

**THE IMPACT OF OBESITY AND FITNESS ON  
ENDOTHELIAL FUNCTION IN POLYCYSTIC  
OVARIAN SYNDROME**

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# **The following tables and figures have been omitted on request of the university –**

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## Abstract

Polycystic ovarian syndrome (PCOS) is a highly prevalent heterogeneous syndrome associated with abdominal obesity, insulin resistance and the metabolic syndrome. This clustering of risk factors could translate into an adverse cardiovascular disease (CVD) risk profile. Endothelial dysfunction, an early barometer of CVD, has been exhibited by women with PCOS; however, it remains unclear whether endothelial dysfunction is independent of CVD risk factors in this population. Exercise training has been found to enhance conduit artery and cutaneous microvessel endothelial function in various populations. Nevertheless, limited research exists regarding the cardiovascular effects of exercise in PCOS, and its impact on endothelial function in conduit arteries and cutaneous microvessels, has not been explored. The primary aim of this thesis was to examine nitric oxide (NO)-mediated endothelial function at different levels of the vascular tree in women with PCOS and to establish whether supervised exercise training induces a therapeutic effect on endothelial function.

A systematic review of published studies comparing FMD in PCOS and control women was conducted. Twenty-one published studies were identified for inclusion (PCOS  $n=908$ ; controls  $n=566$ ). Differences in FMD between PCOS and controls were synthesised and meta-regressed against BMI and age. The pooled mean FMD was 3.5% lower (95% CI=3.4, 3.7%;  $P<0.001$ ) in women with PCOS compared with controls; and the PCOS-mediated reduction in FMD was most evident in studies involving less obese women.

PCOS [ $n=35$ , 28y (95% CI=26, 30), 31kg/m<sup>2</sup> (95% CI=27, 35)] and control women [ $n=16$ , 32y (95% CI=30, 35), 30kg/m<sup>2</sup> (95% CI=25, 32)] were recruited. Brachial artery endothelial function was assessed using flow-mediated dilation (FMD). Internal adipose tissue (IAT), subcutaneous (SAT), visceral (VAT) and abdominal SAT was quantified using whole body magnetic resonance imaging and <sup>1</sup>H magnetic resonance spectroscopy quantified liver and skeletal muscle fat. Cardiorespiratory fitness, glycaemic control, reproductive hormone and lipid profiles were also assessed. FMD was impaired in PCOS when compared with control women [-4.5% (95% CI=-6.3, -2.8),  $P<0.001$ ]. When FMD was adjusted for individual differences in IAT [-4.3% (95% CI=-6.1, -2.4),  $P<0.001$ ], VAT [-4.4% (95% CI=-6.3, -2.5),  $P<0.001$ ] and insulin resistance [-3.9% (95% CI=-5.6, -2.1),  $P<0.001$ ], the difference in FMD between groups remained.

Ten women with PCOS [27y (95% CI=23, 32), 31 kg/m<sup>2</sup> (95% CI=28, 34)] completed a 16-week supervised exercise programme while 7 women with PCOS [29y (95% CI=24, 35), 35kg/m<sup>2</sup> (95%CI=31, 40)] opted for conventional care and followed simple lifestyle advice. Exercise training improved FMD to a greater degree than conventional care [3.4% (95% CI=1.8, 5.1),  $P>0.0005$ ] and in parallel greater improvements in cardiorespiratory fitness were observed with exercise [4.7ml/kg/min (95% CI=1.4, 7.9),  $P=0.005$ ]. These changes with exercise occurred independently of changes in VAT, SAT or insulin resistance.

NO-mediated vasodilation in the cutaneous microvessels was examined in 11 PCOS [29y (95% CI=25, 34), 34kg/m<sup>2</sup> (95% CI=30, 38)] and 6 control women [29y (95% CI=21, 37),

34kg/m<sup>2</sup> (95% CI=28, 39)] using laser Doppler flowmetry combined with intra-dermal microdialysis of L-N<sup>G</sup>-monomethyl arginine to assay the NO dilator system in response to incremental local heating of the forearm. Six women with PCOS [30y (95% CI=22, 37), 31kg/m<sup>2</sup> (95% CI=25, 37)] then undertook a 16-week exercise-training programme. Nitric oxide contribution was attenuated in women with PCOS at peak heating [-16.0CVC<sub>max</sub> (95% CI=-32.5, 0.6), *P*=0.05] and during prolonged maximal heating [-15.4CVC<sub>max</sub> (95% CI=-29.6, -1.3), *P*=0.04], compared with control women. Cardiorespiratory fitness improved by 5.0ml/kg/min (95% CI=0.9, 9.2) following exercise training (*P*=0.03). This was accompanied by increased NO contribution to cutaneous blood flow between 36.5-42°C (*P*<0.05), at peak heating [19.6CVC<sub>max</sub> (95% CI=4.3, 34.9), *P*=0.02] and during prolonged maximal heating [17.1CVC<sub>max</sub> (95% CI=2.2, 32.2), *P*=0.03].

The findings from this thesis suggest that endothelial dysfunction is an intrinsic characteristic of PCOS and that supervised exercise training enhances endothelial function in both conduit vessels and cutaneous microvessels, independent of adiposity or traditional CVD risk factors. The direct impact of exercise training on the vasculature of women with PCOS may decrease the risk of CVD morbidities, such as hypertension, and consequently reduce cardiovascular mortality in post-menopausal years.

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## Declaration

I declare that the work contained within this thesis is entirely my own.

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## **List of abbreviations**

<b>ACh</b>	Acetylcholine
<b>ALT</b>	Alanine transaminase
<b>ANCOVA</b>	Analysis of covariance
<b>ANOVA</b>	Analysis of variance
<b>AUC</b>	Area under the curve
<b>AST</b>	Aspartate transaminase
<b>BMI</b>	Body mass index
<b>BP</b>	Blood pressure
<b>CAD</b>	Coronary artery disease
<b>CHD</b>	Coronary heart disease
<b>CI</b>	Confidence interval
<b>CVC</b>	Cutaneous vascular conductance
<b>CVD</b>	Cardiovascular disease
<b>DBP</b>	Diastolic blood pressure
<b>DICOM</b>	Digital imaging and communications in medicine
<b>eNOS</b>	Endothelial nitric oxide synthase
<b>FFA</b>	Free fatty acid
<b>FMD</b>	Flow mediated dilatation
<b>FSH</b>	Follicle stimulating hormone
<b>GTN</b>	Glycerl trinitrate
<b>HDL</b>	High density lipoprotein
<b>HOMA</b>	Homeostatic model assessment
<b>HR</b>	Heart rate
<b>HRR</b>	Heart rate reserve
<b>Hz</b>	Hertz
<b>IAT</b>	Internal adipose tissue

<b>IMCL</b>	Intramyocellular lipid
<b>IMT</b>	Intima-media thickness
<b>LDF</b>	Laser Doppler flowmetry
<b>LDL</b>	Low density lipoprotein
<b>L-NAME</b>	<i>N</i> -nitro- <i>L</i> -arginine methyl ester
<b>L-NMMA</b>	<i>N</i> <sup>G</sup> -monomethyl- <i>L</i> -arginine
<b>LH</b>	Luteinizing hormone
<b>MAP</b>	Mean arterial pressure
<b>MRI</b>	Magnetic resonance imaging
<b>NAFLD</b>	Non-alcoholic fatty liver disease
<b>NO</b>	Nitric oxide
<b>PCOS</b>	Polycystic ovarian syndrome
<b>PU</b>	Arbitrary perfusion units
<b>RPE</b>	Rate of perceived exertion
<b>RER</b>	Respiratory exchange ratio
<b>ROI</b>	Region of interest
<b>ROS</b>	Reactive oxygen species
<b>SAT</b>	Subcutaneous adipose tissue
<b>SBP</b>	Systolic blood pressure
<b>SD</b>	Standard deviation
<b>SKBF</b>	Skin blood flow
<b>SNP</b>	Sodium nitroprusside
<b>SR</b>	Shear rate
<b>VAT</b>	Visceral adipose tissue
<b>VLDL</b>	Very low density lipoprotein
<b>VO<sub>2peak</sub></b>	Maximal oxygen consumption
<b><sup>1</sup>H-MRS</b>	Proton magnetic resonance spectroscopy

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**CHAPTER 1**  
**INTRODUCTION**

Polycystic Ovarian Syndrome (PCOS) is a condition characterised by clinical or biochemical hyperandrogenism, oligo-/amenorrhea and polycystic appearance of the ovaries upon ultrasound examination (Goodarzi *et al.*, 2011). Although traditionally considered a reproductive disorder, in recent years, the complex heterogeneous phenotype of PCOS has been found to encompass obesity, insulin resistance and androgen excess. Therefore, PCOS has become an increasing concern for many aspects of women's health. Nevertheless, the precise aetiology of PCOS remains unclear with both genetic predisposition and environmental influences being implicated (Legro, 1995). Alarming, PCOS is the single most common endocrinopathy in females, affecting approximately 10% of premenopausal women globally.

Women with PCOS are generally more obese than their age matched non-PCOS counterparts, evidenced by an elevated body mass index (BMI) and waist circumference (Talbot *et al.*, 1995; Gambineri *et al.*, 2002). Specifically, when compared with weight matched controls, women with PCOS display a similar volume of total body fat but a higher volume of central abdominal fat (Carmina *et al.*, 2007b) and elevated visceral adipose tissue (VAT) (Yildirim *et al.*, 2003). Central obesity is a well-established risk factor for metabolic dysfunction in PCOS (Lord *et al.*, 2006) and, furthermore, increased VAT is closely associated with insulin resistance (Hutchison *et al.*, 2011) and seems to be predictive of cardiovascular disease (CVD) (Casella *et al.*, 2008a), specifically atherosclerosis (Grundy, 2002). This CVD risk profile is further exacerbated by atypical blood lipid profiles, with suppressed high density lipoproteins and elevated triglycerides, frequently being expressed in women with PCOS (Wild *et al.*, 1985; Talbot *et al.*, 1995). Given the range and prevalence of co-morbidities evident in women with PCOS, the condition has been described as a gender-specific phenotype of the metabolic syndrome (Expert Panel on Detection/Evaluation, 2001), which is associated with a ~2 fold increased risk of cardiovascular mortality (Alberti, 2006).

Over the past two decades an association between PCOS and CVD has been recognised. This relationship is evidenced by a systematic review and meta-analysis performed by de Groot *et al.* (2011) who investigated the risk of CVD and cardiovascular events in women with PCOS. The study classified PCOS as an independent risk factor for CVD and identified a ~2 fold increased risk of coronary heart disease and stroke. Indeed, PCOS has been acknowledged as a risk factor for CVD by the American Society for Reproductive Medicine Practice Committee (2008b). Nonetheless, while evidence is suggestive of increased CVD risk (Talbot *et al.*, 2000; Wild *et al.*, 2000), the association between PCOS and CVD mortality remains equivocal, since evidence of increased cardiovascular events is limited.

Several risk factors evident in women with PCOS, are associated with endothelial dysfunction. Endothelial dysfunction is an important early event in the development of atherosclerotic disease, which precedes gross morphological signs and clinical symptoms. Specifically, endothelial dysfunction is characterised by impaired bioavailability of the anti-atherogenic molecule nitric oxide (NO), a critical component in endogenous protection against atherosclerosis. Endothelial dysfunction in conduit vessels of women with PCOS, manifest as reduced flow-mediated dilation (FMD), has been observed in some (Casella *et al.*, 2008a), but not all, previous studies (Beckman *et al.*, 2007). In the few studies investigating cutaneous endothelial function in women with PCOS, cutaneous microvessel dysfunction has been observed (Lakhani *et al.*, 2005; Alexandraki *et al.*, 2006) although, more recently, Ketel *et al.* (2008) found similar cutaneous microvessel vasodilator function in obese women with PCOS and controls. Moreover, the underlying mechanism(s) that directly mediate conduit artery and cutaneous microvessel endothelial dysfunction in PCOS are incompletely understood.

Treatment of PCOS is complex, as clinical manifestations vary considerably and thus current pharmacological treatments are limited. Therefore, lifestyle modification, including exercise training, is recommended as a primary prevention strategy of CVD by the androgen excess-PCOS consensus statement (Wild *et al.*, 2010; Harrison *et al.*, 2011). Exercise has been shown to improve the structure and function of vascular endothelial cells and thus offset the development of atherosclerosis in individuals with type 2 diabetes (Maiorana *et al.*, 2001), obesity (Watts *et al.*, 2004), coronary artery disease (Walsh *et al.*, 2003) and metabolic syndrome (Hamdy *et al.*, 2003). Nevertheless, no previous research has compared the therapeutic effect of exercise interventions alone with that of the general lifestyle advice (weight loss via increased physical activity and diet) provided as part of conventional clinical care. Moreover, the effects of exercise training on NO-mediated microvessel endothelial function in women with PCOS have yet to be explored.

In summary, there is some evidence of endothelial dysfunction in women with PCOS. Nevertheless, there is considerable disparity within the existing literature that may relate to considerable inter-subject variability, with different definitions of diagnosis, disease duration, disease severities, and co-morbidities or to poorly matched control groups. Therefore, the potential underlying mechanisms contributing to endothelial function in this population are incompletely understood. Despite the high prevalence of CVD risk (Wild *et al.*, 2000), which extends into the post-menopausal years (de Groot *et al.*, 2011), no study has, to date, investigated the effects of exercise training on NO-mediated conduit artery or cutaneous microvessel endothelial function in women with PCOS.

## **Aims**

Thus, the aims of this thesis are to:

1. Examine the prevalence and extent of endothelial dysfunction, measured using the FMD technique, in women with PCOS according to pre-existing scientific literature, and explore the influence of between-study moderators, such as age, BMI

and diagnostic criteria, on the difference in FMD between PCOS and control women.

2. Investigate the impact of adipose tissue volumes on endothelial function in women with PCOS.
3. Compare the effect of a supervised 16-week moderate-intensity aerobic exercise training programme with conventional clinical care on conduit artery endothelial function in women with PCOS.
4. Investigate cutaneous NO-mediated endothelial function in women with PCOS compared with matched controls.
5. Examine the effect of a supervised 16-week moderate-intensity aerobic exercise training programme on NO-mediated cutaneous microvessel endothelial function in women with PCOS.

**CHAPTER 2**  
**LITERATURE REVIEW**

## 2.1 Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS), originally known as the Stein-Leventhal syndrome, was first described in the 1930's. Stein and Leventhal detailed findings based on seven women with amenorrhea, hirsutism, and polycystic appearance of the ovaries and, in doing so, were the first to depict the triad of clinical characteristics that typify PCOS in its complete phenotype (1935). Today, PCOS is regarded as a heterogeneous syndrome consisting of hyperandrogenism, menstrual dysfunction and the presence of ovarian cysts upon ultrasound examination (Ehrmann, 2005; Franks, 2006).

### 2.1.1 Aetiology and Pathogenesis

Traditionally, PCOS was primarily considered a reproductive disorder. Polycystic ovaries, anovulation and consequent sub-fertility is common among women with PCOS (2008a) and can translate to an increased risk of miscarriage (Cano *et al.*, 1997) or infertility (Norman *et al.*, 2004). In recent times, a complex heterogeneous phenotype of PCOS, encompassing obesity, insulin resistance and androgen excess, has emerged. Currently, the precise aetiology of PCOS remains unclear with both genetic predisposition and environmental influences being implicated (Legro, 1995); therefore, theories alluding to the pathogenesis of PCOS remain hypothetical.

Women with PCOS are more obese than age-matched controls, demonstrating not only an elevated body mass index (BMI), a proxy of global obesity, but also a greater waist: hip ratio (WHR), an anthropometric measure of central obesity (Talbot *et al.*, 1995). Women with PCOS generally display similar volumes of total body fat, but have been found to exhibit increased visceral adipose tissue (VAT) (Yildirim *et al.*, 2003). VAT is adipose tissue that is stored around the internal organs located in the abdomen and is strongly associated with insulin resistance in women with PCOS (Hutchison *et al.*, 2011). Insulin resistance is a common pathophysiological feature of PCOS, which is potentially a

consequence of elevated adipose tissue, and specifically of VAT. However, the molecular mechanisms underlying insulin resistance in PCOS are incompletely understood, although defects in insulin mediated glucose transport (Ciaraldi *et al.*, 1992), GLUT4 production (Ciaraldi *et al.*, 2009) and insulin-regulated lipolysis in adipocytes (Ciaraldi, 2000) have been reported. Insulin resistance results in compensatory hyperinsulinemia to maintain normal glucose levels but, in doing so, adversely affects ovarian androgen production.

More recently, insulin resistance has been considered a pivotal feature of PCOS with approximately 50-70% of women with PCOS exhibiting insulin resistance beyond that usually predicted by BMI (DeUgarte *et al.*, 2005). Insulin resistance causes pancreatic  $\beta$  cells to secrete more insulin in order to maintain euglycaemia, resulting in compensatory hyperinsulinemia which drives many of the phenotypic features of PCOS (Zawadzki & Dunaif, 1992). Notably, hyperinsulinemia promotes ovarian hyperandrogenism (Moggetti *et al.*, 2000) and amplifies the secretion of LH. Insulin acts synergistically with LH to enhance androgen production and inhibits hepatic synthesis of sex hormone binding globulin (SHBG) (a circulating hormone that binds to testosterone), leading to androgen excess (Bergh *et al.*, 1993). This atypical ovarian paracrine signalling disrupts follicle growth and causes menstrual irregularities with resultant chronic anovulation and the accumulation of small follicles on the periphery of the ovary (polycystic morphology) (Balen *et al.*, 1995). Hyperandrogenism is also considered as a central feature of PCOS, as approximately 85% of hyperandrogenic women suffer from the syndrome (Azziz *et al.*, 2009). Androgen excess can be assessed biochemically although cutaneous manifestations of hyperandrogenemia are also apparent in women with PCOS including acne, hirsutism and male pattern alopecia (Ehrmann, 2005).

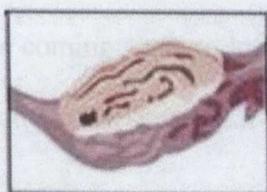
Obese and insulin resistant women with PCOS often exhibit a concomitant set of risk factors, which together epitomise the metabolic syndrome. Indeed, PCOS has been

described as a gender-specific phenotype of the metabolic syndrome (Expert Panel on Detection/Evaluation, 2001). This clustering of risk factors (including metabolic, biochemical and physiological abnormalities) confer substantial cardiovascular risk which exceeds the sum of the risk associated with each abnormality alone (Golden *et al.*, 2002).

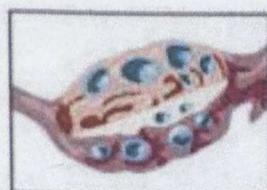
### 2.1.2 Diagnosis and Clinical Manifestations

The symptoms of PCOS typically become apparent at the onset of menarche (Franks, 2002). Traditional diagnosis of PCOS was based on polycystic or enlarged ovaries (Figure 2.1) and the identification of abnormal gonadotropin secretion that invariably manifests in women with PCOS (Waldstreicher *et al.*, 1988). Gonadotropins are protein hormones secreted by gonadotrope cells of the pituitary gland, including follicle stimulating hormone (FSH) and luteinizing hormone (LH) which fluctuate significantly over the course of the menstrual cycle. Ultrasound assessment and accurate diagnosis of polycystic ovaries is complex and the most commonly used criteria are those proposed by Jonard *et al.* (2003). Polycystic ovaries can be established when at least one ovary demonstrates an enlarged ovarian volume ( $>10\text{cm}^3$ ) or  $>12$  follicles measuring between 2-9mm in diameter.

Normal Ovary



Polycystic Ovary



**Figure 2.1** Illustration of a healthy and polycystic ovary and polycystic appearance of the ovary upon ultrasound examination.

Still today, there is not a universal diagnostic criterion for defining PCOS, as symptoms are heterogeneous, vary significantly over time and differ in severity from patient to patient.

Currently, there is one biological definition of PCOS, published by the World Health Organisation (Organization, 1973), and three consensus definitions in widespread use (Table 2.1). The first was defined at proceedings sponsored by National Institute of Health (NIH) and summarised by Zawadski and Dunaif (1992). This definition identifies hyperandrogenism as the key feature of PCOS (following exclusion of other hyperandrogenic disorders) with accompanying oligo-ovulation. In 2003, an expert convention was convened in Rotterdam and concluded that PCOS should be diagnosed based on the presence of two of the following three features i) clinical or biochemical hyperandrogenism (having excluded other possible causes by appropriate biochemical assessments), ii) oligo-/amenorrhoea, iii) polycystic ovaries (2004). More recently, the androgen excess society (AES) defined PCOS as an androgen excess disorder (Azziz *et al.*, 2006). The task force recognised four key features of PCOS including, (i) ovulatory and menstrual dysfunction, (ii) hyperandrogenemia, (iii) clinical features of hyperandrogenism and (iv) polycystic ovaries. Despite lengthy debate among clinicians and academics regarding the definition of PCOS, it is widely accepted that the definition will continue to evolve in coming years to incorporate new research findings. Current clinical guidelines in the UK utilise the Rotterdam criteria for diagnosing PCOS.

