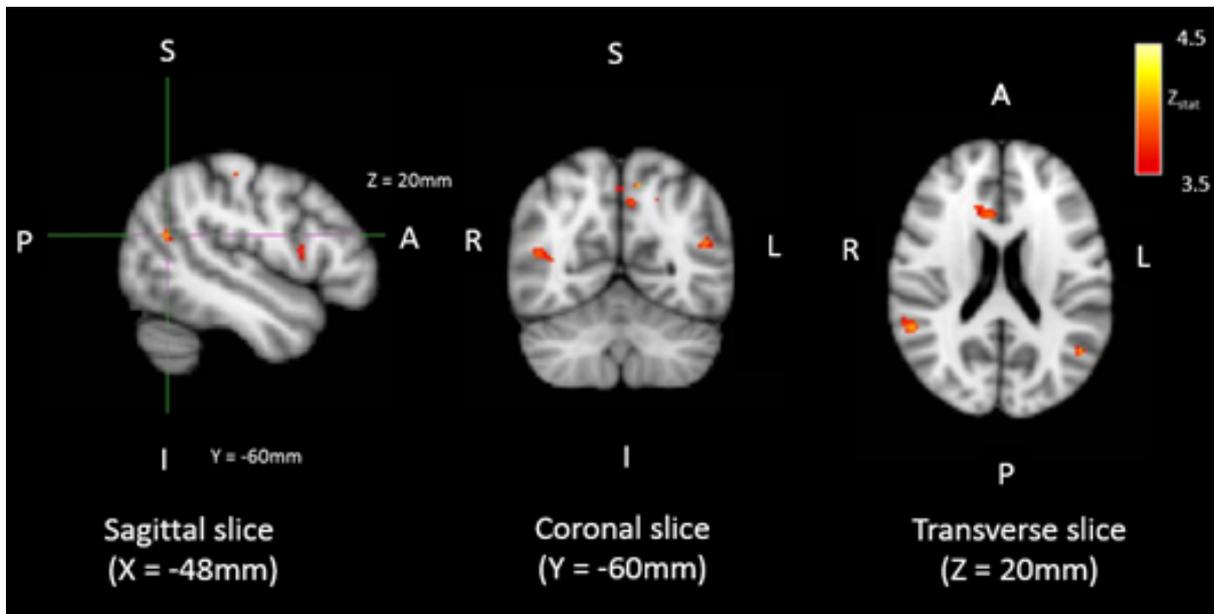


Figure 4.2 (b)

BOLD signal activation (modelled for blood pressure) greater in PCOS (n=15) versus controls (n=15) in left angular gyrus and lateral occipital cortex. The significant region is displayed with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$



### 4.3 MOTOR ACTIVATION

The scan was undertaken as outlined in chapter 2, section 2.8.5. An in-house pneumatic handgrip device was positioned in the dominant hand and connected to a pressure transducer. Subjects followed visual instructions presented on the screen as to the rest and squeeze periods, with a target bar showing when 30% IFC squeeze had been achieved. Subjects performed the block paradigm (chapter 2, Figure 2.3) on two occasions with time to rest between the runs. The 'squeeze' pattern for each individual was saved as a text file and used in each of the Feat analyses for each individual.

The significant area of BOLD signal change occurred in the left cerebral white matter in the PCOS group ( $p < 0.0005$ ), and left cerebral white matter in the control group (lingual gyrus, temporal fusiform cortex posterior division) ( $p < 0.0005$ ) (Figure 4.3 (a) and (b)). There was no statistically significant difference in the areas of BOLD signal in motor activation when comparing groups.

Figure 4.3(a)

BOLD activation in the PCOS group (n=20) in left cerebral white matter during IFC. Significant region displayed with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$

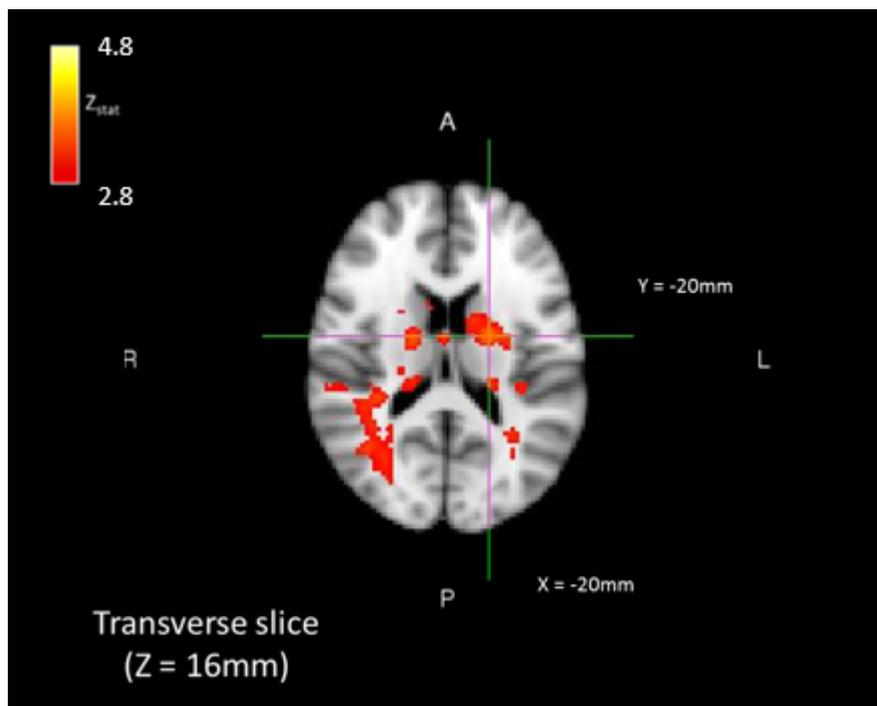
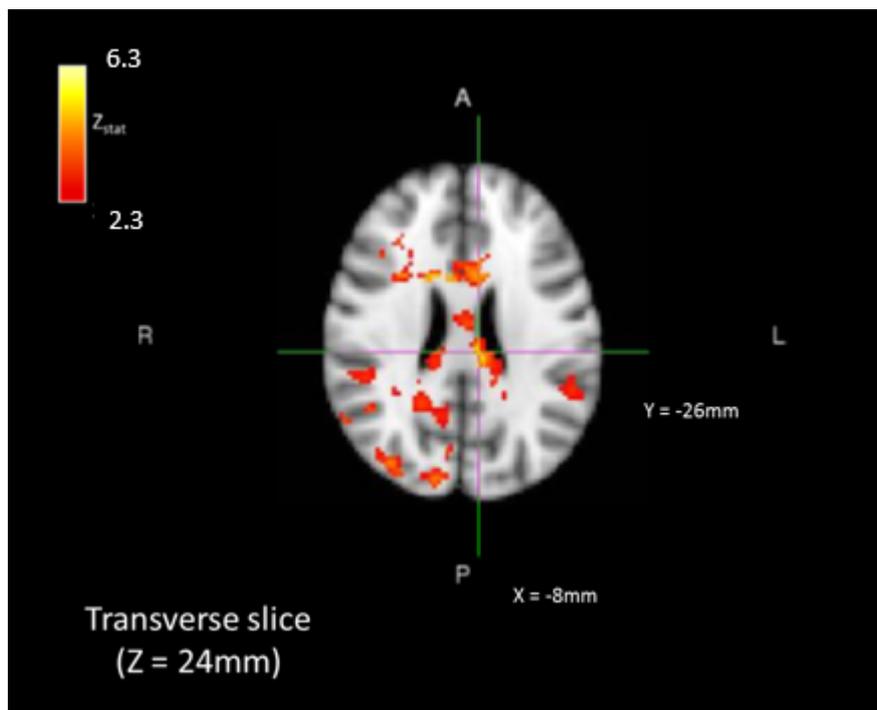


Figure 4.3(b)

BOLD activation in the control group (n=20) in left cerebral white matter during IFC. Significant region displayed with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$



#### 4.4 VISUAL ACTIVATION

The experimental protocol included visual stimuli in the form of a flashing check-board (2Hz lasting 15s) that appeared twice at the end of both rest periods shown in Figure 2.3, chapter 2, i.e. at 6 minutes 15 seconds and 11 minutes 45 seconds. A text file was used for each subject in the Feat analysis with these two 15 second visual stimuli as a regressor.

The visual stimulus increased BOLD signal in the occipital cortex in both groups. In the PCOS group, the BOLD signal peak was in the lateral occipital cortex (cluster  $p < 0.0005$ ) and in the control group, the occipital pole (cluster  $p < 0.0005$ ) (Figure 4.4(a) and (b)). There was no significant difference in areas of BOLD signal activation during visual stimulus when I compared the two groups.

Figure 4.4(a)

BOLD activation during visual stimulus in the PCOS group (n=20). Significant region displayed in lateral occipital cortex with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$

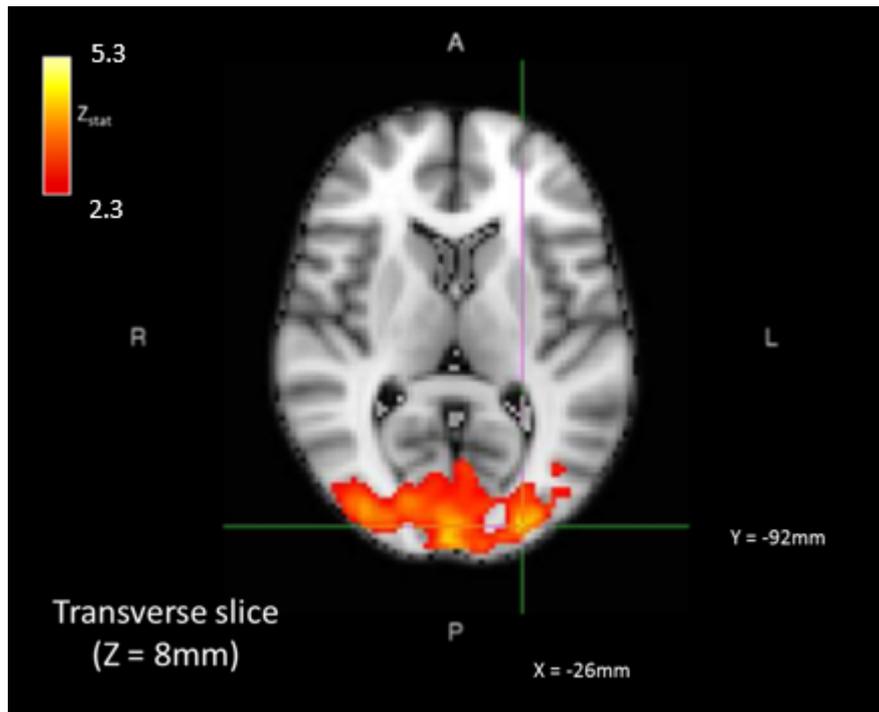
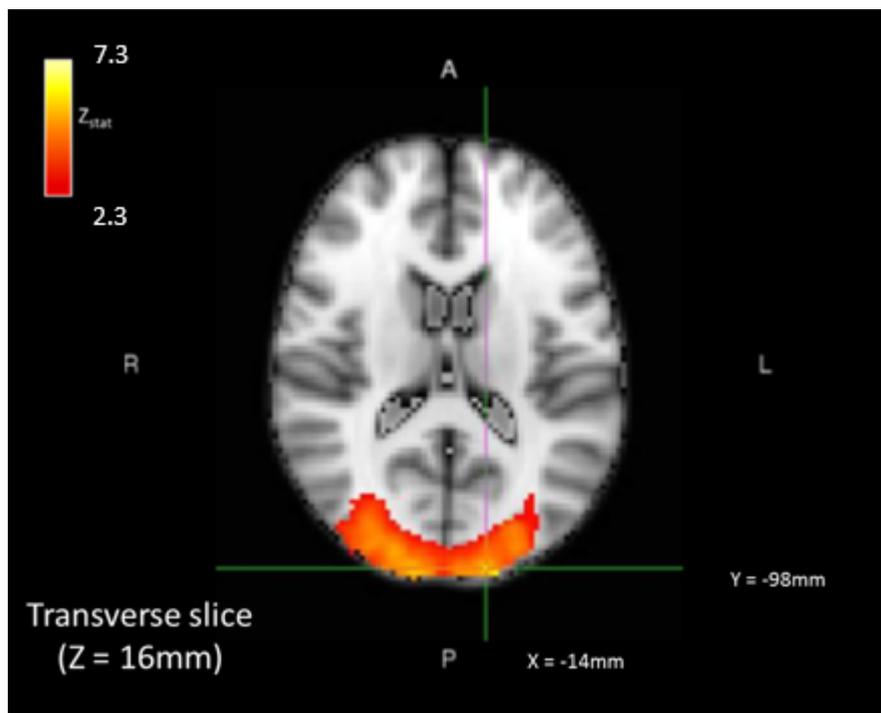


Figure 4.4(b)

**BOLD activation during visual stimulus in the control group (n=20). Significant region displayed in occipital cortex pole with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$**



#### 4.5 INFLUENCE OF METABOLIC PARAMETERS

##### 4.5.1 Testosterone and Insulin Sensitivity

The BOLD signal change modelled for haemodynamic response was accounted for variance associated with testosterone, using testosterone as a covariate at the group

level. BOLD activation in the right orbitofrontal cortex was still greater in the PCOS group as compared to controls ( $p < 0.0001$ ) (as shown in Figure 4.2(a)).

When the BOLD signal was separately corrected for insulin sensitivity using HOMA-IR, the BOLD signal in the right orbitofrontal cortex in the PCOS compared to the control group was no longer significant but the left angular gyrus and lateral occipital cortex remained significant.

#### **4.5.2 BMI**

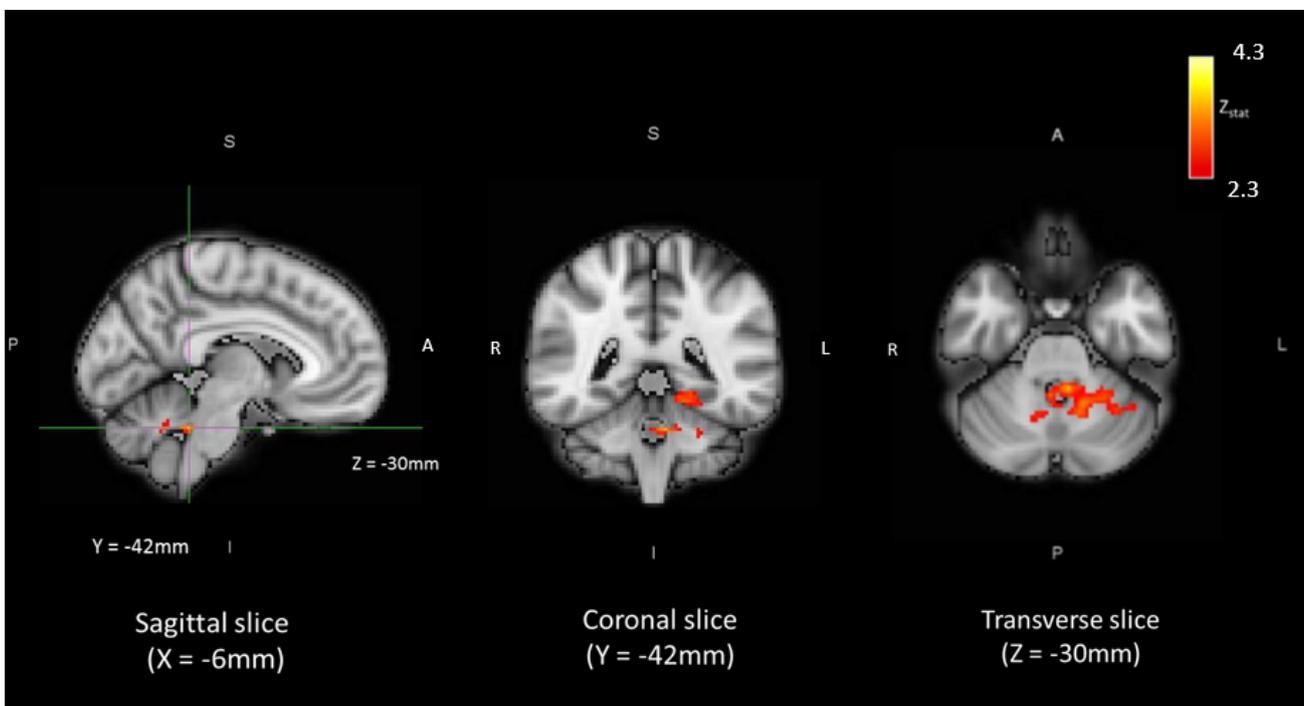
In order to examine the effect of BMI alone on the BOLD signal change for the haemodynamic response, I combined the PCOS and controls into one group. These 30 subjects with data available for the haemodynamic response model were subdivided into a normal BMI group ( $\text{BMI} \leq 25 \text{Kg/m}^2$ ) or overweight-obese group ( $\text{BMI} > 25 \text{Kg/m}^2$ ). 13 subjects were in the overweight-obese group and 17 in the normal BMI group. The mean BMI in the normal group was  $22.5 \pm 1.3 \text{Kg/m}^2$  versus  $29.1 \pm 3.5 \text{Kg/m}^2$  in the overweight-obese group ( $p < 0.005$ ), with mean ages of  $29.7 \pm 3.8$  yrs and  $30.1 \pm 6.1$  yrs ( $p = 0.84$ ) respectively. Insulin AUC and HOMA-IR were greater in the overweight-obese group compared to normal BMI group, but the difference was not

significant ( $47707.50 \pm 34599.11$  vs  $35482.06 \pm 18882.62$  pmol min/L,  $p=0.23$  and  $1.14 \pm 0.71$  vs  $0.94 \pm 0.59$ ,  $p=0.40$ ).

BOLD signal activation was greater in the brainstem in the normal BMI group as compared to controls ( $p=0.009$ ), shown in Figure 4.5. When this was corrected for HOMA-IR, this difference between groups did not persist.

Figure 4.5

**BOLD activation (modelled for blood pressure) greater in normal BMI group (n=17) compared to overweight-obese group (n=13) in the brainstem. The significant region is displayed with a threshold of  $Z > 2.3$ , with a cluster probability threshold of  $p < 0.05$**



#### 4.6 INFLUENCE OF PCOS PHENOTYPE

As there were no differences in the blood pressure responses to IFC between the hyperandrogenic (H) and non-hyperandrogenic (non-H) groups, it did not seem valid

to analyse the BOLD fMRI responses to IFC and modelled blood pressure responses according to these subgroups.