**Table 2.1** A comparison of consensus diagnostic criteria for PCOS (Lujan *et al.*, 2008).

### *2.1.3 Prevalence & Epidemiology*

Worldwide, PCOS affects up to 10% of women of reproductive age (Azziz *et al.*, 2004; Manfredi *et al.*, 2006) according to the ‘classical’ NIH criteria (Gazelle *et al.*, 1994); and up to 20% of women according to the broader Rotterdam criteria (Broekmans *et al.*, 2006). Based on this statistic, PCOS is the single most common endocrinopathy in women of reproductive age. Interestingly, the prevalence of PCOS is similar in various populations worldwide (Diamanti-Kandarakis *et al.*, 1999; Asuncion *et al.*, 2000; Azziz *et al.*, 2004; Moran *et al.*, 2010a) suggesting that PCOS is a unique disorder, that manifested prior to human migration, and is not solely lifestyle-induced.

PCOS appears to be more prevalent among family members than in the general population (Legro *et al.*, 1998; Kahsar-Miller *et al.*, 2001). Heritability for PCOS of 0.71 was reported in Dutch twins suggesting that genetic factors do influence the prevalence of the syndrome (Vink *et al.*, 2006). Heritability of a population is an estimate of how much genetics contribute to the prevalence of a characteristic. PCOS appears to be inherited as a complex disorder, wherein several genetic variants are implicated in the development of the disorder

(Goodarzi *et al.*, 2011). The phenotypic expression of PCOS can be exacerbated by lifestyle and environmental influences (Moran & Teede, 2009) which ultimately increases the likelihood of cardiovascular and metabolic complication (Carmina *et al.*, 2005).

## **2.2 Metabolic features of PCOS**

The long term morbidities associated with PCOS extend far beyond the reproductive axis; PCOS is associated with several cardiovascular and metabolic pathologies including obesity, insulin resistance, impaired glucose tolerance, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), dyslipidaemia and hypertension (Goodarzi *et al.*, 2011). Consequently, it might be expected that cardiovascular disease (CVD) would be highly prevalent in women with PCOS (Glueck *et al.*, 2003). The clustering of CVD risk factors that characterises the metabolic syndrome (see section 2.2.3 *The Metabolic Syndrome*) is thought to be the driving force behind the CVD epidemic in Western society. Indeed, it is estimated that individuals with the metabolic syndrome encompass a ~5 fold greater risk of developing type 2 diabetes and are ~3 times more at risk of mortality due to myocardial infarction or stroke, when compared to individuals without the syndrome (Stern *et al.*, 2004). The high prevalence of metabolic co-morbidities in women with PCOS is not surprising, since insulin resistance and central obesity are fundamental pathogenic features of both PCOS and the metabolic syndrome.

### *2.2.1 Obesity and Adipose Tissue*

Women with PCOS generally display elevated global (BMI) and central obesity (waist circumference) compared with matched control women. This is associated with several risk factors for coronary heart disease (CHD) and atherosclerosis (Talbot *et al.*, 1995; Gambineri *et al.*, 2002). The incidence of obesity in PCOS varies geographically, the obese phenotype being most common in Westernised society (Knochenhauer *et al.*, 1998). When compared with weight-matched controls, women with PCOS display a similar volume of

total body fat but a higher volume of central abdominal fat (Carmina *et al.*, 2007b) and VAT (Yildirim *et al.*, 2003). Yildirim *et al.* (2003) assessed VAT in 30 healthy weight PCOS and 30 matched control women and reported a significantly higher volume in the PCOS cohort. This study utilised ultrasound for the assessment of VAT which, although it has well accepted limitations, has been reported to correlate with computed tomography (Armellini *et al.*, 1990). There was also evidence of glucose intolerance, hyperinsulemia and atypical blood profiles, namely raised triglycerides and reduced high density lipoproteins (HDL), despite the absence of obesity. Interestingly, multiple regression analysis revealed that VAT significantly contributed to hyperinsulinemia and triglyceride concentration. Based on Yildirim's findings, it could be hypothesised that VAT accumulation increases circulating triglycerides, which act as a stimulus to increase hepatic secretion of glucose and therein contribute to hyperinsulemia in women with PCOS. Thus, VAT is considered a pivotal and early feature of metabolic disturbance in women with PCOS, independent of obesity. However, using the non-invasive gold standard MRI, VAT has been reported to be similar among overweight and obese PCOS and weight-matched women (Barber *et al.*, 2008).

Although subcutaneous adipose tissue (SAT) accounts for a greater volume of total body fat, VAT is more metabolically active and therefore more susceptible to lipolysis (Montague & O'Rahilly, 2000); hence VAT has been described as an endocrine source of circulating free fatty acids (FFA). A unique anatomical feature of VAT is that it is drained via the portal vein, whereas SAT secretes adipokines into the systemic circulation (Matsuzawa *et al.*, 1995). Thus, VAT has been hypothesised to deliver high concentrations of FFA directly to the liver (see section 2.2.4 *Non-Alcoholic Fatty Liver Disease*) thereby contributing to hyperinsulinaemia by driving hepatic gluconeogenesis (Fujioka *et al.*, 1987). Indeed, excess VAT accumulation is also responsible for the up-regulation of  $\beta$  oxidation which results in subsequent oxidative stress, the release of inflammatory

cytokines (fibrinogen, WBC, CRP & PAI-1) and adipokines (leptin, visfatin & TNF $\alpha$ ) and a reduction of endogenous adiponectin, an anti-inflammatory substance (Carmina *et al.*, 2006; Cascella *et al.*, 2008a; Carmina *et al.*, 2009; Pepene, 2012).

Recently, Hutchison *et al.* (2011) confirmed the relationship between VAT and insulin resistance, following statistical adjustment for age, in women with PCOS. This relationship was identified using the gold standard technique for assessing insulin resistance (euglycaemic clamp) and computer tomography assessment of VAT. A hyperinsulinaemic euglycaemic clamp quantifies insulin resistance by measuring the amount of glucose required to compensate for increased insulin levels. The concentration of insulin can be manipulated to assess either peripheral or hepatic insulin action. Moreover, it was reported that elevated VAT is predictive of CVD in post-menopausal women and obesity has been specifically linked to an increased likelihood of atherosclerosis in both men and women (Grundy, 2002). Furthermore, raised glycohaemoglobin, which reflects insulin resistance, was observed in men and women exhibiting greater adiposity (McGill *et al.*, 2002). Taken together, these findings support the hypothesis that obesity is a risk factor for atherosclerosis in women and that emerging risk factors related to obesity, such as insulin resistance, are atherogenic.

### 2.2.2 *Insulin resistance*

Insulin resistance is often associated with the pathogenesis of PCOS and can be intensified by co-existent obesity (Dunaif *et al.*, 1989). PCOS and obesity together attribute the greatest impairment in insulin sensitivity, indeed, insulin resistance is apparent in 50-70% of women with PCOS and in 95% of obese women with PCOS (Carmina & Lobo, 2004). This augmented insulin resistance has been identified as a potential contributor to the development of type 2 diabetes (Norman *et al.*, 2001) and subsequent CVD risk within this patient group (Meyer *et al.*, 2005c). Both insulin resistance and the progression to type 2

diabetes imply changes in lipid metabolism and a plethora of cardiovascular risk factors. This association is depicted by Folsom *et al.* (1997) who demonstrated a linear increase in CVD risk across quintiles of fasting insulin in non-diabetic women (Figure 2.2).

**Figure 2.2** Sex-specific, age- and race-adjusted incidence of CHD in relation to fasting serum insulin concentrations among people without diabetes in the atherosclerotic risk in communities study. Values within the bars represent the relative risk of CHD for that insulin category referenced to the lowest category (Folsom *et al.*, 1997).

Boudreaux *et al.* (2006) conducted an 8-year follow up in women with PCOS to determine the magnitude of risk in developing type 2 diabetes. Women with PCOS, defined using the Rotterdam criteria, demonstrated an increased relative risk of type 2 diabetes of ~2.3 compared with matched control women. Increasing age and weight gain significantly worsened glycaemic control as obese women with PCOS were ~5 times more likely to develop type 2 diabetes, following statistical adjustment for age. These findings were in agreement with a systematic review and meta-analysis of 35 studies investigating the prevalence of glucose intolerance and type 2 diabetes in women with PCOS (Moran *et al.*, 2010b). The authors found an increased prevalence of glucose intolerance, type 2 diabetes

and the metabolic syndrome in both BMI-matched and unmatched studies. Specifically, PCOS was associated with a ~2.5 fold increased prevalence of impaired glucose tolerance and a ~4 fold increased prevalence of type 2 diabetes, suggesting that insulin resistance is an inherent feature of PCOS, independent of obesity.

### 2.2.3 The Metabolic Syndrome

The relationship between PCOS, type 2 diabetes and CVD is well established. The cluster of CVD risk factors which characterises the metabolic syndrome are highly prevalent in both PCOS and type 2 diabetic patients (Alberti, 2006). According to the latest International Diabetes Federation definition (2006), for an individual to have the metabolic syndrome they must demonstrate central obesity (defined as waist circumference with specific ethnicity values) plus any two of the following; raised triglycerides ( $\geq 1.7\text{mmol/l}$ ), reduced HDL cholesterol ( $< 1.29\text{mmol/l}$  in females), raised blood pressure (SBP $\geq 130\text{mmHg}$ , DBP $\geq 85\text{mmHg}$ ) and/or raised fasting glucose ( $5.6\text{mmol/l}$ ). Hu *et al.* (2004) conducted a thorough review based on 11 prospective European cohort studies (with a median follow-up of 8.8 years) comprising 6156 men and 5356 women, without diabetes, aged 30-89 years. Presence of the metabolic syndrome was based on a revised WHO definition (1998) and the authors found that the more components of the metabolic syndrome evident in a given individual, the higher the rate of cardiovascular related mortality. Components of the metabolic syndrome observed in women with PCOS include; abdominal obesity (Valenzuela *et al.*, 2006), insulin resistance (Teede *et al.*, 2007), dyslipidaemia (Legro *et al.*, 2001) and hypertension (Meyer *et al.*, 2005b). Therefore, rather unsurprisingly, women with PCOS encompass an adverse cardiovascular risk profile (Diamanti-Kandarakis *et al.*, 2006; Ketel *et al.*, 2010).

Dyslipidaemia is the most common metabolic pathology of PCOS with a prevalence of up to 70% (Legro *et al.*, 2001). In particular, suppressed HDL and elevated triglycerides is an

atypical blood lipid profile frequently expressed in women with PCOS (Wild *et al.*, 1985; Talbott *et al.*, 1995), which is indicative of the metabolic syndrome. This dyslipidaemia is thought to stem from increased adiposity, particularly visceral adiposity, which can be reflected by an increased waist circumference (Expert Panel on Detection/Evaluation, 2001). Conversely, the presence of raised blood pressure in young women with PCOS is unclear (Conway *et al.*, 1992; Sampson *et al.*, 1996). Schmidt and colleagues (2011) recently conducted a 21-year follow up study and reported a higher prevalence of hypertension in PCOS compared with age- and BMI-matched control women during the post-menopausal period, suggesting that this component may occur with increasing age.

#### *2.2.4 Non-Alcoholic Fatty Liver Disease*

Women with PCOS have a higher prevalence of fat deposition in the liver, a condition coined Non-Alcoholic Fatty Liver Disease (NAFLD) (Brzozowska *et al.*, 2009). NAFLD represents a disease spectrum, ranging from hepatic steatosis, characterised by deposition of triglycerides in the hepatocytes, through to non-alcoholic steatohepatitis (NASH), characterised by inflammation and fibrosis, which can in turn progress to cirrhosis (Cohen *et al.*, 2011). NAFLD is the most common form of liver disease in the western world, with a prevalence of up to ~70-90% in obese individuals and individuals displaying components of the metabolic syndrome (Marchesini *et al.*, 2005), and encompasses significant CVD risk (Ekstedt *et al.*, 2006). Based on the prevalence of clinical biomarkers such as liver transaminases or ultrasonography to infer the presence of hepatic steatosis, a number of studies have demonstrated a high risk of hepatic steatosis in women with PCOS (Setji *et al.*, 2006; Cerda *et al.*, 2007; Vassilatou *et al.*, 2010; Baranova *et al.*, 2011). Gutierrez *et al.* (2010) identified an increased probability of NALFD in women with PCOS compared with pre- and postmenopausal women (Figure 2.3). PCOS and NAFLD are both associated with cardiovascular risk factors including central obesity, dyslipidaemia and hypertension, but the most common pathology present in both conditions is insulin resistance.

**Figure 2.3** The predicted probability of NAFLD (95% confidence intervals) in premenopausal, postmenopausal and women with PCOS, statistically adjusted for the presence of the metabolic syndrome (Gutierrez-Grobe *et al.*, 2010).

Fat accumulation within the liver is associated with both hepatic (Bugianesi *et al.*, 2005) and adipocyte insulin resistance (Fabbrini *et al.*, 2009). In an insulin resistant state, the inhibitory effect of insulin on peripheral adipose lipolysis is diminished and thereby causes an influx in circulatory FFA and subsequent absorption of FFA by the liver. Additionally, hyperinsulinaemia provokes an increase in *de novo* lipogenesis (Fabbrini *et al.*, 2009), which further adds to FFA accumulation in the liver. The current body of evidence suggests that insulin resistance, a key pathophysiological feature of PCOS, promotes liver fat accumulation. Alarmingly, excess liver fat accumulation can also exacerbate peripheral insulin resistance, via enhanced basal insulin secretion and decreased suppression of hepatic glucose output, and thus generate a vicious cycle that can ultimately lead to diabetes (Taylor, 2008) and, in turn, CVD.

### **2.3 PCOS and Cardiovascular Disease**

The prevention of increasingly prevalent diseases such as CVD is a major public health challenge. In the UK alone, 2.7 million people are living with CVD, which is currently the

main cause of mortality accounting for approximately 191,000 deaths each year (Allender *et al.*, 2011). PCOS has been acknowledged as a risk factor for CVD by the American Society for Reproductive Medicine (ASRM) Practice Committee (2008b). Further supporting evidence for this association came from a systematic review and meta-analysis performed by de Groot *et al.* (2011) investigating cardiovascular events in women with PCOS. The authors identified PCOS as a risk factor for CVD and reported a ~2 fold increased risk of CHD and stroke. Moreover, this increased risk remained in the studies that were statistically adjusted for BMI, demonstrating that increased obesity is not the sole contributing factor of CVD risk in women with PCOS. Nevertheless, while existing epidemiological evidence is suggestive of increased CVD risk (Talbot *et al.*, 2000; Wild *et al.*, 2000) it is yet to be established whether PCOS *per se* and/or the presence of associated cardiovascular risk factors translates into increased CVD mortality.

In 1992, Dahlgren and colleagues (1992) predicted a ~7 fold increased risk of myocardial infarction in women with PCOS, based on a metabolic risk factor model. However, this research group have recently reported findings from a 21 year follow up study based on the same cohort and reported an increased prevalence of CVD risk factors, including hypertension and raised triglycerides, but a similar incidence of myocardial infarction and diabetes related mortality in PCOS and age-matched control women (Schmidt *et al.*, 2011). Wild *et al.* (2000) conducted a 31 year follow up study whereby the research team collated morbidity data from general practice records for 319 women diagnosed with PCOS (prior to 1979) and 1060 age-matched control women. The authors did not report an increased prevalence of either fatal or non-fatal cardiovascular events in women with PCOS, although the prevalence of cardiovascular risk factors and the rate of non-fatal cerebrovascular disease were higher. A much larger scale study found that 15% of 82,439 women experienced menstrual irregularity between the ages of 20-35y. Those with a history of menstrual dysfunction exhibited an increased relative risk of fatal and non-fatal

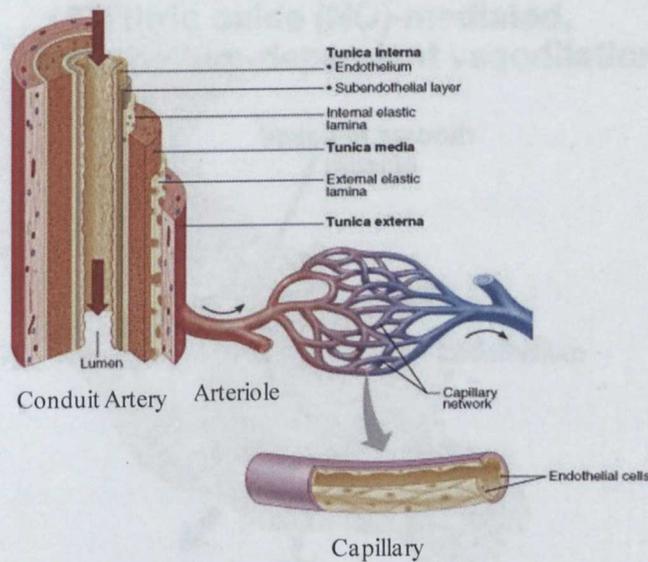
CHD of 1.25 and 1.67, respectively, and this relative risk remained following statistical adjustment for BMI (Solomon *et al.*, 2002). Furthermore, the Women's Ischemia Evaluation Society reported an increased prevalence of angiographic coronary artery disease (CAD) and an elevated risk of adverse cardiovascular events in post-menopausal women displaying clinical features consistent with PCOS (Shaw *et al.*, 2008).

It is of paramount importance to note that epidemiological studies on women with PCOS are fundamentally limited by several recurring factors such as small sample sizes and relatively short follow up periods resulting in a low mean age of the cohort. Furthermore, not all studies have utilised the same diagnostic criteria or indeed any consensus definition of PCOS. It is well accepted that PCOS phenotypes and subsequent risk profiles differ, and thus findings from epidemiological studies must be interpreted with caution. Few studies monitor treatment strategies utilised by women with PCOS during the course of the follow-up period or comment on changing clinical phenotypes over time. Whether the predicted increased risk of CVD mortality in women with PCOS is accurate remains to be confirmed as studies investigating morbidity and mortality well into the postmenopausal period are considerably lacking.

#### **2.4 The Role of the Vascular Endothelium in Conduit Vessels**

The arterial tree consists of an intricate network of blood vessels branching from the heart to conduit arteries, arterioles and capillaries. Conduit vessels are characterised by a thick tunica media that contains a greater amount of smooth muscle cells compared to other branches of the arterial tree (Figure 2.4). The contractility of these vessels allows them to actively vasodilate, under the influence of circulating vasoactive substances and under the control of the sympathetic division of the autonomic nervous system. Arterioles have much smaller internal diameters, which change in response to either sympathetic or endocrine stimulation. Arterioles branch out from conduit vessels and extend to the capillaries (5-

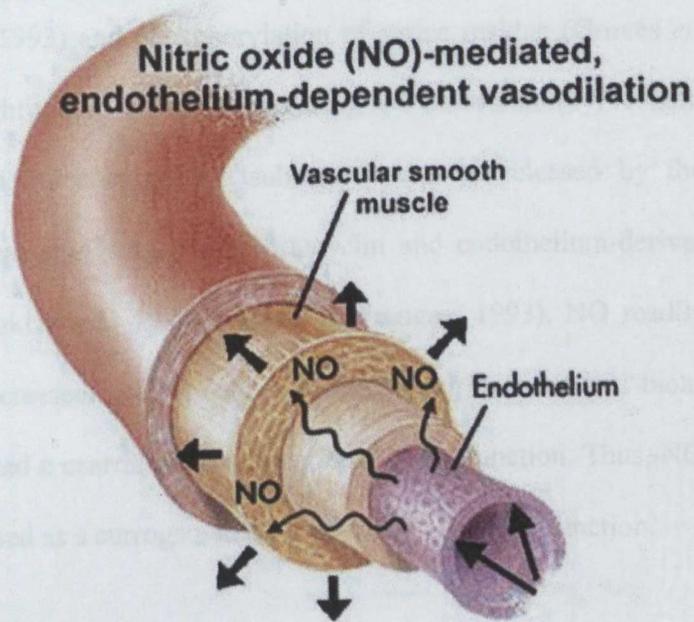
10 $\mu$ m in diameter). Capillaries form an interconnected network, or bed, containing direct connections between arterioles and venules and represent the only component of the arterial tree which permits the diffusion of water, small solutes and lipid-soluble materials into the surrounding interstitial fluid.