## **4.7 DISCUSSION**

### **4.7.1 Brain activity correlating with blood pressure variation**

For a subset of 30 subjects (15 PCOS and 15 controls), matched by age and BMI, the key finding was that in response to the modelled blood pressure changes, BOLD signal activity was observed in the right cerebral cortex, right pallidum, right thalamus and right parietal operculum cortex in the PCOS group ( $p < 0.0001$ ), and in the control group in the intracalcarine cortex and lingual gyrus ( $p = 0.003$ ). Furthermore, BOLD signal activation was greater in the PCOS group versus controls in the right orbitofrontal cortex ( $p < 0.0001$ ), left angular gyrus and lateral occipital cortex ( $p = 0.04$ ).

This is the first study to use BOLD fMRI to identify any higher brain structures or brainstem correlating with modelled blood pressure responses to an IFC task in PCOS. Although other studies have examined the pressor response to IFC in PCOS [Saranya 2014], no reported studies have sought to locate areas of central command associated with this reflex in PCOS.

The role of central command in the pressor response to muscle contraction, and the metaboreflex, has been of increasing interest. The insular cortex and anterior cingulate cortex are two such regions that have been strongly implicated in this from a number of studies [Williamson 2003, Williamson 1997, Critchley 2000(b)]. Williamson *et al.* used single-photon emission computed tomography (SPECT) to demonstrate that during 3 minutes of IFC, followed by 100 seconds of PEI, that there was a marked increase in blood flow in the sensorimotor cortex, left anterior insular cortex, and anterior cingulate cortex during the contraction phase, and in the sensorimotor and right anterior insular cortex during the period of post-exercise ischaemia (PEI). There were no changes noted within the brain stem [Williamson 2003].

Other studies have suggested a role of the brainstem and areas of the cerebral cortex in blood pressure control. Critchley *et al* used PET scanning to correlate the rise in MAP from mental stressor tasks and physical exercise to areas of localised increased cerebral blood flow. They found that these areas included the dominant anterior cingulate, postcentral gyrus, cerebellum, insular cortex and orbitofrontal cortex [Critchley *et al* 2000].

My study strengthens these findings, and highlights areas of the cerebral cortex involved in the pressor response to IFC. In particular, it shows areas of differential activation in PCOS versus healthy volunteers.

Our findings suggest activation of the right cerebral cortex, right pallidum, right thalamus and right parietal operculum cortex in the PCOS group. It is not known why there was activation in the ipsilateral cerebral cortex during the IFC task, but these BOLD signal changes concur with changes seen in previous studies in the sensorimotor cortex [Wong SW 2007a, Wong SW 2007b, Sander 2010] and thalamus [Gianaros 2005]. In addition, the parietal operculum cortex covers the insular and lies proximal to the insular cortex which, as has been mentioned, has significant evidence to support its central role in the pressor response to IFC [Williamson 2003, Williamson 1997, Critchley 2000(b), Wong SW 2007a, Wong SW 2007b, Sander 2010, Nagai 2010].

In the control group the intracalcarine cortex and lingual gyrus showed significant increase in BOLD signal during the pressor response to IFC. These regions are in the visual cortex and have a role in vision and word processing. Neither of these regions have been directly implicated in the pressor response previously. One possible reason

may be that participants were doing a visual task with the squeeze, i.e. looking at the target bar on the screen to see when 30% IFC had been achieved.

When both groups in my study were compared, there was significantly greater BOLD signal activation in the PCOS group compared to controls in the right orbitofrontal cortex, left angular gyrus and lateral occipital cortex. The left angular gyrus and lateral occipital cortex have not been reported previously as having a central role in the pressor response to IFC. The angular gyrus is usually associated with complex language functions and the lateral occipital cortex with human object recognition. It could be that these findings are false positives. The orbitofrontal cortex, however, has been identified as an area of significance.

In a previous fMRI study of 16 participants that used a behavioural stressor to induce systolic blood pressure rises, the orbitofrontal cortex was a region activated during these changes, along with the anterior cingulate, insula, posterior parietal, and the dorsolateral prefrontal regions of the cortex, the thalamus, and the cerebellum [Gianaros 2005]. Furthermore Harper *et al* used fMRI in healthy volunteers to report increased activity of the orbitofrontal cortex, amygdalo-hippocampal complex, hypothalamus and cerebellum during hypertension elicited by cold pressor stimuli and performance of the Valsalva manoeuvre [Harper 1998], also suggesting a central role

of the orbitofrontal cortex in SNS activation and blood pressure regulation. More recently Krämer et al investigated the central regions involved in baroreflex regulation using MSNA in 15 healthy men in PET experiments. MSNA was measured at rest and baroreflex unloading, induced by lower body negative pressure (LBNP). As compared with the control condition, LBNP was associated with increased PET regional glucose metabolism bilaterally in the orbitofrontal cortex. Related to the rise of MSNA burst frequency there was increased activation of the left orbitofrontal cortex and related to the rise of burst incidence there was increased activation of the brainstem corresponding to the rostral ventrolateral medulla. This led to their reasoning that there is a role for the ventrolateral medulla and the orbitofrontal cortex in baroreflex-mediated control of MSNA in humans [Kramer 2014].

It is important to question why no brainstem activation was noted in the PCOS or control group during the IFC task. In Coulson's study 12 young adults underwent BOLD fMRI, performing IFC at 40% of maximum grip, showing a significant increase in MAP and a correlating brainstem BOLD signal change localised to the ventrolateral medulla [Coulson 2015]. Similarly other studies discussed have shown areas of BOLD signal activation localised to the brainstem and specifically the rostral ventrolateral medulla [Sander 2010, Macefield 2009, Wong SW 2007a, Wong SW 2007b]. These findings sit with the known role of the RVLM in cardiovascular regulation and control.

There may be several reasons why no brainstem activity was found in our cohort. First, the scans were corrected for physiological noise (heart rate and respiratory volume per time) in only a proportion of cases, as the physiological data collected during scanning was only recorded for 5 of the 30 participants. Therefore, physiological factors (respiration, cardiac output, etc) may have caused more noise in the data and affected the brainstem analysis. In addition, the blood pressure data used as an input in the Feat analysis was derived from the IFC task performed out-of-scanner. Moreover, we could also only measure blood pressure every 30 seconds (rather than a continuous tracing) and therefore the blood pressure regressor may not have accurately reflected the actual pressor response during the scan sessions. Related to this, blood pressure changes were used to map BOLD signal changes, whereas MSNA responses (ideally 'in-scanner' recordings) from subjects would have better helped to identify brainstem areas associated with sympathetic outflow.

#### **4.7.2 Potential mediators**

When the BOLD signal change modelled for haemodynamic response was corrected using testosterone as a covariate. BOLD activation in the right orbitofrontal cortex was still greater in the PCOS group as compared to controls ( $p < 0.0001$ ). In correcting the BOLD signal for insulin sensitivity using HOMA-IR, BOLD signal in the right

orbitofrontal cortex in PCOS compared to the control group was no longer significant. This would suggest a potential role of insulin in regulating the BOLD signal in the orbitofrontal cortex.

A study in 25 healthy lean and 23 overweight/obese participants performed magnetic resonance imaging to measure cerebral blood flow (CBF) before, 15 and 30 min after application of intranasal insulin or placebo. Additionally, participants explicitly rated pictures of high-caloric savory and sweet food 60 min after the spray for wanting and liking. They observed a differential response in the lean compared with the overweight/obese group in the prefrontal cortex, resulting in an insulin-induced CBF reduction in lean participants only. This prefrontal cortex response significantly correlated with peripheral insulin sensitivity and suggested that brain insulin action was selectively impaired in the prefrontal cortex in overweight and obese adults [Kullmann 2015]. More recently, a further fMRI study examined brain activation in response to a 75-g OGTT in 24 volunteers (12 lean; 12 obese) and specifically the effect of glucagon-like peptide-1 (GLP-1), a gut incretin peptide. They found that significant increase in GLP-1 levels negatively correlated with a change in the food cue-induced brain activity in the orbitofrontal cortex which was independent of simultaneous alterations in insulin and glucose concentrations. The association was present in lean and overweight participants. They concluded that GLP-1, released

after eating, may have an effect on the orbitofrontal cortex, and food intake [Henri 2015].

The orbitofrontal cortex, therefore, may be affected by insulin action, and may explain why in the PCOS group there was increased activation in response to IFC compared to controls.

When I additionally subdivided the subjects into normal or overweight-obese groups, BOLD signal activation was greater in the brainstem in the normal BMI group as compared to overweight-obese ( $p=0.009$ ). This difference between groups was not present when corrected for HOMA-IR, suggesting a role of insulin sensitivity in the brainstem BOLD signal changes in the normal BMI versus overweight-obese group. This could suggest that insulin signalling is intact in lean but not overweight individuals in the brainstem.

It is difficult to accurately locate BOLD fMRI signal changes in the brainstem and this is an acknowledged problem [Beissner *et al.* 2011]. One of the major limitations in the brainstem is the limited resolution which is similar to the size of nuclei of interest and accurate registration in this area is small. In addition, the inability of the study to define the location of the BOLD signal more accurately may reflect the diffuse location of

these structures in humans. Alternatively, the changes in BOLD signal may represent a high degree of background physiological “noise” [Wise *et al.* 2007]. However, the BOLD signal changes may relate to activation in the medulla. The medulla has been associated with an alteration in insulin sensitivity [Janetta 2010] and our findings may suggest a role in autonomic control in the brainstem. However, these findings need to be viewed carefully as the PCOS and healthy volunteer groups were ‘combined’ into normal versus overweight/obese groups.

#### **4.7.3 Motor activation**

Significant changes in BOLD fMRI signal activity were found in the left cerebral white matter in both the control and PCOS group ( $p < 0.0005$ ). There was no difference in areas of BOLD signal activation between groups when the motor IFC task was performed. The areas identified are potentially artefact in that they are not likely to be related to neural activation as they are deep in white matter and could even be related to task head motion.

The motor task was used as a positive control in the experiment to show that the IFC task was associated with an expected BOLD response in the cerebral cortex. There

are no known neuroanatomical reasons why any difference in brain regions in motor tasks should be different between young women with PCOS and healthy controls.

#### **4.7.4 Visual activation**

The visual stimulus (flashing chequer board) was also used during the BOLD fMRI experiment as a positive control to show that there was expected BOLD activation in the visual cortex corresponding with the visual stimulus. It is a common positive control in BOLD fMRI experiments [Matthews 2004]. It was noted that in both groups there was increased BOLD signal in the occipital (visual) cortex, as would be expected. There are no obvious reasons why there would have been any significant differences between the two groups in terms of the area of the visual cortex showing BOLD activation during the task. No neuroanatomical differences in the visual pathways are known between PCOS and controls.

#### **4.7.5 Strengths and limitations**

My study has a number of strengths and limitations with respect to the central command BOLD fMRI aspects. A good number of PCOS and controls were recruited and scanned to note any changes in higher centres in response to IFC. In addition,

the experimental IFC protocol was run twice in the scan session, and the final results derived from an average of these 2 scans.

However, several limitations are also present. As has been mentioned previously, the PCOS group were not significantly more androgenised or insulin resistant compared to the control group so interpretation of the results depends heavily on the original definition of the study population. Any subjects who were unable to undergo an MRI scan were excluded from the study, which may introduce bias. Furthermore, a significant limitation was that the blood pressure responses were recorded out-of-scanner and not in a continuous manner, thus the actual model used in the Feat analysis may not be accurate. As has been mentioned, not all scans were corrected for physiological noise, due to an error in saving all the physiological data in the scan sessions. This, in turn, may have affected the results obtained, particularly the lack of BOLD signal change seen in the brainstem. A final area of weakness is that the MSNA was only recorded in a subgroup of participants. In a study that aimed to look at the role of higher centres in SNS activation in PCOS versus controls, it would have been ideal to have recorded MSNA in all subjects during the experimental IFC protocol. Ideally this should have been performed during the MRI scan sessions and then used as an input in the Feat analysis to correlate MSNA with BOLD signal changes, as used in other studies [Macefield 2013]. This presents practical challenges due to the need

for an MRI-compatible set-up. In addition, there are haemodynamic effects that could confound interpretation. With the blood pressure changes, if the autoregulation is different between the patients and controls a BOLD signal difference could result that would not be neuronal in origin. However this is not very likely because the blood pressure change is fairly gradual so autoregulation should keep up and autoregulatory problems have not been reported in PCOS.

#### **4.8 CONCLUSION**

In my study, when modelling the blood pressure response to IFC, a greater response in BOLD activation in the right orbitofrontal cortex, left angular gyrus and lateral occipital cortex was noted in PCOS subjects compared with controls. The right orbitofrontal cortex activation persisted when corrected for testosterone but not when the data were adjusted for HOMA-IR.

The IFC task in right-handed subjects is associated with BOLD activation in the left cerebral cortex. There was no difference between PCOS and control groups in any areas of the cerebral cortex activated.

The visual task evoked a BOLD response in the occipital cortex for both PCOS and control groups, with no difference in areas of the cerebral cortex activated between groups.

Finally when subjects were subdivided in normal and overweight-obese BMI groups, greater BOLD activation was noted in the brainstem during IFC in the normal BMI compared to overweight-obese group, and this difference was no longer significant when corrected for HOMA-IR.

# **CHAPTER 5**

## **CONCLUSION AND FUTURE DIRECTIONS**

## 5.1 RELEVANCE OF RESEARCH UNDERTAKEN

Polycystic ovary syndrome (PCOS) is an established metabolic disorder associated with insulin resistance, dyslipidaemia and hypertension [Dunaif 1997, Talbott 1995, Kim 2013, Joham 2015]. Longitudinal studies have shown that there is a risk intrinsic to PCOS in developing Type 2 Diabetes Mellitus which increases with BMI [Moran 2010, Morgan 2012, Pasquali 2013]. Furthermore, there is increasing evidence that insulin resistance, obesity, hypertension and PCOS *per se* are all associated with increased sympathetic tone [Masuo 2003, Alvarez 2002, Grassi and Mancia 2004, Sverrisdottir 2008].

Higher centres have been identified from imaging studies looking at regions of central sympathetic activation and blood pressure control, including the dominant anterior cingulate, postcentral gyrus, cerebellum, insular cortex and orbitofrontal cortex [Critchley *et al* 2000, Kramer 2014]. Higher centres have therefore emerged as playing a key part in sympathetic drive and blood pressure control in humans. The higher

regional brain centres involved in such sympathoexcitation in PCOS are unknown. As fMRI has the potential to reveal some of these regions, the present study sought to identify higher centres involved in blood pressure control in PCOS and how these may be influenced by obesity, hyperandrogenism and insulin resistance.

## **5.2 CONTRIBUTION OF MY FINDINGS TO THE FIELD OF PCOS**

### **5.2.1 PCOS and SNS activation**

PCOS is associated with increased SNS activation as demonstrated by augmented MSNA burst frequency to static exercise compared to controls. To my knowledge, mine is the first study to observe the effect of static exercise on MSNA response in PCOS.

Previous studies have sought to examine the autonomic neural control of the cardiovascular system in response to exercise in PCOS. Tekin *et al* compared 26 young women with PCOS and 24 matched healthy volunteers who were matched

according to age, BMI and physical activity. Their blood pressure, heart rate and heart rate recovery (HRR) responses to an exercise tolerance test were studied. It was noted that following exercise there was an augmented rise in systolic blood pressure during exercise in the PCOS group, which remained significantly elevated in recovery as compared to controls and there was also an attenuated HRR. This led them to conclude that after the onset of exercise in PCOS, there is an initial increase in heart rate that results from a decline in parasympathetic tone and an increase in sympathetic activity that results in an increase in systolic blood pressure and contributes to the increase in heart rate [Tekin 2008]. This study did not employ MSNA to measure SNS activity and indeed no such previous study in the field of PCOS has used MSNA to monitor the acute response to an exercise stimulus.

### **5.2.2 Blood pressure changes in PCOS and BOLD signal activation**

Blood pressure changes associated with static exercise in PCOS correlate with significant BOLD signal activation in the ipsilateral orbitofrontal cortex as compared to controls. This could suggest that different neural pathways are involved in the blood

pressure response to static exercise in PCOS compared with healthy volunteers, or alternatively there may be circuit upregulation involving the orbitofrontal cortex in PCOS compared to controls. This is the first study to use BOLD fMRI to identify any areas of the higher brain structures or brainstem correlating with the IFC task in PCOS. Although other studies have examined the pressor response to IFC in PCOS [Saranya 2014], no reported studies have sought to locate areas of central command involved in this reflex in PCOS.

Previous fMRI studies have demonstrated activation of the orbitofrontal cortex in association with blood pressure rises [Gianaros 2005, Harper 1998]. In addition, Critchley and colleagues studied cerebral blood flow during 40% IFC in 6 young healthy men using positron emission tomography (PET). IFC was associated with a significant rise in blood pressure, and led to increased regional cerebral blood flow (rCBF) activity in the right anterior cingulate, left postcentral gyrus, bilateral cerebellum and cerebellar vermis, right posterior insula and adjacent transverse temporal gyrus, and right orbitofrontal cortex. Conversely, there was significant negative covariation between rCBF and HR evident in the right middle frontal gyrus, right anterior and

posterior cingulate, bilateral insula, bilateral orbitofrontal cortex, left cerebellum and left amygdala [Critchley 2000(a)].

Furthermore it is of interest that the orbitofrontal cortex has been shown to have neuronal connections in monkey models to the insular cortex, which is one of the key areas in the higher pressor response to exercise [Cavada 2000]. There may therefore be significant connections between the orbitofrontal cortex, insular and ventrolateral medulla that are key in regulating the sympathetic and blood pressure response to exercise in PCOS.

### **5.2.3 Insulin resistance and orbitofrontal cortex BOLD signal change**

Potential mediators of the BOLD signal change in the orbitofrontal cortex in PCOS may include insulin resistance and hyperandrogenism, the two key metabolic findings characteristic of this condition. Whilst I found no evidence of an association of hyperandrogenism with this activation, in correcting the BOLD signal for insulin sensitivity (by HOMA-IR), the BOLD signal difference in the right orbitofrontal cortex

between the PCOS group and controls became no longer significant. This would suggest a potential role of insulin sensitivity in regulating the BOLD signal in the orbitofrontal cortex, although causation cannot be ascertained from these findings.