**Figure 2.4** The arterial tree from conduit vessels (arteries) through to capillaries.

A squamous epithelial layer lines the inner surface of all blood vessels, this is known as the endothelium (Figure 2.4). Once considered a passive layer of inert cells, the vascular endothelium is now recognised as large endocrine organ that lines the entire vascular compartment at the interface between blood and the vessel wall which is responsible for the maintenance of vascular tone. A healthy endothelium mediates anti-atherogenic properties that protect against vasoconstriction, smooth muscle cell growth and inflammatory responses (Davignon & Ganz, 2004). A healthy endothelium produces numerous paracrine substances, including nitric oxide (NO), which help maintain the health of the vascular wall and regulate vasomotor function (Green *et al.*, 2004). NO is a labile, lipid soluble gas synthesised in endothelial cells from the amino acid L-arginine through the action of endothelial nitric oxide synthase (eNOS) (Palmer *et al.*, 1988). It rapidly diffuses into the vascular smooth muscle of the tunica media where it binds to the

enzyme guanylate cyclase (Ignarro *et al.*, 1986), resulting in an increase in cyclic guanosine monophosphate, which induces smooth muscle relaxation and subsequent vasodilation (Furchgott & Jothianandan, 1991) (Figure 2.5).



**Figure 2.5** Nitric oxide (NO) mediated endothelium-dependent vasodilation.

Additionally, by releasing NO, the endothelium inhibits platelet and leukocyte activation, inflammation and thrombosis while maintaining the vascular smooth muscle in a non-proliferative state (Davignon & Ganz, 2004). Efficient endothelial function is therefore essential in order to maintain the health of vessel walls throughout the arterial tree and NO, in particular, is a vital component in the endogenous defence against atherosclerosis.

NO is tonically secreted by the endothelium and contributes approximately 50% to basal vascular tone (Vallance *et al.*, 1989). NO production can be up-regulated via physiological stimuli such as increased flow and consequent shear stress, or pharmacologically using receptor agonists such as acetylcholine (ACh), to induce acute arterial vasodilation. Vasodilation is a physiological response to an acute release of NO during periods of increased flow (Hutcheson & Griffith, 1991). Presently, the signalling cascade linking

mechanical stimulation to the secretion of NO is incompletely understood; however, several mechanisms are thought to be involved. Increased flow and arterial shear stress have been reported to induce endothelial potassium channel activation (Oleson & Johnson, 1988), calcium influx in endothelial cells (Dull & Davies, 1991), release of bradykinin (Hecker *et al.*, 1993) and phosphorylation of serine residue (Groves *et al.*, 1995); all of which are thought to play a role in increasing NO bioavailability. Nonetheless, it is crucial to acknowledge other vasoactive substances can be released by the endothelium in response to shear stress, such as prostacyclin and endothelium-derived hyperpolarizing factor (Grabowski *et al.*, 1985; Kuchan & Frangos, 1993). NO readily reacts with free radicals, and increased oxygen degradation resulting in a reduced bioavailability of NO. This is considered a central feature of endothelial dysfunction. Thus, NO bioavailability is commonly utilised as a surrogate marker of endothelial (dys)function.

Atherosclerosis is a progressive disease, caused by the deposition of plaques and fatty deposits resulting in thickening of arterial walls, which precedes overt CVD. It is characterised by vascular inflammation and infiltration of lipids, cholesterol, calcium and cellular debris into the sub-intima of the arterial wall, resulting in vascular remodelling, plaque formation, acute and chronic luminal obstruction and consequent blood flow abnormalities (Stary *et al.*, 1995). Clinical manifestations of CVD (e.g. myocardial infarction, angina and stroke) are increasingly apparent with age and the prevalence of risk factors. However, the onset of the atherosclerotic disease process precedes both evidence of traditional cardiovascular risk factors and clinical events (Chan *et al.*, 2003). Endothelial dysfunction is the earliest detectable event in the development of atherosclerosis that precedes gross morphological signs and clinical symptoms (Celermajer *et al.*, 1992; Green *et al.*, 2011). In the presence of endothelial dysfunction, the endothelium may adopt a phenotype that promotes inflammation, thrombosis, vasoconstriction and atherosclerotic lesion formation.

## 2.5 Measurement of Conduit Artery Endothelial Function

Non-invasive assessment of conduit artery endothelial function *in vivo* is commonly utilised to assess NO-mediated vasodilator function. In the 1990's, high-frequency ultrasonographic imaging of the brachial artery was developed to assess NO-mediated endothelial function, using a technique called flow-mediated dilation (FMD). This method involves direct assessment of conduit artery dilator responses to reactive hyperaemia induced by a brief period of limb ischemia (Green *et al.*, 2004). This stimulus causes a shear stress that provokes the endothelium to release NO, which induces a subsequent vasodilation (i.e. increase in arterial diameter) that can be imaged using duplex ultrasonography (Dijkhorst-Oei *et al.*, 1999). On the assumption that the occluding cuff, which induces the ischemia, is positioned distal to the imaged artery (Doshi *et al.*, 2001) and that the period of ischemia is not greater than five minutes (Mullen *et al.*, 2001), the vasomotor response of the vessel to this stimulus is largely mediated by NO (Joannides *et al.*, 1995; Doshi *et al.*, 2001). The FMD response can therefore provide an index of conduit artery endothelium-dependent NO function (Ganz & Vita, 2003).

FMD was first described by Schretzenmayr (1933), however, it was not until 1986 that the importance of the endothelium in mediating this response was demonstrated (Pohl *et al.*, 1986). Pohl and colleagues (1986) demonstrated that the femoral artery FMD response of canines is significantly attenuated if the endothelium is denuded. A follow on study was conducted by Sinoway *et al.* (1989), who first described the phenomenon of FMD *in vivo* within humans, reporting a delayed dilation of the brachial artery after the time of peak blood flow, following a reactive hyperaemic challenge. Soon after, Celermajer *et al.* (1992) demonstrated that an impaired FMD is exhibited in populations with CVD risk factors. Importantly, endothelium-derived NO was confirmed as the primary mediator of FMD in humans by Joannides *et al.* (1995), who reported an attenuated FMD response in the radial artery during the infusion of NO blocker; L-N<sup>G</sup>-monomethyl Arginine (L-NMMA). This

finding has also been demonstrated in the brachial (Doshi *et al.*, 2001; Mullen *et al.*, 2001) and femoral artery (Kooijman *et al.*, 2008).

Doshi *et al.* (2001) highlighted the importance of cuff placement during the FMD technique to ensure a NO-mediated vasodilator response. This study demonstrated that a NO blockade almost completely abolished the vasodilator response to FMD when the cuff was placed distal to the imaged artery (Figure 2.6). Distinct from this was the observation that when the cuff was positioned proximally, NO blockade had much less of an effect on the vasodilator response. Similarly, the duration of the hyperaemic stimulus is an important factor when considering the validity of FMD. Indeed, Mullen *et al.* (2001) reported that the FMD response to transient reactive hyperaemia (5 minutes) was almost completely abolished by NO blockade, however, following a period of sustained reactive hyperaemia (>5 minutes) the subsequent FMD response was unaffected by NO blockade. Thus, in order to produce a vasomotor response that accurately reflects the bioavailability of NO and is less subject to operator bias, FMD must adhere to stringent guidelines, with any deviation in protocol potentially increasing the contribution of alternative vasodilator pathways (Thijssen *et al.*, 2011).

**Figure 2.6** NO contribution to FMD using proximal and distal cuff occlusion during control and L-NMMA infusion (Doshi *et al.*, 2001).

## **2.6 Prognostic Relevance of Flow-Mediated Dilation**

The observation that FMD provides a direct assessment of arterial wall function has enthused interest in the prognostic value of the technique. Indeed, recent evidence suggests that FMD may possess prognostic value in patients at high risk of CVD (Green *et al.*, 2011) and, specifically, FMD has been reported to be an accurate independent predictor of occult CAD (Mutlu *et al.*, 2011a). Moreover, the independent prognostic information provided by FMD may exceed that of traditional risk factors in clinical populations (Naghavi *et al.*, 2003). Takase *et al.* (1998) induced endothelium-dependent vasodilation in both the coronary and brachial artery as a result of increased flow and subsequent shear stress in response to either 20 $\mu$ g adenosine triphosphate disodium (ATP) infusion or reactive hyperaemia. This group reported a robust relationship between coronary and brachial artery endothelial function ( $r=0.79$ ,  $P<0.001$ ) and proposed that brachial artery FMD could be utilised as a prognostic marker for coronary artery endothelial function (Figure 2.7). As well as being strongly and independently associated with CVD risk and mortality in CAD

(Takase *et al.*, 1998; Chan *et al.*, 2003); FMD has also been reported as an accurate prognostic tool in CVD (Gokce *et al.*, 2003), peripheral vascular disease (Brevetti *et al.*, 2003; Gokce *et al.*, 2003) and chronic heart failure (Meyer *et al.*, 2005a). Such studies suggest that impaired FMD response in clinical groups is potentially indicative of future cardiovascular events.

**Figure 2.7** The relationship between flow-mediated dilation in a coronary artery stimulated by 20 $\mu$ g of ATP and flow-mediated dilation in a brachial artery stimulated by hyperaemia (Takase *et al.*, 1998).

Interestingly, less data exist regarding the prognostic value of FMD in asymptomatic individuals; some existing studies support the independent prognostic value of the technique (Shechter *et al.*, 2009) while others imply no difference in the capacity of FMD to predict CVD risk compared to traditional risk factor assessment (Shimbo *et al.*, 2007). Yeboah and colleagues (2007) examined the prognostic value of FMD in an asymptomatic aged (72-98 years) population and found FMD to be an accurate independent predictor of cardiovascular event rate. However, the prognostic value of FMD observed in this study only amounted to a further ~1% predictive accuracy compared with that of traditional cardiovascular risk factors. This finding is in agreement with previous research suggesting

that FMD may be a less accurate predictor of CVD risk in older cohorts (Witte *et al.*, 2005). Nevertheless, a more recent large scale multi-ethnic population-based study in 3026 younger asymptomatic participants reported that FMD added further prognostic information regarding cardiovascular event incidence than is provided by the Framingham risk score, when classifying patients into risk categories (Yeboah *et al.*, 2009). Importantly, this finding is indicative that the prognostic value of FMD may be stronger than traditional CVD risk screening in individuals with no overt evidence of CVD. A recent meta-analysis of 14 heterogeneous observational studies examining the relationship between brachial artery FMD and cardiovascular events suggested that impairment of brachial artery FMD is significantly associated with future cardiovascular events (Inaba *et al.*, 2010). The authors published robust risk ratios (calculated from individual studies and then pooled using random-effects models) indicating that that the risk of a cardiovascular event increases by 21% for every 1 standard deviation (=3.5%) decrease in FMD.

FMD is traditionally considered as an index of NO-mediated vasodilator function and thus a marker of NO bioavailability, although protocols utilised to induce an FMD response in humans are not all equally NO dependent (Corretti *et al.*, 2002; Green *et al.*, 2005). Nonetheless, a recent meta-analysis conducted by Green *et al.* (2011) revealed that the FMD response to proximal cuff occlusion, which is thought to be less NO-mediated than the response to distal cuff occlusion (Doshi *et al.*, 2001), is in fact equally predictive of future cardiovascular events. Whilst this does not diminish the predictive power of FMD, these data suggest that NO may not be the sole contributor to the prognostic value of the technique. In addition, as atherosclerosis is systemic in its development, FMD provides the opportunity to gain insight into coronary artery health using this direct, accurate and non-invasive technique.

## 2.7 Reproductive Hormones and Conduit Artery Function

Given that PCOS is an endocrine disorder, primarily due to dysfunction in reproductive hormone production, it is important to note that female reproductive hormones, namely oestrogen, may confer some protection on the vascular system. The biosynthesis of oestrogen is primarily influenced by FSH, which regulates the rate-limiting enzyme aromatase that is responsible for converting oestrogens from androgens. In women with PCOS, the ratio of LH (regulates androgen synthesis) to FSH is decreased due to an accelerated hypothalamic gonadotropin-releasing hormone (GnRH) frequency (Ehrmann, 2005). As a result, aromatase activity is diminished which in turn reduces the biosynthesis of oestrogen, and androgenic biosynthesis is up-regulated due to increased LH secretion. Therefore, oestrogen is depleted and testosterone is elevated in women with PCOS compared with non- PCOS women.

Williams *et al.* (2001) studied changes in FMD during the menstrual cycle in 15 healthy normally menstruating women during the early follicular, late follicular, early luteal, and late luteal phases (Figure 2.8). The authors reported that FMD was higher in the follicular phase than the menstrual or luteal phase, which corroborates previous findings (Hashimoto *et al.*, 1995). It is hypothesised that this inherent variability in FMD response throughout the course of the menstrual cycle is due to changes in oestrogen, which progressively rises from menstruation through the follicular phase then proceeds to fall during the luteal phase (Guyton & Hall, 1991). Herrington *et al.* (2001) assessed brachial artery FMD in 1636 post-menopausal women, 512 of whom were taking oestrogen replacement. There was an association between oestrogen and improved FMD only in women who did not demonstrate overt CVD, suggesting that oestrogen is more effective for the maintenance of vascular health than for the treatment of established vascular disease. The influence of oestrogen to promote vasodilation appears to be due to its ability to up-regulate eNOS activity in the peripheral vasculature. This effect was clearly demonstrated by Hayashi *et al.*

(1995) who observed increased activity of eNOS in cultured human endothelial cells following 8 hours of oestrogen administration.

**Figure 2.8** FMD of the brachial artery with reactive hyperaemia across the four phases of the menstrual cycle. Individual data are shown together with the mean $\pm$ SEM. \*Significant difference from one phase to another ( $P<0.05$ ). Endothelium-independent changes in brachial artery vasodilation did not significantly change across the four phases of the menstrual cycle (Williams *et al.*, 2001).

The effect of androgens on vascular reactivity is less studied than the cardioprotective effect of oestrogen. Numerous studies have confirmed an increased prevalence of premature atherosclerotic disease in men (Lerner & Kannel, 1986; Wingard *et al.*, 1989); however, there has been no consistent association between androgen levels within sex-specific physiological ranges and cardiovascular event rates. Androgen deprivation in men is associated with enhanced vascular reactivity (Herman *et al.*, 1997; Empen *et al.*, 2012) and testosterone replacement therapy has been shown to decrease FMD of the brachial

artery in hypogonadal men (Bernini *et al.*, 2006). Similarly, high dose androgen treatment in women is associated with a reduced brachial artery FMD (McCredie *et al.*, 1998). However, the high dose treatment utilised in this study was in female-to-male transsexuals and as such testosterone levels in these subjects were highly elevated (~15mmol/l) compared with that observed in hyperandrogenic women with PCOS (>2.5mmol/l). The underlying mechanism(s) relating to androgens and impaired vascular reactivity remains unknown. In some studies, testosterone therapy has been found to decrease HDL cholesterol (Thompson *et al.*, 1989), however, the existing data concerning the relationship between androgens and lipoproteins have not consistently shown detrimental effects on endothelial function (Barrett-Connor, 1995). It is plausible that androgens impose direct effects on the vessel wall, as steroid receptors exist in the vasculature, but this hypothesis remains unfounded.

## **2.8 PCOS and Conduit Artery Function**

Endothelial dysfunction, manifest as reduced FMD, has been observed in women with PCOS when compared to control women in some (Casella *et al.*, 2008a), but not all, previous studies (Beckman *et al.*, 2007). On balance, the weight of evidence supports the presence of endothelial dysfunction in women with PCOS and inconsistencies in the extant literature may be related to the considerable variability between subjects or the heterogeneity of PCOS itself, with different definitions of diagnosis, disease duration, disease severities, and co-morbidities.

Meyer *et al.* (2005c) evidenced impaired endothelial dysfunction in obese and insulin resistant women with PCOS compared with age- and BMI-matched controls and reported that FMD was significantly correlated with both insulin resistance and fasting insulin, which corroborated previous findings (Sorensen *et al.*, 2006; Soyman *et al.*, 2011). Impaired insulin regulation is known to decrease the activation of eNOS and subsequent

release of NO (Steinberg & Baron, 2002) suggesting that insulin resistance contributes to endothelial dysfunction in women with PCOS. Nevertheless, several studies have reported normal endothelial function in PCOS (Mather *et al.*, 2000; Brinkworth *et al.*, 2006). Arikan *et al.* (2009), for example, compared brachial artery FMD and carotid artery intima media thickness (cIMT, an established structural marker for early atherosclerotic disease) in 39 healthy weight women with PCOS with evidence of insulin resistance and 30 age- and BMI-matched controls. Despite the presence of insulin resistance determined via numerous methods (QUICKI index, Matsuda index and insulin glucose disposal AUC) there was no difference in either FMD or cIMT between PCOS or control women. Moreover, there was no correlation between insulin resistance and FMD. There are several potential mechanisms that could explain these differences. Firstly, the PCOS cohort utilised in the study by Arikan *et al.* (2009) were ~10 years younger than those in Meyer's study and thus an effect of age or disease duration could account for inconsistencies. Secondly, although insulin resistance was evident in both studies, hyperinsulinaemia was only apparent in obese women with PCOS, suggesting that fasting insulin may be a more accurate predictor of endothelial function. Thirdly, studies have utilised women with PCOS with ranging BMI; it has been postulated that the presence of obesity in PCOS may further increase the risk of CVD as obesity contributes to a more severe metabolic phenotype of PCOS (Carmina *et al.*, 2005; Carmina *et al.*, 2006).

Pepene *et al.* (2011) recorded significantly lower brachial artery FMD in women with PCOS compared with controls although the women with PCOS in this study displayed significantly greater BMI and total fat mass. A recent meta-analysis identified BMI as a contributor to the risk of CHD and stroke in women with PCOS (de Groot *et al.*, 2011), and therefore BMI must be considered as a moderating factor on FMD in women with PCOS. Nonetheless, it is important to note that BMI is not the most accurate marker of obesity as it cannot distinguish between adipose and lean tissue (Oreopoulos *et al.*, 2011).

Women with PCOS demonstrate similar total and trunk fat volumes, but a higher relative quantity of central abdominal fat (Carmina *et al.*, 2007b) and visceral adiposity (Casella *et al.*, 2008a) compared with BMI-matched controls. Casella *et al.* (2008a) assessed brachial artery FMD and VAT in 200 overweight women with PCOS and 100 matched controls. The authors demonstrated impaired FMD and elevated VAT in the PCOS cohort despite being matched for BMI. A significant relationship between elevated VAT and impaired FMD response was also evident. Although this relationship has been described previously (Hashimoto *et al.*, 1998), this is the first study to demonstrate this relationship in women with PCOS. VAT is associated with insulin resistance and adverse lipid profiles, therefore further studies are required in order to determine the underlying mechanisms associated with VAT that influence endothelial function in this population.

Depending on the definition utilised for diagnosis, the characterisation of PCOS can differ significantly. Hyperandrogenism is a fundamental feature of 'classic' PCOS (Zawadzki & Dunaif, 1992) and these patients also exhibit greater menstrual irregularity, total and abdominal obesity, insulin resistance and demonstrate more severe risk factors for type 2 diabetes and CVD (Carmina *et al.*, 2005; Welt *et al.*, 2006; Carmina *et al.*, 2009; Moran & Teede, 2009). Although, El-Kannishy *et al.* (2010a) reported significantly reduced FMD in lean and obese women with PCOS, diagnosed using the Rotterdam criteria and compared with a lean, age-matched control group. The authors reported no relationship between FMD and insulin resistance or BMI but the data did support the role of hyperandrogenism in vascular reactivity. Previous studies have outlined the role of sex hormones in the maintenance of arterial health in PCOS (Golden *et al.*, 2002; Westerveld *et al.*, 2008) although the role of androgens in relation to endothelial function remains unclear. Interestingly, the authors described "interplay" between androgens, dyslipidaemia, adiposity, insulin resistance and endothelial function. Further research is required in order

to establish the specific effect these co-morbidities exert on endothelial function in women with PCOS.

## **2.9 The Role of the Vascular Endothelium in Cutaneous Microvessels**

Primarily, the assessment of endothelial dysfunction as a surrogate marker of CVD has focussed on conduit vessel function. However, a growing body of evidence has emerged suggesting that the microcirculation (network of microvessels) may be the initial site of endothelial damage preceding structural changes and endothelial dysfunction in macrovessels (Brodsky *et al.*, 2004). The microcirculation is taken to include vessels <150µm in diameter (De Boer *et al.*, 2012). The skin is the largest and most accessible organ in humans and as such cutaneous circulation has emerged as an accessible and representative vascular bed for investigating microvascular endothelial function and disease status (Holowatz & Kenney, 2007). Although cutaneous microvessels share common characteristics with vessels elsewhere within the vascular tree they possess distinct physiological and anatomical features. For example, non-acral skin plays a crucial role in thermoregulation through which blood flow can fluctuate between near zero (hyperthermia) to ~60% cardiac output during extreme heat stress (Kellogg, 2006).

Cutaneous microvascular function correlates with arterial endothelial function (Khan, 2008) and several other traditional cardiovascular risk factors which frequently manifest in women with PCOS including obesity (de Jongh *et al.*, 2004) and hypertension (Rizzoni *et al.*, 2003). Systemic microvascular endothelial dysfunction is a key component in the inherent pathogenic complications associated with diseases such as type 2 diabetes, hypertension, CAD and hypercholesterolemia (Joannides *et al.*, 2006). Evidence for this claim comes from the highly documented prevalence of microvascular complications exhibited by type 2 diabetic patients, such as neuropathy. Thus, it is imperative to independently assess the health and function of the microvasculature in clinical

populations such as PCOS, in which insulin resistance and type 2 diabetes are common pathologies.