A previous fMRI study performed resting-state scans in 17 healthy lean female subjects to assess intrinsic brain activity by fractional amplitude of low-frequency fluctuations before, 30 and 90 min after application of intranasal insulin. They showed that insulin decreases intrinsic brain activity in the hypothalamus and left orbitofrontal cortex, suggesting insulin may reduce food intake by modifying the reward and prefrontal circuitry of the human brain [Kullmann S 2013]. Although their study focused on the potential acute effect of insulin on food intake behaviour, the change in activity in the orbitofrontal cortex in response to insulin fits with our current findings. The orbitofrontal cortex, therefore, may be affected by insulin action, and may explain why in the PCOS group there was increased activation in response to IFC compared to controls. It may be that PCOS subjects retain insulin sensitivity in the neurons in the orbitofrontal cortex, in contrast to systemic insulin resistance in this condition, and the neurons there respond normally to insulin. The task-induced signal change here could

be greater in the PCOS group because they are hyperinsulinaemic (secondary to the systemic insulin resistance) compared to controls. It has been shown that insulin resistance in PCOS is selective, with reduced sensitivity in skeletal muscle and adipose tissue, but preserved insulin signalling in the ovary, resulting in hyperandrogenic anovulation [Ciaraldi TP 2009, Dunaif 1992, Wu 2014]. A hypothesis is that part of this selective insulin sensitivity in PCOS may include preserved insulin sensitivity in neuronal circuits in the brain, as suggested by my findings in relation to the orbitofrontal cortex.

### **5.3 SUGGESTIONS FOR FUTURE RESEARCH**

#### **5.3.1 Future research on SNS activation in PCOS**

The research can be developed in a number of ways. First, it would be interesting to compare a significantly hyperandrogenised, insulin-resistant group of women with PCOS with controls. As has been discussed, neither group differed significantly in this respect, so it would be useful to repeat the study in several PCOS phenotypes to see

if my findings are observed in all subjects with the condition or only in certain subtypes.

Along with this, more accurately measuring visceral adiposity by MR or CT imaging, and performing a euglycaemic hyperinsulinaemic clamp study in the subjects would better characterise the cohort.

Second, methods of measuring the pressor response to IFC could be modified. The blood pressure and heart rate responses were measured using measurements at intervals throughout the experiment, whereas continuous monitoring of cardiovascular parameters may be useful. In addition, measurement of the catecholamine response by the isotope dilution method would be a possibility, although difficult to undertake outside specialised lab environments. Future studies might also include microneurography in all subjects, ideally undertaken simultaneously with the fMRI scanning. Again, this presents practical challenges due to the invasive nature of this work, and the need for an MRI-compatible set-up. To examine underlying mechanisms of SNS activation in PCOS during the IFC task, a PEI period could also be built into the experiments.

### 5.3.2 Future research on higher centres in SNS activation in PCOS

Finally, a key task should be to try and explore the mediators of higher centre SNS activation in PCOS. My data suggest that insulin resistance may be potentially mechanistically relevant in this regard. An interventional study aimed at clarifying the role of insulin in this response might incorporate (a) the use of a hyperinsulinaemic euglycaemic clamp in an MRI scanner to artificially induce high insulin levels, then undertaking the study IFC protocol pre- and post-insulin infusion to see if the signal changes in either group are altered in response to insulin, and (b) the use of a mixed meal challenge to physiologically elevate insulin levels (although this would also elevate glucose hence an adjustment would have to be made for this).

Furthermore, using an intervention, such as weight loss, insulin-sensitisers (e.g. metformin) or centrally acting sympatholytics (e.g. moxonidine) to note changes in BOLD signal to IFC pre- and post-intervention could be an interesting component of future studies. Such interventions may be helpful in identifying mechanisms driving

central neural SNS pathways in PCOS, notably insulin resistance, and how their activation may be modified.

Additionally, brain tissue and neurons from animal models with induced-PCOS could be studied in acute and chronic hyperinsulinaemic states, to see if any signalling pathways are altered in these states. This in turn may help to identify any underlying mechanisms which could explain the current findings.

Finally, with respect to improving image quality in future studies, fMRI could be performed at 7T with enhanced signal and spatial resolution. This could help to better identify specific higher centres associated with blood pressure changes and SNS activation in PCOS, especially in the brainstem [Deistung 2013].

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# **APPENDIX 1**

## **Letter of approval from South East Wales Research Ethics Committee**



Gwasanaeth  
Moeseg  
Ymchwil | **RES** | Research  
Ethics  
Service

South East Wales Research Ethics  
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South East Wales Research Ethics Committee - Panel D

18 October 2012

Dr Aled Rees  
Senior Lecturer/Consultant Endocrinologist  
Cardiff University  
Centre for Endocrine and Diabetes Sciences  
School of Medicine  
Cardiff University, Cardiff  
CF14 4XN

Dear Dr Rees

**Study title:** Anthropometric and metabolic correlates of Sympathetic  
Nervous system activation in women with Polycystic  
Ovary Syndrome  
**REC reference:** 12/WA/0239  
**Protocol number:** SPON1103-12

Thank you for your letter of 17 October 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair, in the absence of the Chair.

#### Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

#### Ethical review of research sites

##### NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

#### 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*

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

#### Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Advertisement	3	01 October 2012
Covering Letter	A Rees	27 July 2012
Evidence of insurance or indemnity	CaRRS	07 June 2012
Evidence of insurance or indemnity	Zurich Municipal/Cardiff University	22 June 2012
Evidence of insurance or indemnity	Zurich Municipal/Cardiff University	10 July 2012
Investigator CV	A Rees	01 July 2012
Investigator CV	A Lansdown	20 September 2012
Letter from Sponsor	Cardiff University	15 June 2012
Other: GP Information Sheet for Healthy Volunteers	1	01 March 2012
Other: GP Information Sheet for Patients with PCOS	1	01 March 2012
Other: Advertisement	2	01 September 2012
Participant Consent Form: Patient	3	01 October 2012
Participant Consent Form: Healthy Volunteer	3	01 October 2012
Participant Information Sheet: Patient	3	01 October 2012
Participant Information Sheet: Healthy Volunteer	3	01 October 2012
Protocol	2	01 September 2012
REC application	3.4	19 July 2012
Response to Request for Further Information		19 September 2012
Response to Request for Further		17 October 2012

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

#### Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/WA/0239
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Please quote this number on all correspondence
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With the Committee's best wishes for the success of this project

Yours sincerely



**Sir D Walters**  
**Alternate Vice Chair**

Email: [jagit.sidhu@wales.nhs.uk](mailto:jagit.sidhu@wales.nhs.uk)

Enclosures: *"After ethical review – guidance for researchers"* [SL-AR2]

Copy to: *R&D Office Cardiff University*  
*R&D Office Cardiff & Vale University Health Board*

# **APPENDIX 2**

## **Patient Information Sheet and Consent Form**

# **SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN POLYCYSTIC OVARIAN SYNDROME**

## **PATIENT INFORMATION SHEET**

(Version 3, October 2012)

**Title of Study:** Anthropometric and metabolic correlates of Sympathetic Nervous system activation in women with Polycystic Ovary Syndrome

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** We'd suggest this should take about 15 minutes

Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear. Take time to consider whether or not you would wish to take part.

Thank you for reading this.

## **PART 1**

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

Polycystic ovary syndrome (PCOS) is the commonest hormone condition in women of reproductive age, affecting up to 10% of the premenopausal population. In addition to its well-recognised effects on weight gain, excessive hair growth and infertility, it is becoming increasingly clear that PCOS is associated with long-term health risks including diabetes and arterial (blood vessel) disease. The reasons for this are not entirely clear but we believe that a part of the nervous system that affects blood pressure and energy balance, called the sympathetic nervous system, may be involved.

We aim to measure sympathetic nervous system activity in women with PCOS and relate this to body composition and changes in blood pressure. This may help in developing future treatments in PCOS.

This study will involve two visits to the Clinical Research Facility at the Heath hospital (each lasting 2-3 hours) and one visit to the Cardiff University Brain Research Imaging Centre (CUBRIC) (lasting about 1-2 hours).

## ***2. Why have I been invited?***

You have been invited to take part in this study as your doctor has identified you as having PCOS. A total of 25 patients and 25 healthy volunteers will be studied.

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

It is up to you to decide to join the study. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

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

The study will take place at the Clinical Research Facility at the University Hospital of Wales and the Cardiff University Brain Research Imaging Centre (CUBRIC).

### ***Visit 1***

On this visit you will be asked to attend in the morning having only had water to drink. We will require you to have a pregnancy test before carrying out any measurements.

### **Body composition measurements**

We will measure your weight, height, blood pressure, waist circumference, hip circumference and take a brief clinical history. We will measure the amount of fat in your body by two techniques: DEXA and CT scanning. The DEXA scan is a simple, rapid and non-invasive technique which is used routinely in clinical practice. This scan does involve a very small dose of radiation, equivalent to what you would be exposed to simply by living in Cardiff for 1 day. A CT scan will then be performed. Unlike CT scans used in medical practice this will be a single image (or 'slice') only, taken at the level of your lower spine. This will give us enough information to measure the amount of fat present, while minimising your exposure to radiation. The amount of radiation you will receive is very small and similar to what you would be exposed to simply by living in Cardiff for 1 week.

### **Blood tests**

We will place a plastic needle (cannula) in a vein in your elbow and take blood samples (approximately four teaspoons) to check levels of your hormones (testosterone, thyroid function and prolactin), 'fat' proteins (called adiponectin, leptin and resistin), and to measure cholesterol, insulin, glucose and CRP (a marker of inflammation in the body). We will then ask you to take a drink containing

a measured amount of glucose (an oral glucose tolerance test [OGTT]) which assesses how good your body is at handling sugar (termed 'insulin sensitivity'). Further blood samples (at 30, 60 and 90 minutes) will be taken during the test and a final test is taken at two hours, equating to about a further four teaspoons.

### **Sleep tests**

Before leaving the facility, you will be asked to complete a questionnaire about your sleep, provided with simple equipment to measure your sleeping pattern at home once overnight and shown how to set this up. Breathing (airflow through the nose and mouth) is measured by a thermister (measures airflow by the temperature change as you breathe in and out) and bands around your chest and abdomen will measure your breathing effort. An oximeter probe measures oxygen levels in your blood. You may find them a bit strange at first, but most people do not find them uncomfortable or an obstacle to falling asleep. We will make every effort to make sure that you are as comfortable as possible. You will be offered a demonstration of how to use the equipment to ensure you are happy using it and able to record the information required.

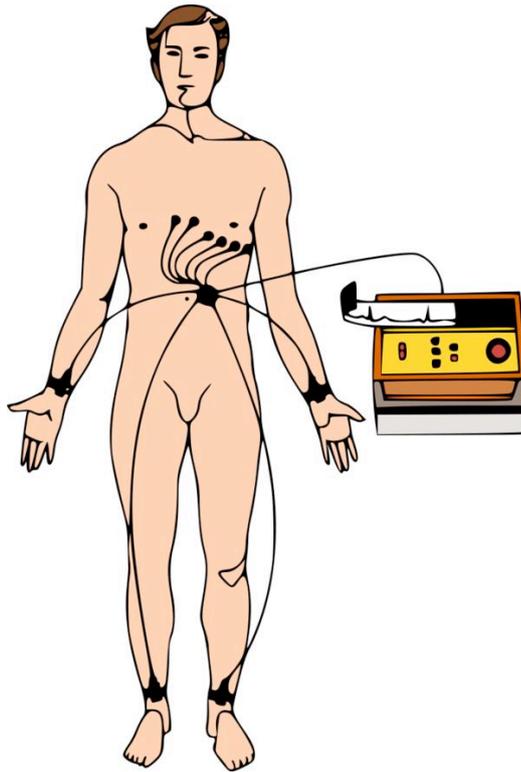
The picture below shows the type of device that you will use for this part of the study.



### **Visit 2**

#### **Nerve and blood pressure tests**

On this visit you will be asked to attend in the morning having only had water to drink. We will place a plastic needle (cannula) in a vein in your elbow. We will place some leads on your chest initially to record an electrocardiogram (ECG) ('a heart tracing') as shown in the diagram below.



We will ask you to rest on a bed and will place a small needle just under the skin on your leg in order to measure nerve activity. A thin wire will be placed at the nerve. The wires are like acupuncture needles, but the tip is very fine (it is the width of a human hair). Once a good nerve recording is found, the electrodes will stay in your leg for approximately 30-60 minutes. You will be asked to keep your leg as still as possible for this duration. You will be asked to squeeze a ball at different intensities while we measure your nerve activity, for 5-minute periods at different grip intensities. A blood sample (about one teaspoon) will be drawn from the cannula in your arm both before and after squeezing the ball. Just before you finish squeezing the ball on one occasion, a blood pressure cuff will be inflated around your upper arm to stop the blood flow to your hand for 2 minutes whilst nerve recordings are made, before being deflated. Your blood pressure and heart rate will be measured at regular intervals throughout. You will also be asked to place your hand in cold water for 2 minutes while nerve recordings are measured.

Finally, while you are resting quietly, a medical doctor will infuse study drugs (called nitroprusside and phenylephrine) through the catheter in your arm to lower and raise your blood pressure (by approximately 10 mmHg in either direction). These small changes in blood pressure are brief (approximately 60 seconds) and reverse quickly; they are no larger than the changes seen during daily life (e.g. standing up, exercising, or urinating).

### **Visit 3**

#### **Brain scan**

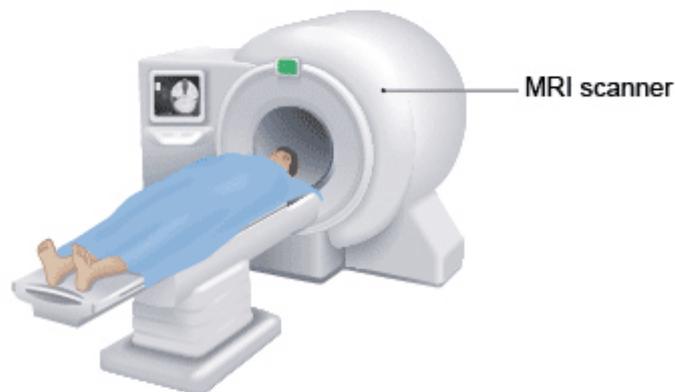
On a third day, we will ask you to attend the Cardiff University Brain Research Imaging Centre (CUBRIC) for a functional Magnetic Resonance Imaging (fMRI) scan. MRI is a method for producing images of the brain. It does not involve any

radiation. MRI can be used to determine which parts of the brain are active during different tasks – this is known as functional MRI or fMRI.

Because the scanner magnet is very powerful, you will be asked to remove metal from your pockets (coins, keys) and also be asked to remove articles of clothing (in a dedicated changing room) that have metal fasteners (belts, bras, etc), as well as most jewellery. Please bring suitable clothing with you for the scan. Sweatshirts and jogging trousers are ideal.

To be scanned, you will lie on your back on a bed on runners, on which you will be moved until your head or the specific region of your body to be scanned is inside the magnet (which is like a long tube). The scanning process itself creates intermittent loud noises, and you must wear ear-plugs or sound-reducing headphones. We are able to talk to you while you are in the scanner through an intercom. It is important that you keep your head as still as possible during the scan, and to help you with this, your head will be partially restrained with padded headrests. We shall ask you to relax your head and keep it still. You will be asked to squeeze a ball again (like previously) at different intensities while we measure your brain activity, for 5-minute periods at different grip intensities. Detailed instructions will be given just before the scan, and will appear on a screen during the scan.

The picture below shows a person having an MRI scan.



**A person having an MRI scan**

The whole procedure will typically take about 1 hour, plus another 15 minutes to discuss with you the purposes of the study and answer any questions about it which you may raise. This is not a diagnostic scan and therefore your scan will not be examined for abnormalities.

Very occasionally, however, when we look at MRI data from subjects, unexpected potential abnormalities are discovered. Once we have looked at the data in some detail, we may ask a Consultant to examine the data, and if appropriate a report can be forwarded to your GP. You should not regard this Research scan as a Medical screening procedure.

If you wish we will be able to reimburse any travelling expenses / car parking fees incurred while attending for the study visits. We will also give you a single payment of £50 to reimburse you for your inconvenience.

### **5. What do I have to do?**

It is important that you take your regular medication in the normal way without altering the dose or timing of these, *except for prior to the nerve and brain test visits* when we would also ask that no 'over the counter' medications (e.g. paracetamol) are taken on the morning of these visits. We also request that you have no alcohol on the day before these visits and the day of the visit and no exercise after 7:00pm the evening before these visits and no exercise on the day of the visit. We ask also that you consume no caffeine (e.g. coffee, coke, red bull) from the evening before these visits. If you are a smoker we request that you avoid smoking on each morning. We request that you report any illnesses to us as they may influence the timing of your test visit. You should inform us of any dose adjustments in any regular medications made during the study. If you are taking any heart or blood pressure medications you should not take part in the study. If unsure please check with the researchers.

You should inform us if there is any possibility of you being pregnant and this will be tested for in all participants. If you are pregnant you will not be able to participate in this study.

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

Studies are carried out by trained researchers who are experienced with all the procedures described, thus reducing possible disadvantages and risks. Potential disadvantages and risks related to the experimental measurements and procedures we are studying include:

*Placement of a thin tube in a vein for blood samples:* Collection of blood may cause possible discomfort (temporary pain, swelling, bruising and rarely infection).

*Placement of the nerve electrode:* The nerve recording procedure occasionally may result in the leg muscles feeling tired. Also, you may have a pins-and-needles feeling or a greater sensitivity to touch in the leg. However, these side effects are rare, affecting approximately 1 in 10 participants, and if they occurred, would only be temporary lasting for a day or two at the most.

*Drugs used to change blood pressure:* The drugs used on the second visit to change blood pressure are nitroprusside and phenylephrine. These are commonly used and safe drugs. Very occasionally nitroprusside can cause you to experience low blood pressure, excessive increase in heart rate, flushing, dizziness, and headache. Similarly phenylephrine can very occasionally cause high blood pressure, excessive increase or decrease in heart rate, dizziness, headache, and rarely abnormal heart beats. The small changes in blood pressure that occurs with these drugs are brief (approximately 60 seconds) and reverse quickly; they are no longer than the changes seen during daily life (e.g. standing up, exercising). A medical doctor will be present to supervise as these drugs are administered.