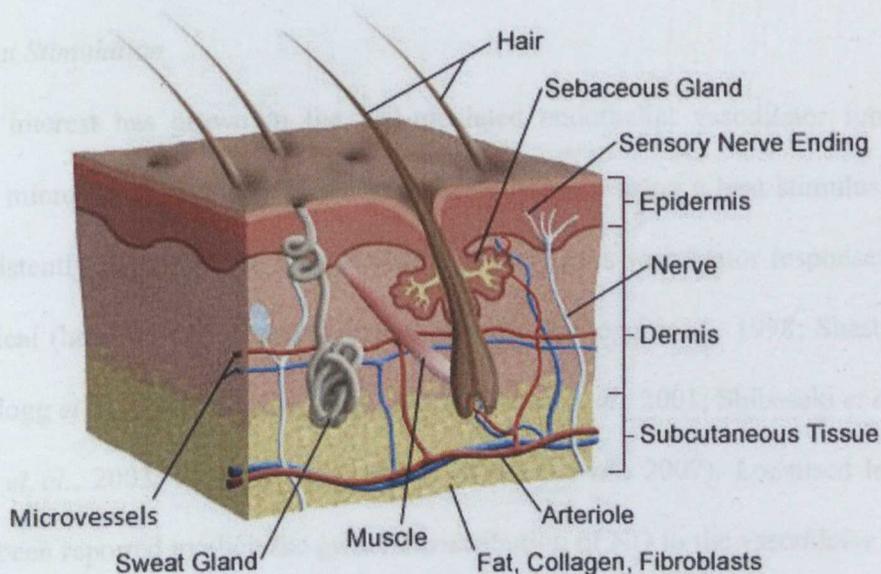
### **2.10 Measurement of Cutaneous Microvessel Endothelial Function**

Laser Doppler flowmetry (LDF) is a versatile technique that allows direct monitoring of the density and velocity of blood cells within a sample volume (flux) both at rest and during maximal provocation. As well as providing an index of maximal dilator capacity of the skin (which is diminished with certain pathologies, see; 2.13. *PCOS and Cutaneous Microvessel Function*), LDF can also be used in combination with techniques such as intradermal microdialysis to comprehensively interrogate the control of cutaneous microvascular endothelial function, by infusing vasoactive agonist and antagonist substances (Figure 2.9).

**Figure 2.9** The microdialysis fibre is inserted ~0.3-1.0mm beneath the epidermal surface. A 10mm semi-permeable membrane enables infusion of pharmacological agents directly into the microvascular bed. A laser Doppler probe is positioned directly above the experimental site to monitor cutaneous blood flow (Cracowski *et al.*, 2006).

### 2.10.1 Intradermal Microdialysis

Intradermal microdialysis is a technique that allows the continuous local delivery of potent pharmacological agents into the epidermis (Figure 2.9) via a semi-permeable membrane, with no systemic effect. Adapted in the mid 1990's for dermatological work, this novel technique is the optimal method for administering L-NMMA (a NO blockade) as well as sodium nitroprusside (SNP, a NO donor) to the interstitial space beneath the epidermal layer (Figure 2.9). Flux over this localised area can be quantified using LDF, therefore, changes in cutaneous blood flow induced by pharmacological stimuli can be recorded (For details regarding the methodology of intradermal microdialysis, see *Chapter 7*). SNP can be administered in conjunction with peak thermal hyperaemia to elicit maximal cutaneous vasodilation that enables flux measures to be expressed as a percentage of a maximal response. Maximal cutaneous vascular conductance ( $CVC_{max}$ ) incorporates mean arterial pressure and laser Doppler flux ( $CVC = \text{laser Doppler flux} / \text{mean arterial pressure}$ ).



**Figure 2.10** A cross sectional diagram of human skin and integrated microvessels.

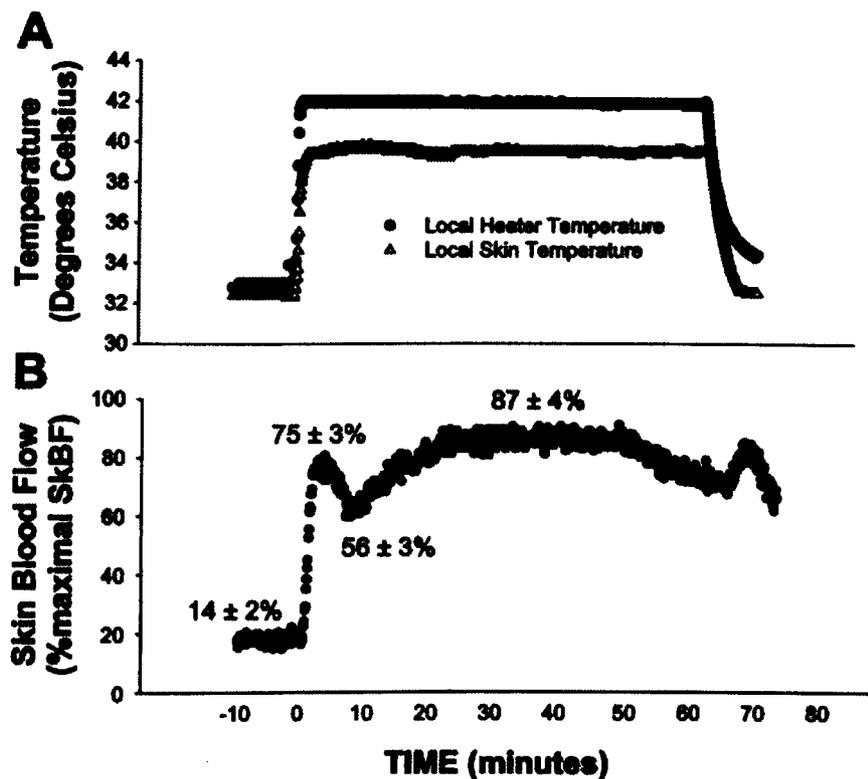
Unlike iontophoresis, another technique to interrogate the cutaneous microvasculature, which utilises opposing electrical currents to deliver charged pharmacological agents to localised areas of skin is microdialysis and this method does not exhibit any confounding

effects of electrical current-induced hyperaemia. Iontophoresis has additional well-accepted limitations such as individual variation in the electrical resistance characteristics of the dermal barrier, which microdialysis has been argued to overcome (Cracowski *et al.*, 2006). Although microdialysis is minimally invasive and only causes minor reversible trauma, the technique is limited by the potential confounding effect of the trauma caused during fibre insertion. Indeed, cutaneous drug delivery can be influenced by probe insertion depth, differences in barrier functions of the skin, lag time (duration of time before the substance enters the skin from time of administration), elimination or metabolism rate of the delivered agent and possibly the volume of distribution (Kreilgaard, 2002). Variability can be minimised through good working practice and careful instrumentation (minimising trauma), whereby the same researcher performs all cannulations; ensuring a consistent cannulation depth is maintained across all participants and time points of the study design.

### 2.10.2 Heat Stimulation

Particular interest has grown in the NO-mediated endothelial vasodilator function of cutaneous microvessels, which has routinely been assessed using a heat stimulus. Studies have consistently shown NO to be a mediator of cutaneous vasodilator responses to both physiological (heating) and pharmacological stimuli (Kellogg *et al.*, 1998; Shastry *et al.*, 1998; Kellogg *et al.*, 1999; Shastry *et al.*, 2000; Minson *et al.*, 2001; Shibasaki *et al.*, 2002; Holowatz *et al.*, 2003; Kellogg *et al.*, 2005; Stewart *et al.*, 2007). Localised heating at 42°C has been reported to elicit the greatest contribution of NO to the vasodilator response (~70%, (Kellogg *et al.*, 1999; Minson *et al.*, 2001)), compared to other stimuli such as ACh administration (~30-60%, (Boutsiouki *et al.*, 2004; Kellogg *et al.*, 2005; Stewart *et al.*, 2007)) and whole body heating (~30-40%, (Shastry *et al.*, 1998; Wilkins *et al.*, 2003)). Localised heating is therefore the most specific methodology currently available for the investigation of NO-mediated cutaneous microvessel vasodilator function.

Localised heating of the skin stimulates a temperature-dependent sustained increase in cutaneous blood flow (Charkoudian, 2003) and achieves maximal vasodilation of cutaneous microvessels between 42°C-44°C (Christen *et al.*, 2004). The utilisation of a rapid heating protocol (0.5°C increase in heater temperature every 5 seconds) has been found to elicit a biphasic response (Minson *et al.*, 2001) (Figure 2.11). This maximal vasodilator response consists of an initial rapid peak in blood flow within the first 10 minutes, which has been reported to occur as a result of axon reflexes of local sensory nerves initiating the release of known vasodilators; calcitonin gene related peptide and substance P which elicit an increase in cutaneous blood flow. This is followed by a secondary rise to a sustained plateau after approximately 20-30 minutes of heating (Minson, 2010). The secondary slow rise and plateau phase of the localised heating response is reported to be largely NO-mediated (Kellogg *et al.*, 1998; Minson *et al.*, 2001), with recent evidence suggesting that NO is generated from eNOS (Kellogg *et al.*, 2008). However, eNOS inhibition by intradermal infusion of L-NMMA does not fully suppress the plateau phase response to localised heating, suggesting that other vasodilators may be involved (Minson, 2010).



**Figure 2.11** A The local heater temperature of 42°C warms the skin to ~40°C which has been shown to elicit maximal cutaneous vasodilatation. B Typical bi-phasic response to the application of local heating, with the initial axon reflex stimulating a response ~75% of the maximal response, and the secondary plateau phase (NO-dependent) ~87% of maximal response (Minson *et al.*, 2001).

To more thoroughly assess the cutaneous NO dilator system, a more gradual heating protocol can be utilised that does not provoke the initial axon reflex-mediated response (0.5°C rise every 5 minutes, Figure 2.12) (Houghton *et al.*, 2006). Consequently, in recent years several studies have adopted this gradual heating protocol to provide a more accurate assessment of cutaneous microvessel NO vasodilator function (Figure 2.12). In addition, this technique has been complimented with infusion of SNP following either peak thermal or ACh-induced hyperaemia, (or a combination) to confirm the attainment of maximal cutaneous vascular conductance (Figure 2.13).

**Figure 2.12** A more gradual applied heating protocol results in a smaller initial axon reflex compared to the fast protocol utilised in Figure 2.8 (Houghton *et al.*, 2006).

**Figure 2.13** A more gradual applied heating protocol avoids provocation of the initial axon reflex mediated response. Peak thermal-induced hyperaemia can be complimented with infusion of SNP to confirm the attainment of maximal cutaneous vascular conductance (Black *et al.*, 2008b).

## **2.11 Reproductive Hormones and Cutaneous Microvessel Function**

Since atherosclerosis is largely limited to conduit arteries, studies have mainly focused on the role of reproductive hormones in these vascular beds. However, there is evidence that female hormones alter thermoregulation and thus the control of cutaneous blood flow (Charkoudian, 2010). Previously, Charkoudian *et al.* (1999) examined the effect of exogenous steroid hormones (oestrogen and progesterone), using the oral contraceptive pill, on the cutaneous vasodilator response to local warming in young women. The authors reported an augmented vasodilator response and hypothesised that this effect was primarily induced by oestrogen, which is in accordance with an abundance of previous research documenting the cardio-protective role of oestrogen on the microvascular endothelium (Cracowski, 2011). More recently, Sokolnicki *et al.* (2007a) examined the effect of oestrogen and testosterone on cutaneous microvessel dilation in response to local heating in elderly males. All participants were simultaneously administered with a GnRH agonist, to suppress endogenous testosterone and oestrogen production, and an aromatase inhibitor to block the conversion of androgens to oestrogen. Thereafter, subjects were divided into four groups whereby (a) received a testosterone gel, (b) received an oestrogen patch, (c) received both forms of hormone replacement and (d) received no hormone replacement. This study reported no consistent influence of either testosterone or oestrogen on cutaneous NO-mediated vasodilation. This surprising finding was attributed to the fact that oestrogen concentrations were relatively low in comparison to those observed in women where an effect on vasodilation was observed. The authors concluded that the effect of testosterone on vasodilator function remains unclear.

## **2.12 PCOS and Cutaneous Microvessel Function**

Microvascular dysfunction can impact both vascular resistance and insulin-mediated glucose disposal in the periphery, thereby contributing to hypertension and insulin resistance, both of which are commonly observed in women with PCOS (Cussons *et al.*,

2006). Therefore, endothelial dysfunction in the peripheral vascular system has been suggested as a primary mechanism for cardiovascular and metabolic risk factors commonly observed in PCOS (De Boer *et al.*, 2012). In the few studies investigating cutaneous endothelial function in PCOS, cutaneous microvessel dysfunction has been observed in response to venous occlusion plethysmography (Paradisi *et al.*, 2001), wire myography (Kelly *et al.*, 2002), iontophoresis-mediated infusion of ACh (Lakhani *et al.*, 2005) and microdialysis-mediated endothelin A and B agonist infusion (Wenner *et al.*, 2011). Paradisi *et al.* (2001) were the first to demonstrate endothelial dysfunction in the microcirculation of women with PCOS using invasive leg plethysmography. The authors measured leg blood flow in response to graded intrafemoral artery infusions of methacholine chloride, an endothelium-dependent vasodilator, and to euglycemic hyperinsulinemia in 12 obese women with PCOS and 13 age- and weight-matched control women. Leg blood flow was observed to be 50% lower in women with PCOS compared with matched controls following methacholine chloride infusion, and increased by only 30% in response to euglycemic hyperinsulinemia as opposed to 60% in controls.

More recently, Lakhani *et al.* (2005) observed microvascular dysfunction in women with PCOS compared with matched controls using iontophoresis-mediated infusion of ACh and SNP. The authors postulated that the abnormal response to ACh observed may have been due to metabolic disturbance in the PCOS cohort, as an impaired response to ACh has been demonstrated in non-insulin dependent diabetic patients (Berghoff *et al.*, 2002). However, some years later, Ketel *et al.* (2008) reported that cutaneous microvascular function was similar in women with PCOS and matched controls. Although ACh-mediated dilation is postulated to reflect NO bioavailability, Holowatz *et al.* (2005) reported the contribution of NO to this response is minimal. More recently, Wenner *et al.* (2011) infused endothelin A and B agonists via the gold standard technique of intradermal microdialysis and measured cutaneous blood flow using LDF during local heating to 42°C. Although, this study

investigated the impact of the vasoconstrictor endothelin-1 in women with PCOS; the authors observed microvessel vasodilator function is compromised in women with PCOS.

Studies have consistently demonstrated that the NO system is a mediator of microvessel vasodilation to a variety of stimuli (Minson *et al.*, 2001; Holowatz *et al.*, 2003) and that augmented cutaneous NO-mediated microvessel function confers anti-atherogenic adaptation (Black *et al.*, 2008b). The NO-mediated vasodilator pathway plays a crucial role in the control of cutaneous endothelial function and studies that have employed the optimal microdialysis technique (Cracowski *et al.*, 2006) to elicit a NO blockade have demonstrated attenuated NO function in aged (Minson *et al.*, 2002) populations. Nonetheless, information is currently lacking on the NO contribution to microvascular endothelial dysfunction in women with PCOS.

### **2.13 Current Treatment Strategies for PCOS**

Treatment of PCOS is complex as clinical manifestations vary considerably and thus current pharmacological treatments are limited. Traditional treatment strategies in PCOS target anovulatory uterine bleeding, hirsutism and acne, all of which can be managed to a certain degree by administration of the oral contraceptive pill. Recent focus has turned to the management of metabolic and cardiovascular complications.

#### **2.13.1 Metformin**

Presently, the pharmacological therapy metformin plays an integral role in the management of PCOS. Metformin is an insulin sensitising agent and has been found to ameliorate endothelial dysfunction in women with PCOS (Diamanti-Kandarakis *et al.*, 2010). Romualdi and colleagues (2008) observed an increased basal diameter of the brachial artery and furthermore an increased FMD response in 13 women with PCOS following three and six months of metformin therapy (1000mg/day). Nevertheless, this study

exclusively recruited healthy weight women with PCOS with no evidence of insulin resistance, therefore, these findings cannot be generalised to the PCOS population at large who inherently exhibit an increased risk of type 2 diabetes (Teede *et al.*, 2007). Diamanti-Kandarkis *et al.* (2005) assessed brachial artery FMD in 20 overweight insulin-resistant women with PCOS, prior to and following 6 months of metformin administration (1700mg/day), and in 20 matched control women. The authors observed impaired FMD in women with PCOS that reversed to control levels following metformin therapy. Such studies support the hypothesis that metformin may reduce the long-term risk of atherosclerosis and CVD in women with PCOS. At a molecular level metformin seems to enhance activation of eNOS in cultured endothelial cells (Davis *et al.*, 2006) and inhibits oxidative stress, thereby increasing the bioavailability of NO. However, metformin administration often results in well documented adverse side effects including nausea and abdominal pain (Diamanti-Kandarakis *et al.*, 2010).

### *2.13.2 Lifestyle Interventions*

As an alternative, non-pharmacological therapies, such as lifestyle modification are advocated (Wild *et al.*, 2010), and have been investigated over recent years as a preventative strategy for CVD in women with PCOS. The hypothesis that type 2 diabetes can be prevented is supported by clinical trials encompassing diet, exercise, or both in persons at high risk for the disease (Tuomilehto *et al.*, 2001). The largest study to investigate the combined effect of diet and exercise was conducted by the diabetes prevention programme research group (2002) who employed a four-year lifestyle intervention in individuals who displayed raised fasting glucose. Participants were randomly assigned to either placebo, metformin (850mg, twice daily) or a lifestyle-modification programme targeting 150 minutes of physical activity per week and healthy eating. The incidence of diabetes was 58% lower in the lifestyle intervention group and 31% lower in the metformin group when compared to the placebo. This study clearly

demonstrated that it is possible to delay the development of type 2 diabetes, and its associated complications such as CAD and CVD, in the absence of pharmacological therapy (Knowler *et al.*, 2002).

A small randomised study demonstrated that nutritional counselling alone ( $n=5$ ) compared with nutritional counselling and exercise ( $n=7$ ; 3 sessions of walking/cycling and 12 resistance exercises) each reduced fasting insulin in women with PCOS (Bruner *et al.*, 2006). Notably, there were increased benefits, evidenced by greater reductions in body fat with exercise, although the authors could not distinguish between the effects of aerobic and resistance exercise training. A second study examined three forms of lifestyle modification in women with PCOS; diet alone, diet and aerobic exercise and diet and aerobic-resistance exercise (Thomson *et al.*, 2008). All three interventions successfully reduced mean body weight, blood pressure, triglycerides, cholesterol, glucose and insulin levels, with more favourable effects in terms of body composition observed in the exercise groups. Specifically, there was a greater reduction in fat mass of ~45% in exercise groups.

More recently, Lass *et al.* (2011) assessed the impact of a 1-year lifestyle intervention on the metabolic syndrome and CVD parameters in 59 obese adolescent PCOS girls (12-18y). The lifestyle intervention was based on nutritional advice, exercise training and behavioural therapy. In PCOS adolescents who achieved significant weight loss during the intervention, cIMT, blood triglycerides, waist circumference and blood pressure improved significantly, and the prevalence of the metabolic syndrome and homeostasis model assessment- insulin resistance (HOMA IR) decreased; whereas no changes were observed in PCOS adolescents with no weight loss. Nevertheless, the authors could not distinguish between the effects of exercise *per se* from diet, behavioural therapy and/or weight loss, nor did they include a control group for comparison.

### 2.13.3 Exercise

The androgen excess (AE)-PCOS consensus statement recommends lifestyle modification, specifically exercise, as a primary prevention strategy of CVD (Wild *et al.*, 2010; Harrison *et al.*, 2011). Despite this, a recent review of exercise intervention studies in PCOS identified 8 eligible studies, only 5 of which were randomised controlled trials and only 6 examined the effect of exercise alone (without dietary restriction). Existing exercise training studies in women with PCOS have reported the consistent beneficial effects of exercise on reproductive function (Palomba *et al.*, 2008), obesity (Moran *et al.*, 2009b), cardiorespiratory fitness (Vigorito *et al.*, 2007) and insulin resistance (Hutchison *et al.*, 2011). Vigorito *et al.* (2007) observed a modest decrease in BMI and WHR in 45 women with PCOS following 12 weeks of moderate-intensity cycling 3 times per week. This reduction in obesity status was associated with a reduction in blood pressure and improved insulin regulation. A modest exercise-induced reduction in BMI and WHR is commonly observed within the existing literature (Giallauria *et al.*, 2008; Palomba *et al.*, 2008) although very few studies have assessed the effect of exercise on specific fat deposition. In particular, examining the change in VAT and SAT, which are closely related to insulin resistance (Despres, 2007), following exercise training would provide insight into the mechanisms associated with insulin resistance and CVD in PCOS. Although modest weight reduction has been reported to improve CVD risk factors in women with PCOS (Pasquali *et al.*, 1994), the effect of exercise on markers of cardiovascular risk is not widely studied. This is surprising given the mounting evidence indicating that women with PCOS are at high risk of CVD coupled with evidence indicating that exercise reduces CVD risk in associated clinical populations including individuals with hypertension (Molmen *et al.*, 2012), the metabolic syndrome (Dunkley *et al.*, 2012) and type 2 diabetes (Melkus, 2011).