*CT scan:* A 'single slice' CT scan and DEXA scan do involve exposure to a small amount of radiation. In addition the CT scan could by chance pick up an unsuspected abnormality in your abdomen, in which case you will be given an opportunity to discuss these findings further with Dr Aled Rees.

*MRI scan:* The fMRI scan involves lying in a relatively confined space so if you are likely to become very uneasy in this situation (suffer from claustrophobia), you should NOT take part in the study. If you do take part and this happens, you will be able to alert the staff by activating an alarm and will then be removed from the scanner quickly. Likewise the scanning process itself creates intermittent loud noises but you will be provided with ear-plugs or sound-reducing headphones to wear.

*Sleep studies:* Sometimes there may be a sensation of warmth where the oximeter probe is attached to your finger. However, this does not generally cause any significant or lasting discomfort.

The MRI, blood and sleep studies may also pick up unsuspected abnormalities in which case you will be given an opportunity to discuss these findings with Dr Rees.

### ***7. What are the potential benefits of taking part?***

There are unlikely to be any direct benefits for you but the study may provide us with important information in determining whether sympathetic nervous system activity could be important in women with PCOS. It may therefore be possible in future to design new treatments for blood pressure and similar complications of PCOS.

### ***8. What if there is a problem?***

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

### ***9. Will my taking part in the study be kept confidential?***

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

## **PART 2**

### **1. What happens if new information becomes available?**

If this happens, your research doctor might consider you should withdraw from the study. He will explain the reasons and arrange for your care to continue.

### **2. What will happen if I don't want to carry on with the study?**

If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up until your withdrawal. A decision to withdraw at any time will not affect the standard of care you receive.

### **3. What if there is a problem?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Dr Aled Rees 02920 7445002 reesda@cf.ac.uk or Dr Andrew Lansdown lansdownaj1@cf.ac.uk). This study is being indemnified by Cardiff University. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

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

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. With your permission your GP will be informed of your participation in this study. With your permission we may also look at sections of your medical notes which are relevant to the research study.

### **5. What will happen to any samples I give?**

The blood samples will be collected and stored securely for later analysis in the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. Only immediate members of the research team will have access to these samples. All identifiable information will be removed from the samples which will be destroyed by incineration once the tests are complete.

### **6. What will happen to the results of the research study?**

The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have

been published in the relevant journal(s). Patients will not be identified in any report/publication.

**7. Will I be able to have the results of my tests?**

We will contact you if there are any unsuspected abnormalities in your test results and you will be given an opportunity to discuss these findings with Dr Aled Rees. If you wish to have all your test results (including normal results), we will be able to provide these for you on your request.

**8. Who is organising and funding the research?**

The study is being organised by Dr Aled Rees (the Principal Investigator) and Dr Andrew Lansdown (Research Registrar) from the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. Funding for the study is provided from funds within the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. The doctors conducting the research are not being paid for including and looking after patients in the study.

**9. Who has reviewed the study?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South Wales Research Ethics Committee.

**10. Contact for further information**

Should you have any further queries regarding this research study, then please do not hesitate to contact me on 02920 7445002 or 07746 780566. You can also contact me via e-mail on reesda@cf.ac.uk or Dr Andrew Lansdown by email lansdownaj1@cf.ac.uk

Thank you for considering taking part in this study.

Dr Aled Rees  
Senior Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

**SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN POLYCYSTIC OVARY SYNDROME**

Patient Identification Number for this study:

**PATIENT CONSENT FORM**

(Version 3 October 2012)

Title of Study: Anthropometric and metabolic correlates of Sympathetic Nervous system activation in women with Polycystic Ovary Syndrome

**Name of Researchers:** Dr Aled Rees, Dr Andrew Lansdown

**Please initial box**

- 1. I confirm that I have read and understood the information sheet dated October 2012 (version 3) for the above study and 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 any reason, without my medical care or legal rights being affected.
  
- 3. I understand that sections of my medical notes may be looked at by the sponsor and responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
  
- 4. I consent to my GP being informed of my participation in the Study.
  
- 5. I consent to a pregnancy test.
  
- 6. I agree to take part in the above study.

Name of patient	Date	Signature
Researcher	Date	Signature
Name of person taking consent	Date	Signature

# **APPENDIX 3**

## **Volunteer Information Sheet and Consent Form**

# **SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN POLYCYSTIC OVARIAN SYNDROME**

## **HEALTHY VOLUNTEER INFORMATION SHEET**

(Version 3, October 2012)

**Title of Study:** Anthropometric and metabolic correlates of Sympathetic Nervous system activation in women with Polycystic Ovary Syndrome

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** We'd suggest this should take about 15 minutes

Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear. Take time to consider whether or not you would wish to take part.

Thank you for reading this.

## **PART 1**

### ***2. What is the purpose of this study?***

Polycystic ovary syndrome (PCOS) is the commonest hormone condition in women of reproductive age, affecting up to 10% of the premenopausal population. In addition to its well-recognised effects on weight gain, excessive hair growth and infertility, it is becoming increasingly clear that PCOS is associated with long-term health risks including diabetes and arterial (blood vessel) disease. The reasons for this are not entirely clear but we believe that a part of the nervous system that affects blood pressure and energy balance, called the sympathetic nervous system, may be involved.

We aim to measure sympathetic nervous system activity in women with PCOS and relate this to body composition and changes in blood pressure. This may help in developing future treatments in PCOS.

This study will involve two visits to the Clinical Research Facility at the Heath hospital (each lasting 2-3 hours) and one visit to the Cardiff University Brain Research Imaging Centre (CUBRIC) (lasting about 1-2 hours).

## ***2. Why have I been invited?***

You have been invited to take part in this study as you are healthy and similar in terms of age to the patients with PCOS. A total of 25 patients and 25 healthy volunteers will be studied.

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

It is up to you to decide to join the study. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

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

The study will take place at the Clinical Research Facility at the University Hospital of Wales and the Cardiff University Brain Research Imaging Centre (CUBRIC).

### ***Visit 1***

On this visit you will be asked to attend in the morning having only had water to drink. We will require you to have a pregnancy test before carrying out any measurements.

### **Body composition measurements**

We will measure your weight, height, blood pressure, waist circumference, hip circumference and take a brief clinical history. We will measure the amount of fat in your body by two techniques: DEXA and CT scanning. The DEXA scan is a simple, rapid and non-invasive technique which is used routinely in clinical practice. This scan does involve a very small dose of radiation, equivalent to what you would be exposed to simply by living in Cardiff for 1 day. A CT scan will then be performed. Unlike CT scans used in medical practice this will be a single image (or 'slice') only, taken at the level of your lower spine. This will give us enough information to measure the amount of fat present, while minimising your exposure to radiation. The amount of radiation you will receive is very small and similar to what you would be exposed to simply by living in Cardiff for 1 week.

### **Blood tests**

We will place a plastic needle (cannula) in a vein in your elbow and take blood samples (approximately four teaspoons) to check levels of your hormones (testosterone, thyroid function and prolactin), 'fat' proteins (called adiponectin, leptin and resistin), and to measure cholesterol, insulin, glucose and CRP (a marker of inflammation in the body). We will then ask you to take a drink containing a measured amount of glucose (an oral glucose tolerance test [OGTT]) which assesses how good your body is at handling sugar (termed 'insulin sensitivity'). Further blood samples (at 30, 60 and 90 minutes) will be taken during the test and a final test is taken at two hours, equating to about a further four teaspoons.

### **Sleep tests**

Before leaving the facility, you will be asked to complete a questionnaire about your sleep, provided with simple equipment to measure your sleeping pattern at home once overnight and shown how to set this up. Breathing (airflow through the nose and mouth) is measured by a thermister (measures airflow by the temperature change as you breathe in and out) and bands around your chest and abdomen will measure your breathing effort. An oximeter probe measures oxygen levels in your blood. You may find them a bit strange at first, but most people do not find them uncomfortable or an obstacle to falling asleep. We will make every effort to make sure that you are as comfortable as possible. You will be offered a demonstration of how to use the equipment to ensure you are happy using it and able to record the information required.

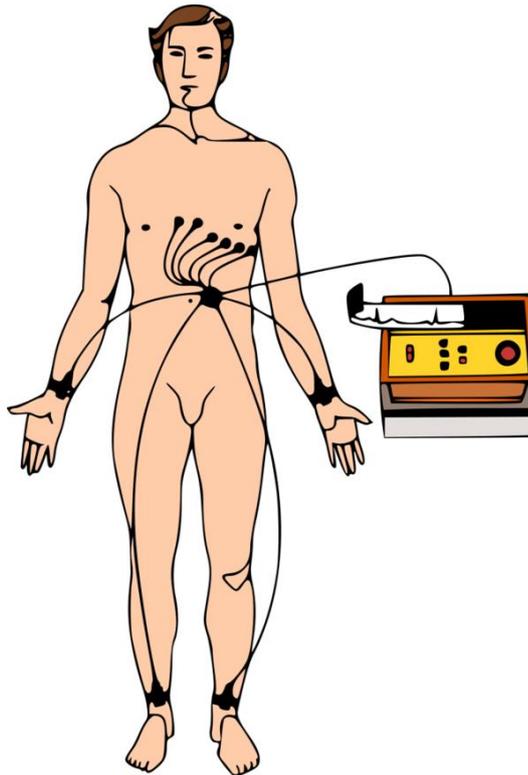
The picture below shows the type of device that you will use for this part of the study.