Hutchinson *et al.* (2011) examined the effect of 12 weeks intensified exercise training on insulin resistance, using the gold standard euglycaemic clamp technique, blood profiles and adiposity in 20 overweight women with PCOS. The exercise-training programme consisted of three 1-hour sessions per week which alternated between moderate-intensity continuous (75-80% HR max) and high-intensity intermittent exercise (6x5minute bouts at 95-100% HR max). A significant decrease in VAT, blood triglycerides and insulin resistance was observed in women with PCOS in the absence of significant weight loss. This study utilised fat density estimates, also known as computer tomography, for the assessment of fat volumes which possesses less sensitivity than MRI but provides the accurate distinction between different fat depots (Müller *et al.*, 2010). The same research group recently administered the same exercise programme to 20 overweight and 20 obese women with PCOS in order to explore the effects of exercise on glycaemic control, with specific focus on its relationship with other cardiovascular and metabolic variables. The authors demonstrated improvements in insulin resistance in the absence of weight loss in both overweight and obese women with PCOS; although, elevated insulin resistance remained post-exercise and there was no relationship between the change in insulin resistance and aerobic fitness. Thus, research investigating the mechanisms accounting for the exercise-induced improvement in glycaemic control in women with PCOS is warranted. Based on the limited data available, exercise appears to exert a beneficial effect on metabolic variables and some traditional cardiovascular risk factors in PCOS. However, several studies are limited by the lack of control groups, assessments of habitual activity and short study durations. That said, the aforementioned findings provide promising evidence that supports the utilisation of exercise training as a treatment strategy in PCOS, yet limited research exists regarding the cardiovascular effects of exercise, and its impact on endothelial function as a predictive and prognostic marker of CVD risk has yet to be explored.

## 2.14 Exercise Training and Flow-Mediated Dilation

Endothelial dysfunction is reversible and numerous interventions that have been reported to modify cardiovascular risk factors and reduce cardiovascular morbidity and concomitantly, improve endothelial function (Maiorana *et al.*, 2003). Exercise, in particular, has been shown to improve the structure and function of the vascular endothelial cells and thus offset the development of atherosclerosis (Kingwell *et al.*, 1997a; Kingwell *et al.*, 1997b). Repeated bouts of exercise involve dramatic changes in haemodynamics and resultant bouts of increased shear stress exerted on the arterial wall. It has been postulated that this shear stress mechanism promotes the bioavailability of the anti-atherogenic substance NO by reducing the number of oxygen free radicals and up-regulating eNOS (Green *et al.*, 2004). As a consequence, these exercise-induced adaptations promote efficient vasomotor function and decrease the risk of atherosclerotic development. The beneficial effect of exercise on endothelial dependent vasodilator function, measured using FMD, has been demonstrated in healthy individuals (Clarkson *et al.*, 1999) and several diseased populations including individuals with type 2 diabetes (Maiorana *et al.*, 2001), obesity (Watts *et al.*, 2004), CAD (Walsh *et al.*, 2003) and the metabolic syndrome (Hamdy *et al.*, 2003). To date, no study has assessed the effect of exercise training on endothelial function in young or post-menopausal women with PCOS.

The utilisation of exercise as a disease-management strategy, specifically in relation to CVD risk, has been successfully employed across several high-risk populations. Walsh and colleagues (2003) employed a randomised crossover design to compare the effect of an 8-week circuit training programme, consisting of a combination of resistance training, cycling and treadmill walking, with no exercise in 10 CAD patients. The authors found an improvement in brachial artery FMD following 8 weeks of exercise training. This study also demonstrated that lower limb exercise induces a sufficient shear stress to enhance brachial artery (upper limb) NO vasodilator function. This is likely to be due to the

heightened haemodynamic stress associated with exercise in large muscle groups. Exercise training has also been reported to improve endothelial function in individuals with the metabolic syndrome (Lavrencic *et al.*, 2000). In this study, 29 individuals with the metabolic syndrome were randomly assigned to either 12 weeks of high intensity cycle-ergometry or to a control group, whereby participants maintained their habitual physical activity levels for 12 weeks. Following the respective interventions, FMD significantly improved in the exercise-trained group, whereas no changes were observed in the control group. Intriguingly, the improvement in FMD occurred in the absence of any changes to the individual components of the metabolic syndrome, indicating that exercise has a direct therapeutic effect on the vasculature.

The beneficial effect of exercise training on FMD in heterogeneous clinical populations demonstrating endothelial dysfunction is irrefutable. Interestingly, exercise-induced improvements in endothelial function occur both prior to, and independent of, changes in traditional markers of cardiovascular risk, such as BMI and blood pressure, in patients with coronary artery disease (Walsh *et al.*, 2003), hypertension (Higashi *et al.*, 1999) and type 2 diabetes (Maiorana *et al.*, 2001). At present, the underlying mechanisms that directly mediate this exercise-induced change in heterogeneous clinical populations remain unclear.

### **2.15 Exercise training and NO-mediated cutaneous microvessel function**

The current body of evidence relating to cutaneous vasodilator function suggests that fitness and/or physical activity promotes increased NO bioavailability in the cutaneous microvasculature of healthy individuals (Lenasi & Strucl, 2004; Wang, 2005). The findings from several studies suggest that endurance exercise training can improve cutaneous microvascular reactivity in older adults (Black *et al.*, 2008b; Tew *et al.*, 2010). For example, Hodges *et al.* (2010) reported an increase in ACh-mediated cutaneous blood flow following 24, 36 and 48 weeks of moderate aerobic intensity exercise in post-menopausal

women. Surprisingly, this study also reported an increase in SNP-mediated skin blood flow after 36 weeks of exercise training, indicative that chronic exercise may induce structural changes to cutaneous microvessels. However, few long-term studies have been performed and little is known regarding the effects of exercise training on cutaneous microvascular reactivity in clinical populations and/or females.

Middlebrooke and colleagues (2006) observed no change in microvascular vasodilator response to iontophoretically applied ACh or localised heating to 42°C in type 2 diabetic patients following six months of aerobic exercise training. However, the exercise-training programme in this study failed to induce an improvement in cardiorespiratory fitness, which poses the question whether the exercise stimulus was adequate in terms of dose. In contrast, Klonizakis and colleagues (2009) reported an augmented cutaneous vasodilator response in response to iontophoresis-mediated infusion of ACh following eight weeks of moderate-intensity exercise training in chronic venous disease patients. Although aged individuals are not considered as a diseased population it is interesting that Tew *et al.* (2012) found reduced cutaneous microvascular reactivity, measured using LDF, in normotensive post-menopausal women during localised heating and post-occlusive reactive hyperaemia. The authors then reassessed cutaneous microvessel vasodilator function following 6, 12, 24, 36, and 48 weeks of endurance exercise training. The training programme was a progressive aerobic exercise programme (30-75% HRR, 3-5 times per week) monitored through the use of exercise diaries. Cutaneous microvessel vasodilator function improved to similar levels of that observed in young women following 24-36 weeks of moderate-intensity aerobic exercise training. Moreover, changes in cutaneous microvascular function correlated with improvements in cardiorespiratory fitness suggesting that exercise is an effective management strategy to improve cutaneous microvascular function in post-menopausal women.

## 2.16 Summary

On balance, the weight of evidence suggests that conduit artery and microvessel endothelial dysfunction, early barometers of atherosclerotic and microvascular disease, is present in women with PCOS (Kravariti *et al.*, 2005; Lakhani *et al.*, 2005). Yet it remains uncertain whether endothelial dysfunction is evident in PCOS independently, or if co-existing morbidities are independent risk factors for endothelial function in this population. Despite the high prevalence of CVD risk (Cussons *et al.*, 2006), which extends into the post-menopausal years (de Groot *et al.*, 2011), no study to date has investigated the effects of exercise training on conduit artery and cutaneous microvessel endothelial function in women with PCOS.

## 2.17 Objectives

The aims outlined in *Chapter 1* will be achieved through the following objectives:

1. Conduct a systematic review of published studies relating to endothelial function measured using the FMD technique in women with PCOS and controls, and to quantify these findings using a formal meta-analytical approach.
2. Utilise valid and novel research techniques to assess conduit artery endothelial function, adiposity, cardiorespiratory fitness and blood profiles in PCOS and matched control women.
3. Examine the relationship between conduit artery endothelial function and potential moderators, such as adipose tissue volumes and insulin resistance, in women with PCOS.
4. Engage women with PCOS in either a 16-week supervised moderate-intensity aerobic exercise training programme or a 16-week period of conventional care (following general lifestyle advice).
5. Compare the effect of exercise and conventional care on conduit artery endothelial function adiposity, cardiorespiratory fitness and blood profiles.
6. Utilise intradermal microdialysis to directly interrogate NO-mediated cutaneous microvessel vasodilatation in response to gradual heating in PCOS and matched control women.

7. Engage women with PCOS in a 16-week supervised moderate-intensity aerobic exercise training programme to assess the effect of exercise on NO-mediated cutaneous microvessel vasodilator function.

## **CHAPTER 3**

# **ENDOTHELIAL FUNCTION MEASURED USING FLOW MEDIATED DILATION IN POLYCYSTIC OVARIAN SYNDROME: A META-ANALYSIS OF THE OBSERVATIONAL STUDIES**

### 3.1 Introduction

Polycystic Ovarian Syndrome (PCOS) is the most common endocrinopathy in females of reproductive age, with a prevalence of up to 20% when utilising the more liberal Rotterdam criteria (Goodarzi *et al.*, 2011). PCOS is characterised by hyperandrogenism, menstrual dysfunction secondary to anovulation and polycystic appearance of the ovaries upon trans-vaginal ultrasound assessment (Goodarzi *et al.*, 2011). This can be identified using three consensus definitions; National Institute of Health (NIH) guidelines (Zawadzki & Dunaif, 1992) and the Androgen Excess Society (AES) consensus, both of which classify PCOS primarily as a hyperandrogenic disorder, or the broader Rotterdam criteria (2004). Importantly, it has been suggested that women with PCOS are at greater risk of cardiovascular disease (CVD) and type 2 diabetes. Despite epidemiological evidence suggesting an increased cardiovascular morbidity and coronary artery mortality in women with PCOS, study findings are equivocal (Wild *et al.*, 2000; Schmidt *et al.*, 2011) and little data are available regarding cardiovascular outcomes (Wild *et al.*, 2000). It is possible that the increased cardiovascular risk could be related to the presence of a number of risk factors evident in women with PCOS such as abdominal obesity, insulin resistance, dyslipidemia and hypertension (Pierpoint *et al.*, 1998). It is yet to be established whether PCOS *per se* and/or the presence of associated cardiovascular risk factors translates into increased CVD.

Several risk factors, evident in women with PCOS, are associated with endothelial dysfunction. Endothelial dysfunction is an important early event in the development of atherosclerosis, which precedes gross morphological signs and clinical symptoms. Non-invasive assessment of endothelial function *in vivo* is commonly undertaken using the FMD technique (Thijssen *et al.*, 2011). This method involves direct assessment of conduit artery dilator responses to reactive hyperaemia induced by a brief period of limb ischaemia

via an occluding cuff placed immediately proximal or distal to the imaged site (Green *et al.*, 2004). The prognostic value of FMD assessment is well established in patients exhibiting an increased risk of CVD (Green *et al.*, 2011) and, specifically, FMD has been found to be an accurate independent predictor of occult coronary artery disease (Mutlu *et al.*, 2011b). Indeed, the independent prognostic information provided via FMD may exceed that of traditional risk factors in clinical groups (Naghavi *et al.*, 2003). Endothelial dysfunction, manifest as reduced FMD, has been observed in women with PCOS when compared to control women in some (Casella *et al.*, 2008b), but not all previous studies (Beckman *et al.*, 2007). Even when women with PCOS were compared with age and BMI matched controls Soares *et al.* (Soares *et al.*, 2009) found no significant difference in FMD, a finding that is consistent with previous studies (Arikan *et al.*, 2009; Moran *et al.*, 2009a). In contrast, Meyer *et al.* (Meyer *et al.*, 2005c) and El-Kannishy *et al.* (El-Kannishy *et al.*, 2010b), demonstrated differences in early functional markers of arterial disease, including FMD, in women with PCOS compared to a matched control group. This disparity in the extant literature may be related to different methods employed to assess FMD, the considerable variability between subjects, with different definitions of diagnosis, disease duration, disease severities, and co-morbidities or to poorly matched control groups.

Therefore, the aim of this study was to systematically review the literature relating to endothelial function, measured using the FMD technique, in PCOS, to obtain a more precise estimate of whether a PCOS-related impairment in FMD exists; and to explore the influence of potential between-study moderators such as age and obesity status on the difference in FMD between PCOS and control women. We also aimed to elucidate whether the different diagnostic criteria for PCOS influences FMD, which is to date an unexplored moderator.

## 3.2 Methods

### 3.2.1 Data Source

A systematic review of peer-reviewed studies was undertaken comparing endothelial function, quantified by means of FMD, in PCOS compared with control women. This literature search was conducted using the Pubmed, BIOSIS, Scopus, CINAHL and WebofScience scholarly databases (2000-2011). Key search words included *Polycystic Ovarian Syndrome*, *vascular function/dysfunction*, *endothelial function/dysfunction* and *flow mediated dilatation*. Reference lists of relevant published works selected were also examined in order to identify additional pertinent studies.

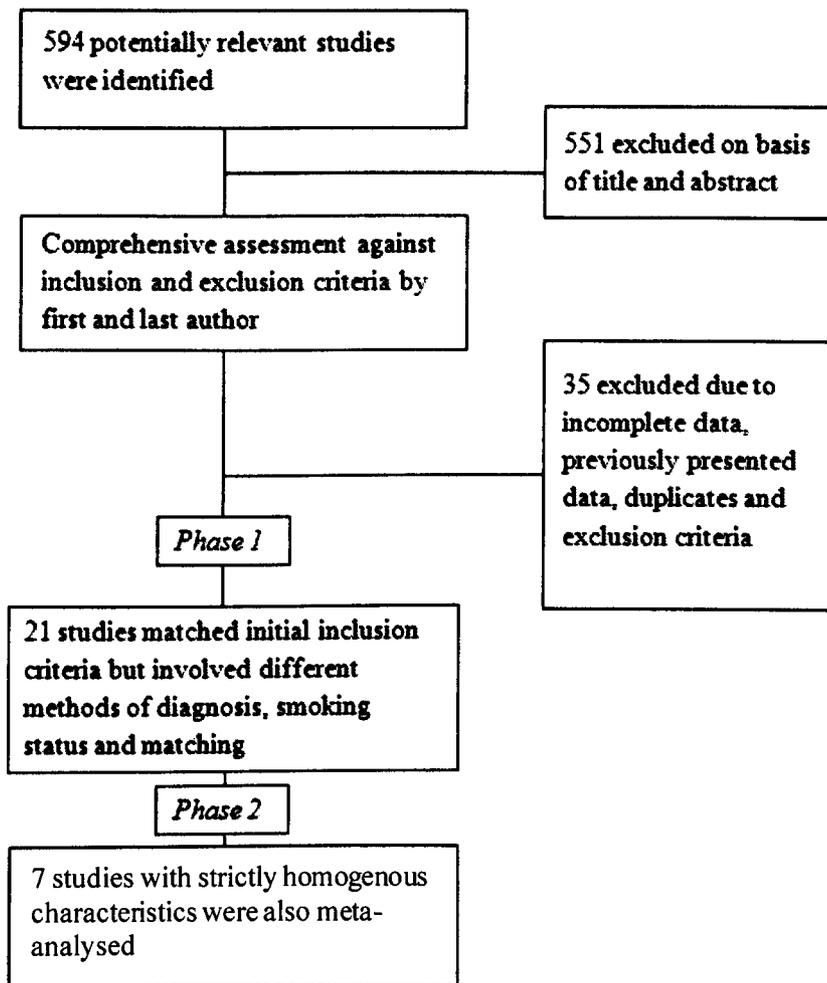
### 3.2.2 Study Selection

Two members of the research team (VSS and HJ) independently selected the studies for inclusion in the meta-analysis and later met to reach a mutual consensus. Twenty-one studies met the initial inclusion criteria, described below, and were incorporated into *phase 1* of the analysis. Subsequently, a sub-set of these studies was created by implementing a more stringent inclusion criterion to form *phase 2* of our analysis. Specifically, for *phase 2* of the analysis, studies were selected that recruited women with PCOS using the Rotterdam criteria (2004) as only one study that met our initial inclusion criteria utilised the AES (Azziz *et al.*, 2006) criteria and only five recruited using NIH guidelines (Zawadzki & Dunaif, 1992).

### 3.2.3 Criteria for Phase 1 Analysis

Published literature relating to endothelial function, assessed via FMD of the brachial artery, in PCOS and non-PCOS women was compiled. In order to minimise measurement error studies were selected based on the utilisation of an analysis technique whereby either an average is derived from multiple diameter measurements determined along a segment of the vessel or the use of automatic edge detection software; and the stimulus to induce

hyperaemia was for 4-5 min in duration. This criterion was devised based upon recommendations from technical guidelines on FMD measurement to minimise variability between studies (Corretti *et al.*, 2002; Thijssen *et al.*, 2011). All studies reported relative FMD (% change from baseline to peak diameter). Studies that recruited women with PCOS via various diagnostic definitions including; Rotterdam (2004), NIH (Zawadzki & Dunaif, 1992) and AES (Azziz *et al.*, 2006) were considered. We also incorporated studies that included smokers. Twenty-one studies were identified during this process (Figure 3.1); at whole study level demographics for women with PCOS ranged from 22-34yrs and 21-37kg/m<sup>2</sup> and for control women were from 23-41yrs and BMI range was 27-37kg/m<sup>2</sup> (PCOS *n*=908; control participants *n*=566). The selected studies excluded individuals based on current use or use within the last three months of insulin-sensitising agents (metformin and/or pioglitazone), weight loss medication, anti-androgens, fertility treatments, glucocorticoids or medication which could alter vascular function such as the oral contraceptive pill.



**Figure 3.1** Summary of study assessment and exclusion stages.

### 3.2.4 Criteria for Phase 2 Analysis

Reviewers selected studies that; 1) specifically recruited PCOS via the Rotterdam criteria ; 2) matched PCOS and control women for age and BMI; 3) recruited participants over 18 years of age; 4) assessed endothelial function in the brachial artery via the FMD technique; 5) excluded smokers; 6) reported only previously unpublished data. Eight published studies were identified during this procedure. One of these studies (Arikan *et al.*, 2009) reported sample mean values of FMD for PCOS and controls of approximately 23-25%. The values were approximately three times higher than the average for the other seven studies. The associated SDs reported in the manuscript by Arikan *et al.* (Arikan *et al.*, 2009) indicated that some FMD measurements were higher than 45%, which is unfeasibly large. Therefore

this study was removed from analysis. The pathway of systematic reviewing is presented in Figure 1. The PCOS demographics in this phase ranged from 23-31yrs and 21-29kg/m<sup>2</sup> and control demographics ranged from 23-34yrs and 21-29kg/m<sup>2</sup> (PCOS  $n=441$ ; control participants  $n=281$ ). All PCOS and control women were normotensive and not taking any medication.

### 3.2.5 Statistical Analyses

Version 2 of the Comprehensive Meta-Analysis software package (Biostat, Englewood, NJ) was utilised to conduct a random-effects (DerSimonian-Laird inverse variance approach) meta-analysis of the mean difference in FMD between PCOS and control women, at whole study level (DerSimonian & Laird, 1986). All studies included in the analyses comprised of two independent groups. Relative weights were assigned to each study mean difference on the basis of respective standard errors. Study cohorts were coded for smoking status (as includes smokers or excludes smokers, studies that did not specify about smoking were coded as includes smokers) and for cuff placement (distal or proximal). Mean age and BMI were included as moderating variables in *Phase 2*. All data are presented as mean (95% confidence intervals).

The primary outcome variable in all of the relevant studies was FMD (%). We found that the sample means of FMD% generally followed a normal distribution. The correlation coefficient between the sample mean differences in FMD and the respective standard errors was found to be 0.2, indicating the absence of substantial heteroscedasticity. Besides, we also analysed the data as standardised effect sizes which should also control for any heteroscedasticity (see below). Across all selected studies (*phase 1*) the average number of women with PCOS was 45 and the average number of control women was 27. Studies were grouped by cuff placement and smoking status to examine the effect of these,

potentially confounding, variables. For *phase 2* the mean number of women with PCOS was 55 and the mean number of control women was 35.