### **Visit 2**

#### **Nerve and blood pressure tests**

On this visit you will be asked to attend in the morning having only had water to drink. We will place a plastic needle (cannula) in a vein in your elbow. We will place some leads on your chest initially to record an electrocardiogram (ECG) ('a heart tracing') as shown in the diagram below.



We will ask you to rest on a bed and will place a small needle just under the skin on your leg in order to measure nerve activity. A thin wire will be placed at the nerve. The wires are like acupuncture needles, but the tip is very fine (it is the width of a human hair). Once a good nerve recording is found, the electrodes will stay in your leg for approximately 30-60 minutes. You will be asked to keep your leg as still as possible for this duration. You will be asked to squeeze a ball at different intensities while we measure your nerve activity, for 5-minute periods at different grip intensities. A blood sample (about one teaspoon) will be drawn from the cannula in your arm both before and after squeezing the ball. Just before you finish squeezing the ball on one occasion, a blood pressure cuff will be inflated around your upper arm to stop the blood flow to your hand for 2 minutes whilst nerve recordings are made, before being deflated. Your blood pressure and heart rate will be measured at regular intervals throughout. You will also be asked to place your hand in cold water for 2 minutes while nerve recordings are measured.

Finally, while you are resting quietly, a medical doctor will infuse study drugs (called nitroprusside and phenylephrine) through the catheter in your arm to lower and raise your blood pressure (by approximately 10 mmHg in either direction). These small changes in blood pressure are brief (approximately 60 seconds) and reverse quickly; they are no larger than the changes seen during daily life (e.g. standing up, exercising, or urinating).

### ***Visit 3***

#### **Brain scan**

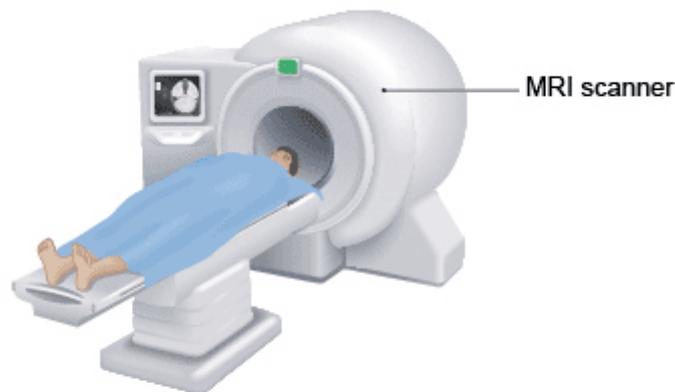
On a third day, we will ask you to attend the Cardiff University Brain Research Imaging Centre (CUBRIC) for a functional Magnetic Resonance Imaging (fMRI) scan. MRI is a method for producing images of the brain. It does not involve any

radiation. MRI can be used to determine which parts of the brain are active during different tasks – this is known as functional MRI or fMRI.

Because the scanner magnet is very powerful, you will be asked to remove metal from your pockets (coins, keys) and also be asked to remove articles of clothing (in a dedicated changing room) that have metal fasteners (belts, bras, etc), as well as most jewellery. Please bring suitable clothing with you for the scan. Sweatshirts and jogging trousers are ideal.

To be scanned, you will lie on your back on a bed on runners, on which you will be moved until your head or the specific region of your body to be scanned is inside the magnet (which is like a long tube). The scanning process itself creates intermittent loud noises, and you must wear ear-plugs or sound-reducing headphones. We are able to talk to you while you are in the scanner through an intercom. It is important that you keep your head as still as possible during the scan, and to help you with this, your head will be partially restrained with padded headrests. We shall ask you to relax your head and keep it still. You will be asked to squeeze a ball again (like previously) at different intensities while we measure your brain activity, for 5-minute periods at different grip intensities. Detailed instructions will be given just before the scan, and will appear on a screen during the scan.

The picture below shows a person having an MRI scan.



**A person having an MRI scan**

The whole procedure will typically take about 1 hour, plus another 15 minutes to discuss with you the purposes of the study and answer any questions about it which you may raise. This is not a diagnostic scan and therefore your scan will not be examined for abnormalities.

Very occasionally, however, when we look at MRI data from healthy subjects, unexpected potential abnormalities are discovered. Once we have looked at the data in some detail, we may ask a Consultant to examine the data, and if appropriate a report can be forwarded to your GP. You should not regard this Research scan as a Medical screening procedure.

If you wish we will be able to reimburse any travelling expenses / car parking fees incurred while attending for the study visits. We will also give you a single payment of £50 to reimburse you for your inconvenience.

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You should inform us if there is any possibility of you being pregnant and this will be tested for in all participants. If you are pregnant you will not be able to participate in this study.

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

Studies are carried out by trained researchers who are experienced with all the procedures described, thus reducing possible disadvantages and risks. Potential disadvantages and risks related to the experimental measurements and procedures we are studying include:

*Placement of a thin tube in a vein for blood samples:* Collection of blood may cause possible discomfort (temporary pain, swelling, bruising and rarely infection).

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*Drugs used to change blood pressure:* The drugs used on the second visit to change blood pressure are nitroprusside and phenylephrine. These are commonly used and safe drugs. Very occasionally nitroprusside can cause you to experience low blood pressure, excessive increase in heart rate, flushing, dizziness, and headache. Similarly phenylephrine can very occasionally cause high blood pressure, excessive increase or decrease in heart rate, dizziness, headache, and rarely abnormal heart beats. The small changes in blood pressure that occurs with these drugs are brief (approximately 60 seconds) and reverse quickly; they are no longer than the changes seen during daily life (e.g. standing up, exercising). A medical doctor will be present to supervise as these drugs are administered.

*CT scan:* A 'single slice' CT scan and DEXA scan do involve exposure to a small amount of radiation. In addition the CT scan could by chance pick up an unsuspected abnormality in your abdomen, in which case you will be given an opportunity to discuss these findings further with Dr Aled Rees.

*MRI scan:* The fMRI scan involves lying in a relatively confined space so if you are likely to become very uneasy in this situation (suffer from claustrophobia), you should NOT take part in the study. If you do take part and this happens, you will be able to alert the staff by activating an alarm and will then be removed from the scanner quickly. Likewise the scanning process itself creates intermittent loud noises but you will be provided with ear-plugs or sound-reducing headphones to wear.

*Sleep studies:* Sometimes there may be a sensation of warmth where the oximeter probe is attached to your finger. However, this does not generally cause any significant or lasting discomfort.

The MRI, blood and sleep studies may also pick up unsuspected abnormalities in which case you will be given an opportunity to discuss these findings with Dr Rees.

### ***7. What are the potential benefits of taking part?***

There are unlikely to be any direct benefits for you but the study may provide us with important information in determining whether sympathetic nervous system activity could be important in women with PCOS. It may therefore be possible in future to design new treatments for blood pressure and similar complications of PCOS.

### ***8. What if there is a problem?***

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

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### ***6. What if there is a problem?***

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Dr Aled Rees 02920 7445002 reesda@cf.ac.uk or Dr Andrew Lansdown lansdownaj1@cf.ac.uk). This study is being indemnified by Cardiff University. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

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The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have

been published in the relevant journal(s). Patients will not be identified in any report/publication.

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We will contact you if there are any unsuspected abnormalities in your test results and you will be given an opportunity to discuss these findings with Dr Aled Rees. If you wish to have all your test results (including normal results), we will be able to provide these for you on your request.

**8. Who is organising and funding the research?**

The study is being organised by Dr Aled Rees (the Principal Investigator) and Dr Andrew Lansdown (Research Registrar) from the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. Funding for the study is provided from funds within the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. The doctors conducting the research are not being paid for including and looking after patients in the study.

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All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South Wales Research Ethics Committee.

**10. Contact for further information**

Should you have any further queries regarding this research study, then please do not hesitate to contact me on 02920 7445002 or 07746 780566. You can also contact me via e-mail on reesda@cf.ac.uk or Dr Andrew Lansdown by email lansdownaj1@cf.ac.uk

Thank you for considering taking part in this study.

Dr Aled Rees  
Senior Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

**SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN POLYCYSTIC OVARY SYNDROME**

Patient Identification Number for this study:

**HEALTHY VOLUNTEER CONSENT FORM**

(Version 3 October 2012)

Title of Study: Anthropometric and metabolic correlates of Sympathetic Nervous system activation in women with Polycystic Ovary Syndrome

**Name of Researchers:** Dr Aled Rees, Dr Andrew Lansdown

**Please initial box**

1. I confirm that I have read and understood the information sheet dated October 2012 (version 3) for the above study and 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 any reason, without my medical care or legal rights being affected.
3. I understand that sections of any medical notes I may have may be looked at by the sponsor and responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
4. I consent to my GP being informed of my participation in the Study.
5. I consent to a pregnancy test.
6. I agree to take part in the above study.

Name of patient Date Signature

Researcher Date Signature

Name of person taking consent Date Signature