### *3.2.6 Exploration of heterogeneity*

Exploration of heterogeneity was examined only in the phase 2 analyses, since specific sources of heterogeneity from inclusion/exclusion criteria were already known to be present in the phase 1 analysis, and then these were removed for the phase 2 analyses. Heterogeneity was explored with the *Q*-test and I-square statistic, which quantifies that variation that is due to heterogeneity rather than sampling error (chance) (Higgins *et al.*, 2003). A *Q* statistic was deemed significant when  $P < 0.05$ . An I-square statistic  $> 50\%$  is deemed to be indicative of substantial heterogeneity. Such information can be used to inform the appropriateness of the choices of a random effects analysis and moderator influence, e.g. via meta-regression approaches. In the event of substantial heterogeneity still being present in phase 2, we performed the following sensitivity analyses; a meta-analysis of the standardised mean differences between PCOS and controls as well as re-analyses with outlier studies removed.

### *3.2.7 Meta-regression analyses of potential moderators of FMD difference between samples*

The study differences in FMD between PCOS and control women were meta-regressed against mean study age and mean study BMI. The study differences in FMD were also compared between studies based on the smoking status of the participants (all non-smokers vs smokers and non-smokers studied together as one sample).

### *3.2.8 Exploration of Publication Bias*

The presence of publication bias was explored with observation of a standard funnel plot of PCOS-control difference vs. study standard error, and quantitatively via Egger's linear

regression test (Egger *et al.*, 1997). If this test is statistically significant, it indicates that there may be a bias of small sample studies which report no significant difference in FMD between PCOS and controls having not been submitted and/or accepted for publication.

### *3.2.9 Quality Assessment*

A systematic appraisal of quality for observational research (SAQOR) was devised, specifically for this analysis, by the research team in order to provide a minimal qualitative assessment of study quality. The SAQOR was adapted from existing quality assessment tools (Ross *et al.*, 2011) and adjusted to assess i) the PCOS sample, ii) the control group, iii) quality of measurement, iv) confounding variables and v) data. Overall, the SAQOR was scored out of 14, quality is deemed better with a greater score.

**Table 3.1** Baseline characteristics of studies included in *Phase 1 meta-analysis*.

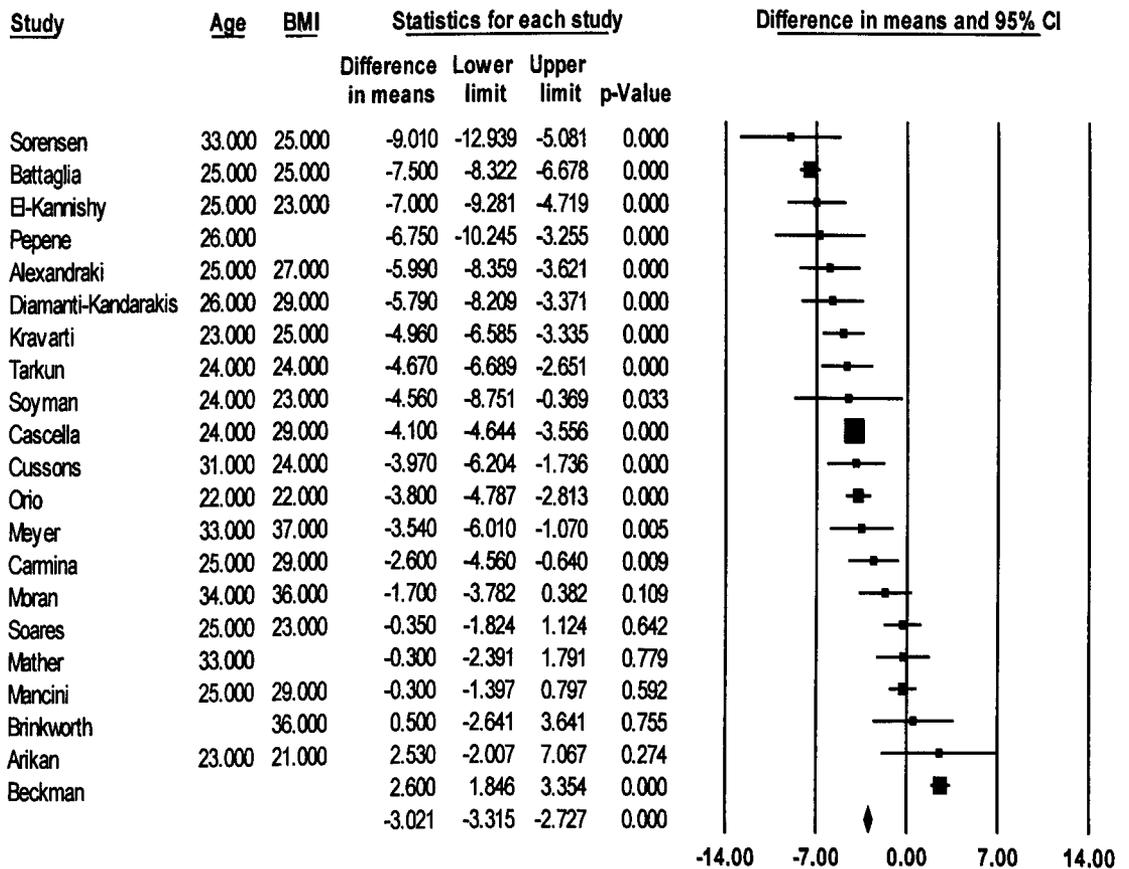
First Author	Year/country	PCOS characteristics	Control characteristics	Smoking status	Cuff placement	Diagnostic criteria	Quality Assessment
Soyman (2011)	Turkey, 2011	n = 30 Age=24, BMI=24	n = 30 Age=23, BMI=23	Excludes smokers	Distal	Rotterdam	12
Kravarti (2005)	Greece, 2005	n = 62 Age=23, BMI=28	n = 17 Age=25, BMI=25	Excludes smokers	Distal	Rotterdam	9
El-Kannishy (2010a)	Egypt, 2010	n = 14 Age=25, BMI=23	n = 10 Age=25, BMI=22	Excludes smokers	Distal	Rotterdam	11
Cascella (2008a)	Italy, 2008	n = 200 Age=25, BMI=29	n = 100 Age=24, BMI=29	Excludes smokers	Proximal	Rotterdam	11
Soares (2009)	Brazil, 2009	n = 40 Age=25, BMI=23	n = 50 Age=25, BMI=23	Excludes smokers	Distal	Rotterdam	12
Tarkun (2004)	Turkey, 2004	n = 37 Age=23, BMI=24	n = 25, Age=24, BMI=23	Excludes smokers	Proximal	Rotterdam	11
Cussons (2009)	Australia, 2009	n = 19 Age=30, BMI=24	n = 19 Age=34, BMI=23	Excludes smokers	Distal	Rotterdam	10
Arikan (2009)	Turkey, 2009	n = 39 Age=23, BMI=21	n = 30 Age=24, BMI=21	Excludes smokers	Distal	Rotterdam	11
Alexandraki (2006)	Greece, 2006	n = 27 Age=25, BMI=27	n = 27 Age=27, BMI=25	Includes smokers	Distal	NIH	9
Orio (2004)	Italy, 2004	n = 30 Age=22, BMI=22	n = 30 Age=23, BMI=22	Excludes smokers	Proximal	NIH	9

First Author	Year/country	PCOS characteristics	Control characteristics	Smoking status	Cuff placement	Diagnostic criteria	Quality Assessment
Moran (2009a)	Australia, 2009	n = 80 Age=34, BMI=36	n = 27 Age=34, BMI=37	Excludes smokers	Proximal	NIH	11
Diamanti-K (2006)	Greece, 2006	n = 25 Age=26, BMI=29	n = 25 Age=28, BMI=26	Excludes smokers	Distal	NIH	11
Beckman (2007)	USA, 2007	n = 10 Age=31, BMI=30	n = 19 Age=41, BMI=26	Excludes smokers	Distal	Non-specific	7
Mather (2000)	Canada, 2000	n = 17 Age=32, BMI=32	n = 19 Age=32, BMI=23	Excludes smokers	Distal	Non-specific	10
Meyer (2005c)	Australia, 2005	n = 100 Age=33, BMI=37	n = 20 Age=33, BMI=37	Excludes smokers	Proximal	Non-specific	8
Battaglia (2008)	Italy, 2008	n = 28 Age=25, BMI=25	n = 15 Age=27, BMI=26	Excludes smokers	Proximal	Non-specific	8
Mancini (2009)	Italy, 2009	n = 10 Age= N/A, BMI=>25	n = 10 Age= N/A, BMI=>25	Excludes smokers	Proximal	Non-specific	9
Carmina (2006)	Italy/USA, 2006	n = 50 Age=25, BMI=29	n = 50 Age=25, BMI=29	Includes smokers	Proximal	Non-specific	5
Pepene (2011)	Romania, 2011	n = 64 Age=24, BMI=30	n = 20 Age=27, BMI=26	Includes smokers	Proximal	AES	9
Brinkworth (2006)	Australia, 2006	n = 12 Age=32, BMI=36	n = 10 Age=37, BMI=34	Includes smokers	Distal	Rotterdam	10
Sorensen (2006)	UK, 2006	n = 14 Age=33, BMI=25	n = 13 Age=33, BMI=25	Excludes smokers	Distal	NIH	13

### 3.3 Results

#### 3.3.1 Phase 1

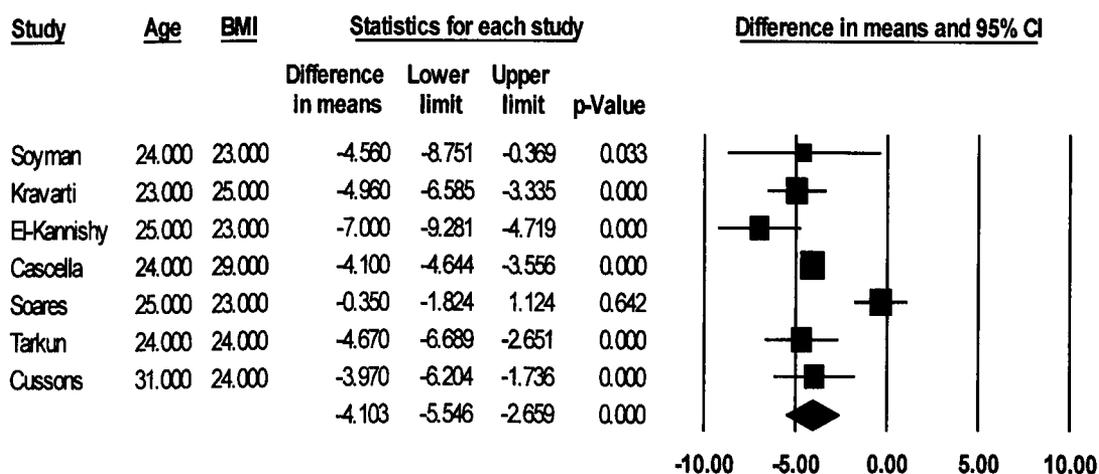
The pooled mean values of FMD were 8.7% (6.7-10.7) and 12.1% (9.8-14.4) for the PCOS and control samples respectively. The pooled mean FMD was found to be 3.4% (95% CI=1.9, 4.9) lower in women with PCOS compared with control women ( $P<0.001$ , Figure 3.2). There was evidence of statistically significant and substantial heterogeneity between studies ( $Q_{20}=445.6$ ,  $P<0.001$ ;  $I^2=96\%$ ). When this meta-analysis was repeated with the standardised difference in FMD as the outcome, the pooled mean standardised reduction in FMD was found to be 0.93 (0.51-1.35), but heterogeneity was still high (I-square = 91%).



**Figure 3.2** Forest plot demonstrating the individual study difference in mean FMD (%) between PCOS and control women (and associated 95% confidence interval) in our Phase 1 analysis. Also shown (in the final row is the random-effects estimate of the pooled mean difference in FMD between PCOS and controls.

### 3.3.2 Phase 2

The pooled mean values of FMD were 8.4% (4.3-12.2) and 12.5% (8.4-16.6) for the PCOS and control samples respectively. The meta-analysed pooled reduction in FMD was found to be 4.1% (95% CI=2.7, 5.5) in women with PCOS diagnosed via the Rotterdam criteria when compared with control women ( $P<0.001$ , Figure 3.3). There was evidence of statistically significant and substantial heterogeneity between studies in this *phase 2* analysis with stricter quality criteria, although the  $I^2$  value was lower than that obtained in the *Phase 1* analysis on the larger more heterogeneous sample of studies ( $I^2=81\%$ ). The mean difference in FMD between PCOS and control women was not influenced by BMI ( $P=0.17$ ) or age ( $P=0.38$ ). As well as being non-significant, the slope of regression for BMI was very shallow with the group difference in mean FMD changing by only 0.13% for every 1 kg/m<sup>2</sup> increase in BMI. Egger's regression intercept revealed that there was no evidence of publication bias (Intercept=-0.22, 95% CI=-3.5, 3.12,  $P=0.90$ ).



**Figure 3.3** Forest plot demonstrating the individual study difference in mean FMD (%) between PCOS and control women (and associated 95% confidence interval) in our Phase 2 analysis. Also shown (in the final row in bold text) is the random-effects estimate of the pooled mean difference in FMD between PCOS and controls.

### 3.4 Discussion

This two-phase meta-analysis encompassing 21 studies with 908 PCOS and 281 control women provides compelling evidence that brachial artery FMD, a strong and independent

predictor of future cardiovascular events is impaired in PCOS. This analysis supports previous evidence suggesting PCOS *per se* infers greater CVD risk but, importantly, also provides information regarding the extent of CVD risk in this population. The authors of a recently-published meta-analysis on the prognostic value of FMD reported that the risk of a cardiovascular event increases by 21% for every 1 standard deviation decrease in FMD (Inaba *et al.*, 2010). This effect size corresponds to an approximate 21% increase in risk for every 3.5% decrease in FMD. Although this previous meta-analysis did not specifically involve PCOS patients, it is interesting to note that we observed a mean FMD reduction of 3.4-4.1% in PCOS compared with controls women, suggesting that the risk of cardiovascular events is higher in women with PCOS.

To our knowledge this is the first meta-analysis investigating the impact of PCOS on FMD, a sub-clinical marker of CVD risk. Previous systematic reviews and meta-analysis studies are suggestive of increased CVD risk in PCOS. Our data corroborate this notion, and provide information that enables comparison of risk ratio's for future cardiovascular events. Another novel aspect of this study is that we have employed a two-staged approach to examine the intricacies of the PCOS diagnostic criteria and the impact of BMI and age on the level of FMD impairment in PCOS. We observed that FMD was consistently impaired in women with PCOS independent of diagnostic criteria. Likewise, in *phase 2* of our analysis, which controlled for the effect of age and BMI, a significant impairment in FMD in women with PCOS remained.

One advantage of this study is that we employed meta-regression analysis to examine the impact of moderating variables on the difference in FMD. Age was not a moderating factor on the difference in FMD response between PCOS and control women, but this could be due to the small age range between participants (23-31years). We found that BMI did not influence the difference in FMD between PCOS and control women with a 0.13% change

in FMD for every  $1\text{kg}/\text{m}^2$  increase in BMI. Intriguingly, when the PCOS and control groups were matched for BMI and only diagnosed using the Rotterdam criteria FMD was impaired in lean women with PCOS as well as overweight and obese women. Although, women diagnosed via the Rotterdam criteria, as illustrated in our analysis, generally exhibit a lower BMI compared to those diagnosed using the 'classical' NIH criteria (Zawadzki & Dunaif, 1992), for which hyperandrogenism is a fundamental diagnostic feature and is associated with increased obesity (Carmina *et al.*, 2005). Our data may therefore suggest that impaired FMD is an inherent attribute in PCOS and advocates the notion that FMD might be a useful prognostic tool for CVD in this population.

Given that BMI was the only consistently reported marker of obesity within the studies included in the present meta-analysis we cannot currently make firm conclusions about the impact of obesity on endothelial dysfunction in women with PCOS. We are aware that BMI cannot distinguish between adipose and lean tissue (Oreopoulos *et al.*, 2011) and central abdominal fat, which is greater in women with PCOS (Carmina *et al.*, 2007a), would have provided a more robust marker of adiposity, which is strongly associated with an increased risk of atherosclerosis (Gasteyger & Tremblay, 2002) and CVD mortality (Dagenais *et al.*, 2005). We also acknowledge visceral adipose tissue (VAT) may influence FMD in women with PCOS. Caescella *et al.* (Cascella *et al.*, 2008a) reported an association between VAT thickness, measured using ultrasound, and impaired FMD in women with PCOS. Furthermore, excess VAT accumulation is responsible for the up regulation of  $\beta$  oxidation which subsequently results in oxidative stress, the release of inflammatory cytokines and adipokines and a reduction of endogenous adiponectin, an anti-inflammatory substance (Carmina *et al.*, 2006; Cascella *et al.*, 2008b; Carmina *et al.*, 2009; Pepene, 2012) all of which could contribute to endothelial dysfunction. Therefore, further investigation of endothelial function related to specific fat deposition in women with PCOS is warranted.

The fundamental aim of this meta-analysis was to obtain a more precise estimate of the PCOS-related impairment in FMD using a large number of previous studies to enhance statistical power and precision. A notable strength of this study is the utilisation of meta-regression analysis to explore the influence of the important between-study moderators of age and BMI between PCOS and control women. Nevertheless, the data reported in this study should be interpreted in line with its limitations. The measurement of FMD is traditionally considered as an index of NO-mediated vasodilator function and thus is a surrogate marker of CVD risk. Since its inception in 1992, FMD has become the most commonly used non-invasive assessment of vascular endothelial function in humans. Advances over the last decade have resulted in a refined understanding of the physiology, analysis, and interpretation of this technique (Harris *et al.*, 2010). Although we are aware of several techniques, which exist for the assessment of endothelial function and other surrogate markers of CVD risk, the primary aim of the study was to focus on the FMD technique. Additionally, not all studies employed a published consensus definition for the diagnosis of PCOS; studies investigating endothelial function to discern between the different phenotypes of PCOS and their associated risk are clearly warranted. We acknowledge that a multitude of factors are exhibited in women with PCOS that could potentially influence FMD, most notably, hyperandrogenism and insulin resistance. Yet, we were unable to include these variables as moderators since a large number of previous studies either did not measure and/or report such data (e.g. waist circumference) or the studies utilised a wide variety of techniques to measure a variables (e.g. insulin resistance, measured via HOMA-IR, QUICKI, MATSUDA, AUCg-AUCi ratio).

Our primary outcome was the percentage change in diameter from a resting baseline to a hyperaemic peak value. This approach to expressing change in diameter is ubiquitous across studies, which enabled the meta-analysis to be undertaken, yet this approach might not be the most appropriate. Percentage change and ratio statistics have been criticised in

many research contexts, since they may not scale consistently over the full range of diameter sizes (Packard & Boardman, 2008). There are other approaches to expressing change while controlling for general anatomical size, involving analysis of covariance and allometric techniques. Unfortunately, these approaches have only been applied to a few recent FMD studies (Birk *et al.*, 2012).

In summary, this meta-analysis suggests that endothelial dysfunction is inherent in women with PCOS, even if they are young and non-obese. Therefore, our in-depth analysis infers that women with PCOS are at greater risk of CVD. Importantly, this FMD impairment and associated CVD risk was not explained by the diagnostic criteria but may be affected by differences in adiposity in terms of fat deposition and/or distribution.

**CHAPTER 4**  
**GENERAL METHODS**

Many of the methods undertaken in this thesis were adopted throughout *Chapters 5-7*. The specific protocols employed for each study can be found within the respective method sections for each chapter.

#### **4.1 Participants**

Women with PCOS were recruited from the gynaecology and reproductive medicine clinic at Liverpool Women's Hospital, Liverpool, UK. PCOS was diagnosed by means of the Rotterdam criteria for diagnosing PCOS (2004), based on the presence of two of the following three criteria; i) clinical (acne/hirsutism) or biochemical hyperandrogenism (testosterone > 2.5mmol/l), ii) oligomenorrhoea or amenorrhoea, iii) polycystic appearance of the ovaries upon ultrasound (enlarged ovarian volume >10cm<sup>3</sup> or >12 follicles between 2-9mm in diameter), having excluded other possible causes by appropriate biochemical tests. Control participants were recruited via local advertisement. Only sedentary individuals were recruited (<2 hours of weekly exercise based on a self-reported questionnaire). All participants were over 18 years of age and had no history of type 2 diabetes, cardiovascular, liver, kidney or respiratory disease and displayed no contraindications to exercise. Current use or use within the last three months of insulin-sensitising agents (metformin and/or pioglitazone), orlistat, anti-androgens, fertility treatments, glucocorticoids or medication which could alter vascular structure and/or function such as the oral contraceptive pill resulted in exclusion. All participants were non-smokers and drank <14 units of alcohol per week. In control women, data was collected during the early follicular phase of their menstrual cycle, and confirmed using reproductive hormone profiles. This was not feasible in women with PCOS, due to the erratic nature of their cycles. Participants were asked to fast for 12 hours, abstain from alcohol and caffeine for 24 hours and refrain from exercise for 48 hours prior to testing sessions. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee (LREC). All participants were informed of the methods verbally and in writing

before providing written informed consent.

#### **4.2 Anthropometric Measurements**

Height was measured in a freestanding position to the nearest 0.5 cm using a measuring device (Seca, Model 220, Germany). Body mass was measured to the nearest 0.05 kg using calibrated electronic digital scales (Seca, Model 767, Germany). From this, body mass index (BMI; mass (Kg) / (height (m)<sup>2</sup>) was calculated. Waist and hip circumference was measured at the level of the umbilicus and greater trochanter, in duplicate, and waist: hip ratio (WHR; waist circumference (cm) / hip circumference (cm)) calculated. Resting blood pressure (mm Hg) and resting heart rate (beats/min) were also determined from an average of three measures using an automated BP monitor (Dinamap, G & E Medical, Tampa, Florida).

#### **4.3 Biochemical Assays and Evaluation**

Following an overnight fast, blood was drawn for biochemical profile, including total cholesterol, triglycerides, high-density (HDL) and low-density (LDL) lipoprotein cholesterol, alanine aminotransferase (ALT), glucose and insulin. Circulating reproductive hormone levels were measured including follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, progesterone, testosterone and sex hormone binding globulin (SHBG).

Total cholesterol, HDL, LDL, triglycerides, glucose and ALT were measured using standardised assays (Olympus A2700). LH, FSH, oestradiol, progesterone, total testosterone and SHBG concentrations were measured by a chemiluminescence method (Siemens Centaur). Free androgen index (FAI) was calculated as  $100 \times [\text{testosterone concentration (nmol/l)} / \text{SHBG concentration (nmol/l)}]$  (normal <7%) (Mathur *et al.*, 1981). Insulin was measured using an ELISA kit (Invitrogen, UK). Using fasting baseline glucose

and insulin concentrations, insulin resistance was calculated by the homeostasis model assessment for insulin resistance (HOMA-IR) (Matthews *et al.*, 1985). Blood samples were analysed by an experienced laboratory technician.

#### **4.4 Vascular Function**

All vascular function assessments were performed in a quiet, temperature-controlled laboratory. Upon arrival, participants rested in the supine position for ~20 minutes to facilitate assessment of baseline mean arterial pressure (MAP) and heart rate (HR). Following the rest period, HR and MAP were determined from an average of three measures on the left arm. Participants were then positioned with their right arm extended and immobilised with foam supports at an angle of ~80° from the torso.

##### *4.4.1 Brachial Artery Flow-Mediated Dilatation*

A 10-MHz multi frequency linear array probe attached to a high resolution ultrasound machine (Siemens Medical Solutions, USA) was utilised to image the brachial artery in the distal one third of the upper right arm. When an optimal image was acquired, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode images of the lumen-arterial wall interface. Continuous Doppler velocity assessment was also obtained using the high resolution ultrasound machine and was collected using a 60° isonation angle. Nitric oxide (NO) mediated endothelial function was assessed by measuring flow-mediated dilation (FMD) in response to a 5 minute ischaemic stimulus, induced by forearm cuff inflation (Thijssen *et al.*, 2011). Baseline images were recorded using specialised recording software (Camtasia). A rapid inflation and deflation pneumatic device (D.E. Hokanson, Bellevue, WA) was used with an inflation cuff placed immediately distal to the olecranon process on the forearm of the imaged arm to provide a stimulus for forearm ischemia (Corretti *et al.*, 2002). A baseline recording lasting 1 minute was acquired before the forearm cuff was inflated (~220 mmHg) for 5 minutes. Artery diameter

and blood flow velocity recordings were resumed 30 seconds prior to cuff deflation and continued for 3 minutes thereafter (Black *et al.*, 2008a). Peak brachial artery diameter and blood flow velocity, and the time taken to reach these peaks following cuff release were recorded.

#### *4.4.2 Brachial Artery Endothelium-Independent Vasodilation*

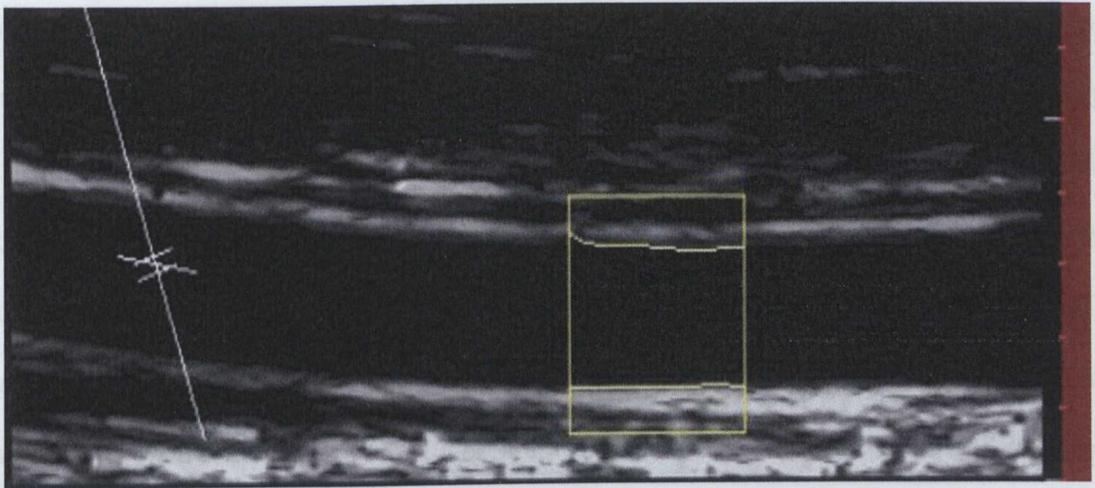
Following a ~15 minute rest period, a 1 minute baseline recording of the brachial artery was acquired. Subsequently, brachial artery endothelium-independent vasodilation was examined following administration of sublingual glyceryl tri-nitrate (GTN, 400 $\mu$ g), a NO donor. GTN provides an endothelial-independent source of NO with which it challenges the integrity of the smooth muscle and thereby provides an insight of smooth muscle sensitivity and a subsequent short-term increase in blood flow velocity and arterial diameter. The brachial artery was imaged for 10-min following administration of GTN.

#### *4.4.3 Brachial artery diameter and blood flow velocity analysis*

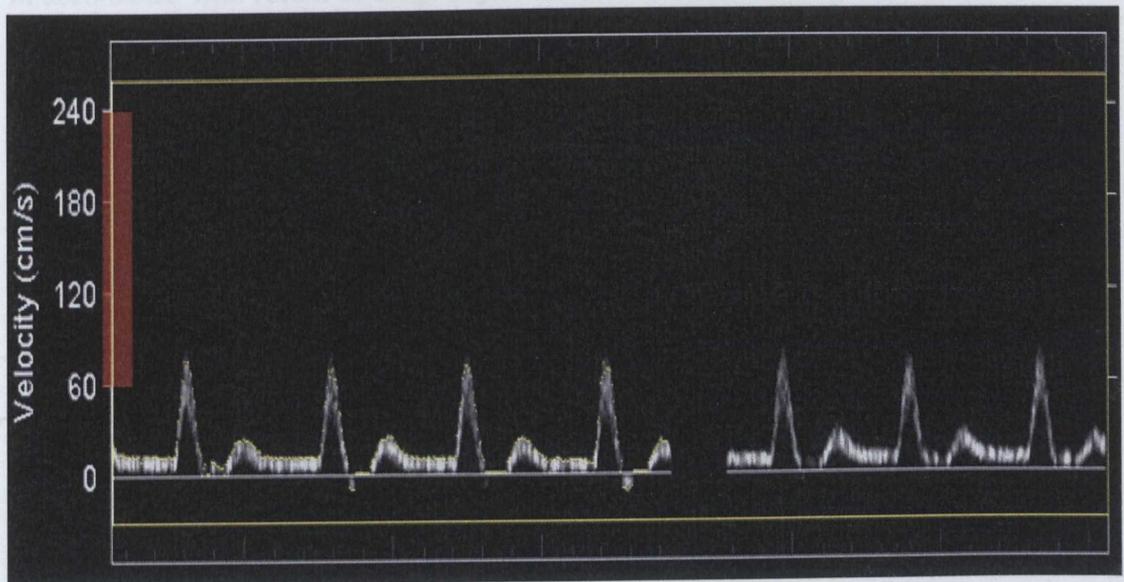
Analysis of brachial artery diameter was achieved using custom-designed edge-detection wall tracking software, which is largely independent of investigator bias (Woodman *et al.*, 2001). The image was taken directly from the ultrasound machine and saved as an AVI file. Subsequent software analysis of this data was performed at 30Hz using an icon-based graphical programming language and toolkit (LabVIEW 6.02, National Instruments). The initial phase of image analysis involved the identification of regions of interest (ROI) on the first frame of every individual study. These ROI's allowed automated calibration for arterial diameter on the B-mode image (Figure 4.1) and velocities (Figure 4.2) on the Doppler strip. A ROI was then drawn around the optimal area of the B-mode image and within this ROI a pixel-density algorithm automatically identified the angle-corrected near and far-wall e-lines for every pixel column within the ROI. The algorithm begins by dividing the ROI into an upper half, containing the near wall lumen-intima interface, and a

lower half containing the far wall interfaces. The near-wall intimal edge is identified by a Rake routine that scans from the bottom to the top of the upper half of the ROI. The position of the edge is established determining the point where the pixel intensity changes most rapidly. Therefore, typical B-mode ROI's contained approximately 200-300 diameter measures per frame, the average of which was calculated and stored. This process transpired at 30 frames per second. A final ROI was drawn around the Doppler waveform and automatically identified the peak of the waveform. The mean diameter measures derived from within the B-mode ROI were then synchronised with the velocity measure derived from the Doppler ROI at 30 Hz. Ultimately, from this synchronised diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity and shear rate (4 times velocity divided by diameter) were calculated at 30 Hz (Black *et al.*, 2008a).

All data were written to file and retrieved for analysis in a custom-designed analysis package. It has been previously demonstrated that reproducibility of diameter measurements using this semi-automated software is significantly better than other manual methods, significantly reduces observer error, and possesses an intra-observer CV of 6.7% (Woodman *et al.*, 2001). Furthermore, our method of blood flow assessment is closely correlated with actual flow through a "phantom" arterial flow system (Green *et al.*, 2002).



**Figure 4.1** 2D B-Mode Ultrasound image recording of the brachial artery in the analysis software with ROI highlighted.



**Figure 4.2** Blood flow velocity trace.

#### 4.4.4 Vascular Data Analysis

Baseline diameter, flow, and shear rate were calculated as the mean of data acquired across the 1-minute baseline period preceding cuff inflation. Peak diameter following cuff deflation was automatically detected according to an algorithm that identified the maximum bracket of data subsequent to performance of a moving window smoothing function. This smoothing routine calculates the median value from 100 consecutive samples before the window shifts to the next bracket of data, which shares 20% overlap

with the preceding bracket. The maximum value of all the calculated median values is then automatically detected and chosen to represent the peak of the post-deflation artery diameter curve. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. The time to peak diameter (in seconds) was calculated from the point of cuff deflation to the maximum post-deflation diameter. Calculation of FMD and time to peak were therefore observer-independent and based on standardised algorithms applied to data that had undergone automated edge-detection and wall-tracking analysis.

In accordance with recent findings (Pyke & Tschakovsky, 2007), we calculated the shear rate stimulus responsible for endothelium-dependent FMD. The post-deflation shear rate data, derived from simultaneously acquired velocity and diameter measures at 30 Hz, was exported to a spread sheet and the area under the shear rate curve (AUC) calculated for data up to the point of maximal post-deflation diameter (FMD) for each individual using the trapezoid rule.

#### **4.5 Magnetic Resonance Methodology**

All women underwent magnetic resonance imaging (MRI) scanning in a 1.5T Siemens Symphony scanner (Siemens Medical Solutions, Erlangen, Germany) in a prone position, being moved through the magnet to acquire full body coverage. Scans were anonymised prior to analysis thus ensuring the observer was blinded to all clinical details.

##### *3.5.1 Volumetric analysis of adipose tissue content*

Total Internal (IAT) and subcutaneous adipose tissue (SAT, fat located immediately below the dermal layer) as well as abdominal SAT and visceral adipose tissue (VAT, fat depots surrounding the internal organs of the abdomen) were calculated from whole body axial

T1-weighted fast spin echo scans (axial scans, 10mm slice thickness followed by a 10mm gap using the integral body coil). The abdominal region was defined as the image slices from the slice containing the femoral heads, to the slice containing the top of the liver/base of the lungs (Figure 4.3). All scans were analysed centrally, and anonymised prior to analysis ensuring blindness to all clinical details.

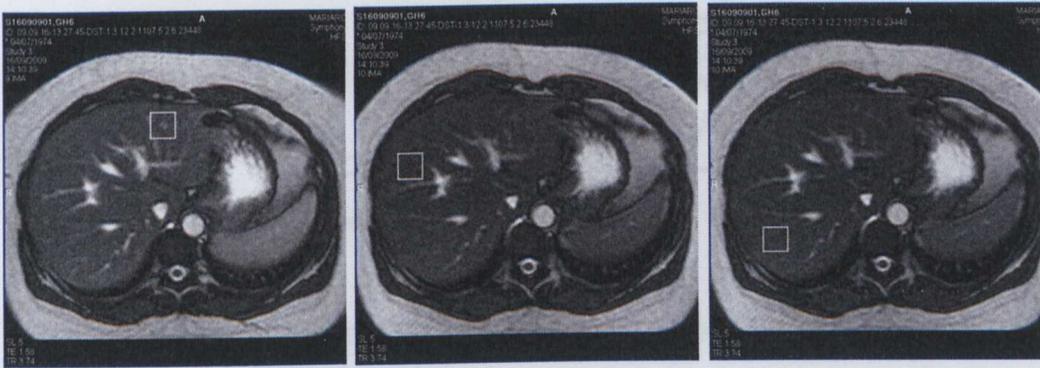


**Figure 4.3** One trans-axial image of abdomen. Area highlighted in green represent abdominal subcutaneous fat and area highlight in red represent abdominal visceral fat.

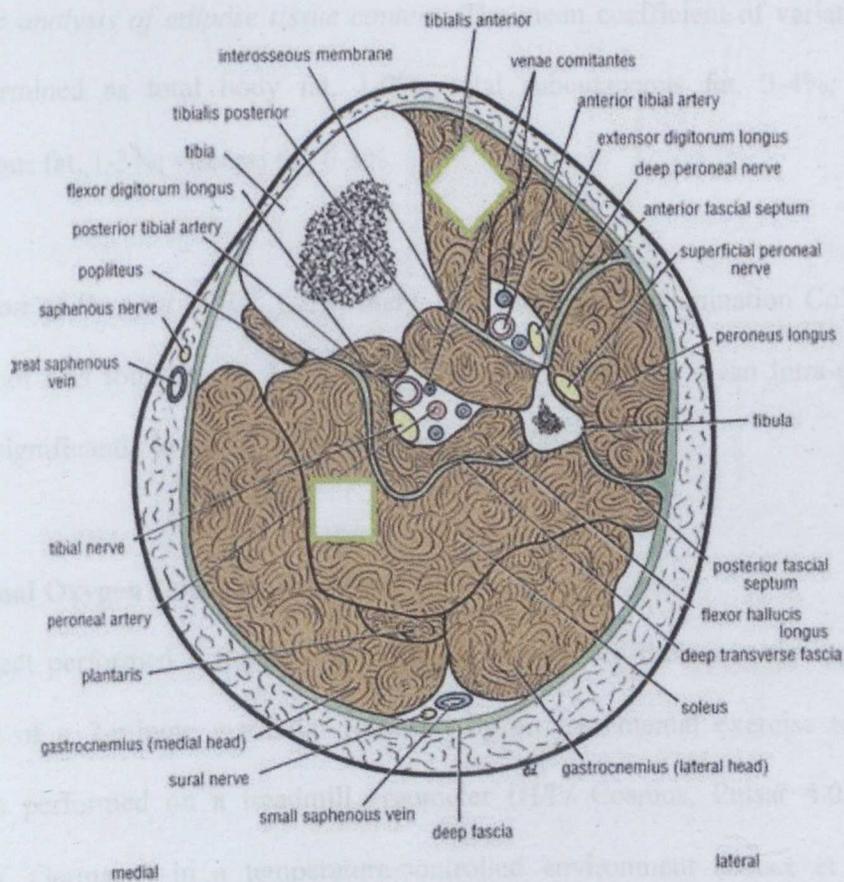
#### 4.5.2 Proton magnetic resonance spectroscopy ( $^1\text{H}$ -MRS)

Three voxels of interest were identified in the liver at standard sites avoiding ducts and vasculature (Figure 4.4). In skeletal muscle a single voxel was identified in each of the tibialis anterior and soleus muscles, avoiding bone, fascia and the neurovascular bundle (Figure 4.5). Single voxel spectroscopy was conducted at each of these five sites. Voxel size was 20x20x20mm, TE 135ms, TR 1500ms, with 64 acquisitions. Where the muscle was too small to allow placement of a 20mm voxel, a 15x15x20mm voxel was placed and the number of acquisitions was increased to 200 to maintain signal-to-noise ratio.  $^1\text{H}$  MR spectra were quantified using the AMARES algorithm in the software package jMRUI-3.0 (Vanhamme *et al.*, 1997; Naressi *et al.*, 2001). As previously described, liver fat is expressed as % of  $\text{CH}_2$  lipid signal amplitude relative to water signal amplitude after

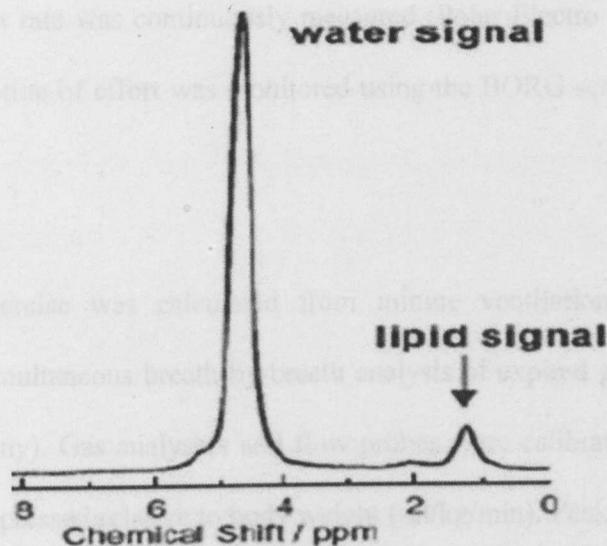
correcting for  $T_1$  and  $T_2$  (Thomas *et al.*, 2005), and intramyocellular lipid (IMCL) is expressed as  $CH_2$  lipid amplitude relative to total creatine amplitude after correcting for  $T_1$  and  $T_2$  (Figure 4.6) (Rico-Sanz *et al.*, 1999).



**Figure 4.4** Example of the voxel positions used during liver spectroscopy.



**Figure 4.5** Example of the voxel positions used during tibialis anterior and soleus spectroscopy.



**Figure 4.6** Percentage ratio of the CH<sup>2</sup> lipid peak area relative to the water peak area.

#### 4.5.3 Reproducibility of MRI and MRS analysis

*Volumetric analysis of adipose tissue content:* The mean coefficient of variations (CoV) were determined as total body fat, 1-2%; total subcutaneous fat, 3-4%; abdominal subcutaneous fat, 1-3%; visceral fat, 6-8%.

*Examination of liver fat (IHCL CH<sub>2</sub>/water):* The mean inter-examination CoV for using this protocol was found to be 7.0% (range 4.0–11.7%) and the mean intra-examination CoV was significantly lower (6.0%) (Thomas *et al.*, 2005).

#### 4.6 Maximal Oxygen Consumption Test

Each subject performed a fitness test (VO<sub>2peak</sub>) to quantify their aerobic capacity. This comprised of a 2-minute warm up followed by an incremental exercise to volitional exhaustion performed on a treadmill ergometer (H/P/ Cosmos, Pulsar 4.0, Nussdorf-Traunstein, Germany) in a temperature-controlled environment (Bruce *et al.*, 1973). Following a 2-minute warm up at 2.2km/h on a flat gradient, the initial workload was set at 2.7km/h at 5° grade. Thereafter, step-wise increments in speed and grade were employed

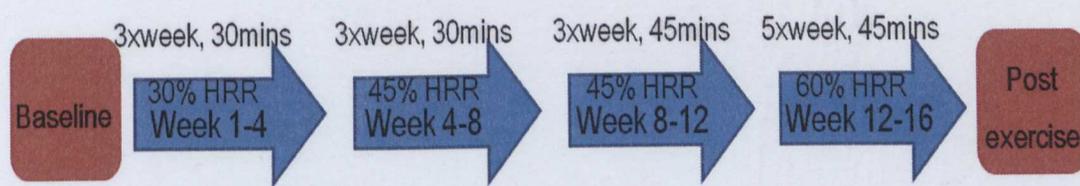
every minute. Heart rate was continuously measured (Polar Electro Oy, Finland) and the participant's perception of effort was monitored using the BORG scale (Burkhalter, 1996; Borg, 1998).

$VO_{2peak}$  during exercise was calculated from minute ventilation, measured using a pneumotach and simultaneous breath-by-breath analysis of expired gas fractions (Oxycon Pro, Jaegar, Germany). Gas analysers and flow probes were calibrated prior to each test. The results were expressed relative to body weight (ml/kg/min). Peak oxygen consumption was calculated as the highest consecutive 15-second period of gas exchange data occurring in the last minute before volitional exhaustion, which usually occurred due to leg fatigue or breathlessness. Physiological criteria for assessment of  $VO_{2peak}$  included a levelling of  $VO_{2peak}$  and/or a respiratory exchange ratio of 1.15 combined with a maximal heart rate at least 90% of the age predicted maximal estimation (220-age) (Miller et al., 1993).

#### **4.7 Supervised Exercise Training Intervention**

Prior to commencing the exercise intervention, all participants attended a thorough familiarisation session at the Research Institute for Sport and Exercise Sciences (RISES) gymnasium. In the first instance, participants were required to undertake 30 minutes of moderate-intensity exercise consisting of treadmill walking/jogging, upright/semi-recumbent cycling, cross-training or rowing. Participants were required to attend the RISES gymnasium at least once a week during which time they wore a heart rate monitor throughout (Polar Fitness, Polar Electro Oy, Finland) and were provided with full supervision and guidance from a trained exercise physiologist (VSS). During these sessions, participants were issued with a weekly progressive exercise programme that was specific to their individual rate of progression. Exercise was primarily directed by heart rate responses and utilisation of Borg's RPE scale (Borg, 1970). Training protocols were specific to each participant's basal fitness level (Hutchison *et al.*, 2011).

During the initial 4 weeks of the intervention, participants underwent 30 minutes of exercise three times a week at 30% of heart rate reserve (HRR). This routine continued from week 5 to week 8, however, during this period exercise workload was modified so that participants maintained a 45% HRR. From week 9 to week 12 participants continued to maintain a workload of 45% HRR and the duration of each session increased to 45 minutes. Finally, from week 13 to week 16 the duration of each session remained at 45 minutes, workload increased to 60% HRR and participants underwent 5 exercise sessions per week (Figure 4.7). Heart rate reserve was calculated as follows;  $((\text{Max HR} - \text{Resting HR}) \times \text{Intensity}) + \text{Resting HR}$ . Maximal and resting HR measures calculated during the maximal oxygen consumption test completed prior to exercise training were utilised.



**Figure 4.7** 16-week moderate-intensity exercise training protocol.

Participants were closely monitored to ensure the maintenance of their individually prescribed rate of perceived exertion and HRR. Furthermore, to facilitate maximum compliance throughout the 16-week period, all participants utilised the Wellness Key system, a software programme that enables remote and accurate tracking of exercise activity. No dietary modifications were made throughout the course of the exercise intervention, confirmed by use of a standard food diary.

#### 4.8 Control Group

Women with PCOS in the control group received typical lifestyle advice provided at clinical consultation. Participants were simply advised by their gynaecologist to modify their lifestyle by losing weight and increasing their physical activity. Control participants

received no contact with the research team at any point during the 16-week intervention period.

## **CHAPTER 5**

# **CONDUIT ARTERY ENDOTHELIAL DYSFUNCTION IN OBESE WOMEN WITH PCOS IS INDEPENDENT OF INTERNAL AND VISCERAL ADIPOSE TISSUE**

## 5.1 Introduction

PCOS is the most common female endocrine disorder associated with menstrual dysfunction and obesity (Goodarzi *et al.*, 2011). Aside from the clinical manifestations, *Chapter 3* provided robust evidence of conduit artery endothelial dysfunction, measured using the flow-mediated dilation (FMD) technique (Thijssen *et al.*, 2011), which appears to be an inherent feature of PCOS. Although, it was difficult to discern between the effects of PCOS, *per se*, and associated co-morbidities; an influence by BMI was evident. Given that BMI cannot distinguish between adipose and lean tissue (Oreopoulos *et al.*, 2011), the direct impact of obesity on endothelial function in women with PCOS, could not be comprehensively assessed.

PCOS is a multi-factorial syndrome with considerable heterogeneity in both clinical characteristics and associated cardiovascular risk factors (Cussons *et al.*, 2006). Insulin resistance, dyslipidaemia and central obesity are common pathological features of PCOS which collectively constitute the metabolic syndrome and thus infer significant cardiovascular disease (CVD) risk (Laws & Reaven, 1993). Specifically, Galvao *et al.* (2012) reported exacerbated endothelial function across rising tertiles of insulin resistance in obese individuals. Nevertheless, a previous study used multiple regression to show that traditional cardiovascular risk factors explained less than 50% of the variation in FMD between PCOS and control women (Sorensen *et al.*, 2006). Yet, the authors only utilised BMI as a marker of obesity.

Indeed, different distributions of body fat, for example increased abdominal fat (Talbot *et al.*, 1995) or visceral adipose tissue (VAT) (Yildirim *et al.*, 2003) in PCOS relative to controls could potentially contribute to the endothelial dysfunction in this patient group. Generally, obesity is associated with a greater volume of subcutaneous adipose tissue (SAT) and VAT. Although SAT accounts for a greater volume of total body adipose tissue,

VAT is more metabolically active (Montague & O'Rahilly, 2000) and closely associated with both insulin resistance (Hutchison *et al.*, 2011) and cardiovascular risk (Cascella *et al.*, 2008a). VAT accumulation is also responsible for the up-regulation of  $\beta$  oxidation which results in subsequent oxidative stress, the release of inflammatory cytokines and adipokines, and a reduction of endogenous adiponectin, an anti-inflammatory substance (Carmina *et al.*, 2006; Cascella *et al.*, 2008a; Carmina *et al.*, 2009; Pepene, 2012). Therefore, it is thought that excess VAT accumulation results in an exacerbated metabolic phenotype of PCOS (Carmina *et al.*, 2005) which may potentially induce a detrimental effect on endothelial function.

In the one study which employed the non-invasive “gold standard” whole body magnetic resonance imaging (MRI) technique to quantify VAT in women with PCOS, similar volumes of VAT were reported between PCOS and weight-matched control women (Barber *et al.*, 2008). Nevertheless, it remains unclear whether endothelial dysfunction is evident in PCOS independent of fat deposition and/or distribution. Therefore, the aim of this study was to examine the impact of adipose tissue volumes on endothelial dysfunction in women with PCOS. It was hypothesised that conduit artery endothelial function would be mediated by VAT.

## **5.2 Methods**

### *5.2.1 Participants*

Thirty five sedentary women with PCOS aged 28y (95% CI=26, 30) of BMI 32kg/m<sup>2</sup> (95% CI=27, 35) and 16 control women aged 32y (95% CI=30, 35) of BMI 30kg/m<sup>2</sup> (95% CI=25, 32) were recruited. For details of the inclusion and exclusion criteria please refer to *Chapter 4, General Methods*. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee. All participants were informed of the methods before providing written informed consent.

### 5.2.2 Research Design

Participants reported to the laboratory on two separate occasions. Visit one included anthropometric measurements, a fasting blood sample, assessment of brachial artery endothelial function and a cardiorespiratory fitness test. Visit two involved whole body magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy (1H MRS). For details of the experimental procedures please refer to *Chapter 4, General Methods*. A 30-year Framingham risk score was calculated for general CVD risk in all participants (Pencina *et al.*, 2009). In control women, data was collected during the early follicular phase of their menstrual cycle, and confirmed using reproductive hormone profiles of follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone and oestrogen. This was not feasible in women with PCOS, due to the erratic nature of their cycles. Participants were asked to fast for 12-hours, abstain from alcohol and caffeine for 24-hours and refrain from exercise for 48-hours prior to testing sessions. All participants were studied at the same time of day to control for the impact of circadian variation (Jones *et al.*, 2010).

### 5.2.3 Statistical Analysis

Analyses were performed using the Statistics Package for Social Sciences for windows, version 17.0 (SPSS Inc. Chicago, IL, USA). The primary outcome variable for this study was FMD (%) and the primary comparison was PCOS vs. control women. Based on the data reported in Cascella *et al.* (2008a) between PCOS ( $n=200$ ) and control women ( $n=100$ ), and using MINITAB 16; a sample size of 6 in each group will have 80% power to detect a standardised difference in means of 2.2SD using a two group *t*-test with a 0.050 one-sided significance level. Firstly, all data were analysed for distribution and transformed appropriately. Differences between PCOS and control women were analysed using independent *t*-tests. Pearson's correlation coefficients (two-tailed) were calculated to evaluate the relationship between FMD and other major variables; followed by a

comparison of slopes to identify differences between groups. Subsequently, FMD were then analysed whilst statistically controlling for internal adipose tissue (IAT), VAT and HOMA-IR as independent covariates. Logarithmically-transformed data were back transformed to the original units and presented in the text as mean (95% CI), unless otherwise stated. Statistical significance was delimited at  $P < 0.05$  and exact  $P$  values are cited (values of  $P$  of “0.000” provided by the statistics package are reported as “ $< 0.001$ ”).

## 5.3 Results

### 5.3.1 Participant Characteristics

The characteristics of all PCOS and control participants are listed in Table 5.1. PCOS and control women were similar in terms of age ( $P=0.14$ ), BMI ( $P=0.10$ ), waist circumference ( $P=0.19$ ) and cardiorespiratory fitness ( $P=0.21$ ). Women with PCOS demonstrated elevated serum testosterone levels [1.0nmol/l (95% CI=0.6, 1.4),  $P < 0.001$ ] and depressed sex hormone binding globulin (SHBG) [-12.2nmol/l (95% CI=-24.3, -0.4),  $P=0.02$ ] compared with controls. Consequently, free androgen index was elevated in the PCOS cohort [5.9% (95% CI=2.6, 9.1),  $P < 0.001$ ]. Lutenizing hormone (LH) was elevated in women with PCOS compared with control women [4.9U/l (95% CI=1.2, 5.6),  $P=0.01$ ].

### 5.3.2 Adipose tissue volume

Control women exhibited lower MRI (total body adipose tissue, IAT, SAT, VAT and abdominal SAT) and  $^1\text{H}$  MRS-derived (liver fat and intramyocellular lipid (IMCL))fat volumes; however, none of these differences were statistically significant ( $P > 0.05$ , Table 5.1).

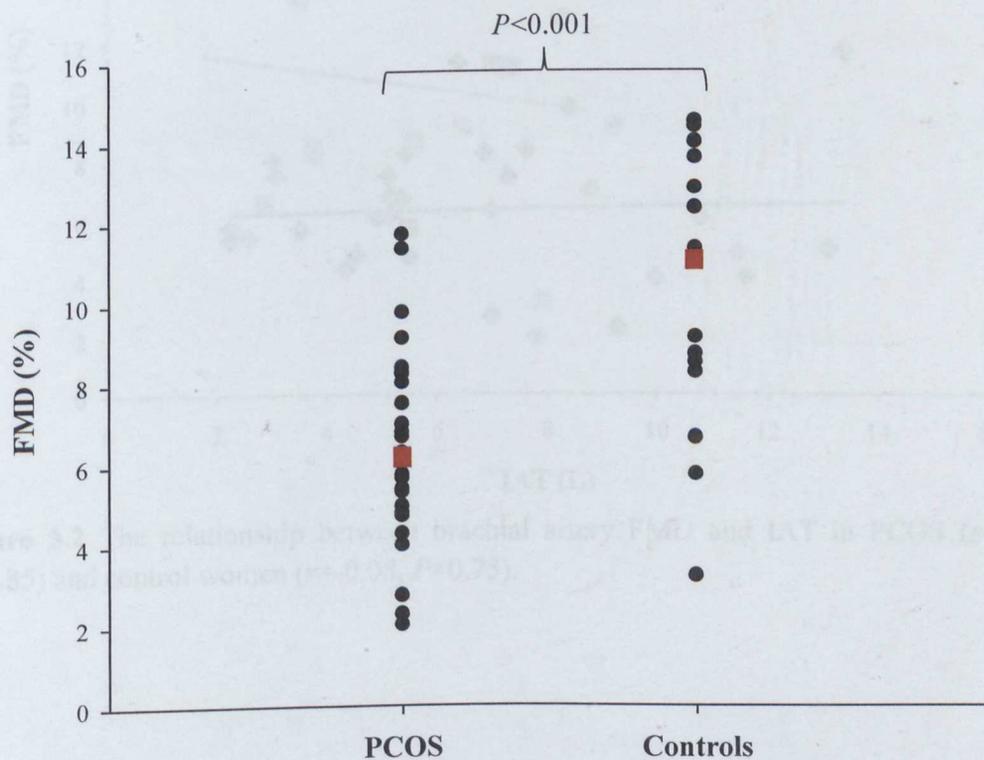
**Table 5.1** Overall comparisons of PCOS and control women.

	PCOS ( <i>n</i> =35)	Control ( <i>n</i> =16)	<i>P</i>
<b>Clinical characteristics</b>			
Age (y)	28(26, 30)	31(27, 35)	0.14
BMI (kg/m <sup>2</sup> )	32(30, 35)	30(25, 32)	0.10
Weight (kg)	90(82, 96)	80(71, 88)	0.10
Waist (cm)	102(96, 108)	95(85, 105)	0.19
VO <sub>2 peak</sub> (ml/kg/min)	26.1(24.0, 28.2)	23.3(18.2, 28.4)	0.21
Testosterone (nmol/l)	2.6(2.3, 2.8)	1.6(1.2, 1.9)	<0.001
SHBG (nmol/l)†	29.4(24.8, 35.0)	42.0(33.7, 52.5)	0.02
FAI†	8.5(6.8, 10.5)	3.7(2.7, 5.0)	<0.001
FSH (U/l)†	5.0(4.4, 5.7)	5.0(4.4, 6.7)	0.48
LH (U/l)†	8.2(6.4, 10.7)	4.8(3.5, 6.7)	0.01
Oestradiol (pmol/l)†	262(218, 315)	253(162, 394)	0.85
Progesterone (nmol/l)†	2.7(2.2, 3.7)	1.9(1.2, 2.8)	0.10
Framingham Risk (CHD 30-year risk) (%)	6.7(5.2, 8.1)	8.7(4.6, 12.8)	0.23
<b>Biochemical and metabolic parameters</b>			
ALT (U/l)†	21.5(17.3, 26.8)	20.0(14.8, 24.3)	0.49
Glucose (mmol/l)	4.6(4.5, 4.8)	4.6(4.3, 4.9)	0.89
Insulin (μU/ml)†	17.3(14.9, 20.0)	20.9(16.3, 26.9)	0.17
HOMA-IR†	3.5(3.0, 4.2)	4.3(3.2, 5.7)	0.22
Cholesterol (mmol/l)	4.9(4.6, 5.3)	4.7(4.2, 5.1)	0.35
Triglycerides (mmol/l)†	3.0(2.6, 3.6)	2.9(2.3, 3.6)	0.70
HDL (mmol/l)	1.4(1.3, 1.5)	1.4(1.2, 1.6)	0.89
LDL (mmol/l)	3.0(2.7, 3.3)	2.8(2.4, 3.1)	0.29
Chol:HDL ratio	3.7(3.3, 4.0)	3.5(2.9, 4.1)	0.47
<b>MRI-measured Adipose Tissue Volumes</b>			
Total body fat (l)	46.2(41.0, 51.4)	38.4(29.7, 47.2)	0.10
IAT (l)	6.8(5.7, 7.9)	5.4(4.2, 6.6)	0.13
SAT (l)	39.4(34.9, 43.9)	33.1(25.1, 41.0)	0.13
IAT:SAT ratio†	0.18(0.15, 0.20)	0.18(0.14, 0.21)	0.97
VAT (l)	3.7(3.0, 4.3)	2.9(2.1, 3.6)	0.16
Abdominal SAT (l)	14.1(12.2, 16.0)	11.0(7.8, 14.1)	0.08
VAT:abdominalSAT ratio†	0.28(0.23, 0.33)	0.29(0.22, 0.37)	0.58
<b><sup>1</sup>H MR Spectroscopy</b>			
Liver fat (% CH <sub>2</sub> /H <sub>2</sub> O)†	4.3(1.8, 8.7)	1.6(0.2, 5.6)	0.17
Soleus IMCL (CH <sub>2</sub> /creatinine)†	10.7(8.6, 13.2)	8.7(5.9, 12.8)	0.31
Tibialis Anterior IMCL (CH <sub>2</sub> /creatinine)†	10.3(7.8, 13.6)	10.4(7.0, 15.3)	0.98
<b>Vascular Function (brachial artery)</b>			
Flow-Mediated Dilation (%)	6.2(5.4, 7.0)	10.7(8.7, 12.8)	<0.001
Baseline Diameter (mm)	0.34(0.32, 0.35)	0.33(0.31, 0.35)	0.80
Peak Diameter (mm)	0.36(0.34, 0.37)	0.37(0.34, 0.39)	0.49
Shear rate AUC (s <sup>-1</sup> ×10 <sup>3</sup> )	20.1(15.4, 24.7)	21.1(13.6, 28.5)	0.81
FMD-Mediated Time to Peak (s)	65.5(54.3, 76.7)	47.5(36.1, 58.9)	0.05
GTN-Mediated Dilation (%)	21.3(18.9, 23.7)	20.2(17.1, 23.3)	0.58
GTN-Mediated Time to Peak (s)	386(350, 423)	343(304, 381)	0.14

† Variables analysed after logarithmic transformation.

### 5.3.3 Measures of Endothelial Function

Brachial artery FMD was impaired in women with PCOS compared with controls [-4.5% (95% CI=-6.3, -2.8),  $P<0.001$ , Figure 5.1]. No differences were observed in baseline diameter, peak diameter or shear rate ( $P>0.49$  Table 5.1). However, the time to reach peak diameter was greater in women with PCOS [18.0s (95% CI=-0.1, 36.1),  $P=0.05$ , Table 5.1]. There was no significant difference between endothelium independent vasodilation [1.1% (95% CI=-3.0, 5.2),  $P=0.58$ , Table 5.1] or time to peak diameter in response to GTN [44s (95% CI=-15, 102),  $P=0.14$ , Table 5.1].

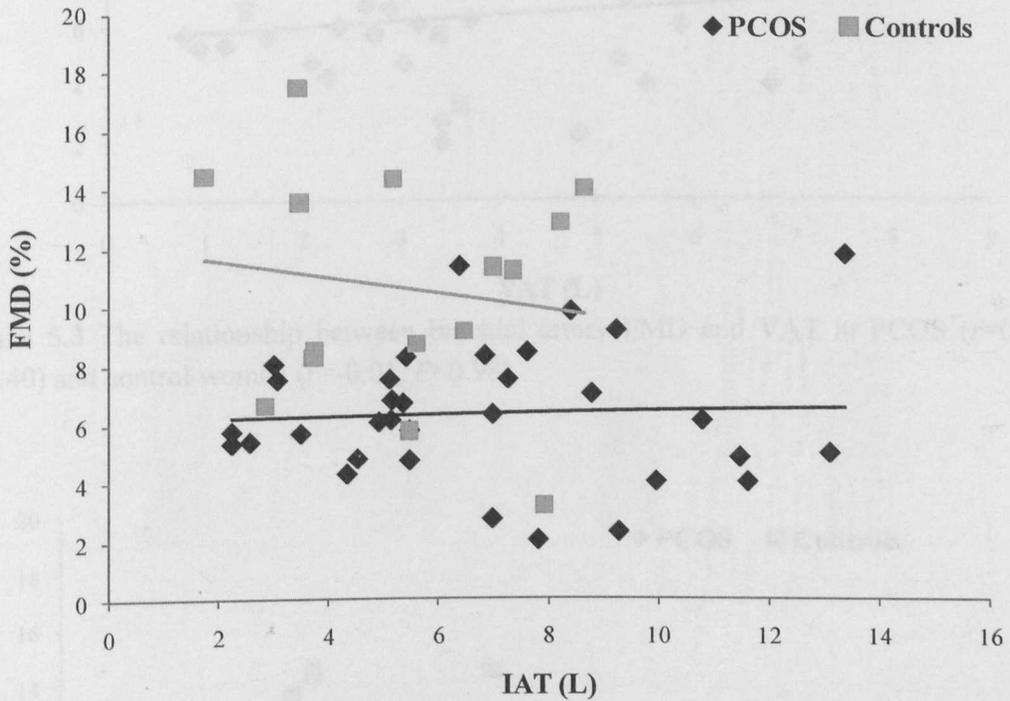


**Figure 5.1** Individual FMD data in women with PCOS and matched controls. Mean FMD% indicated by red points.

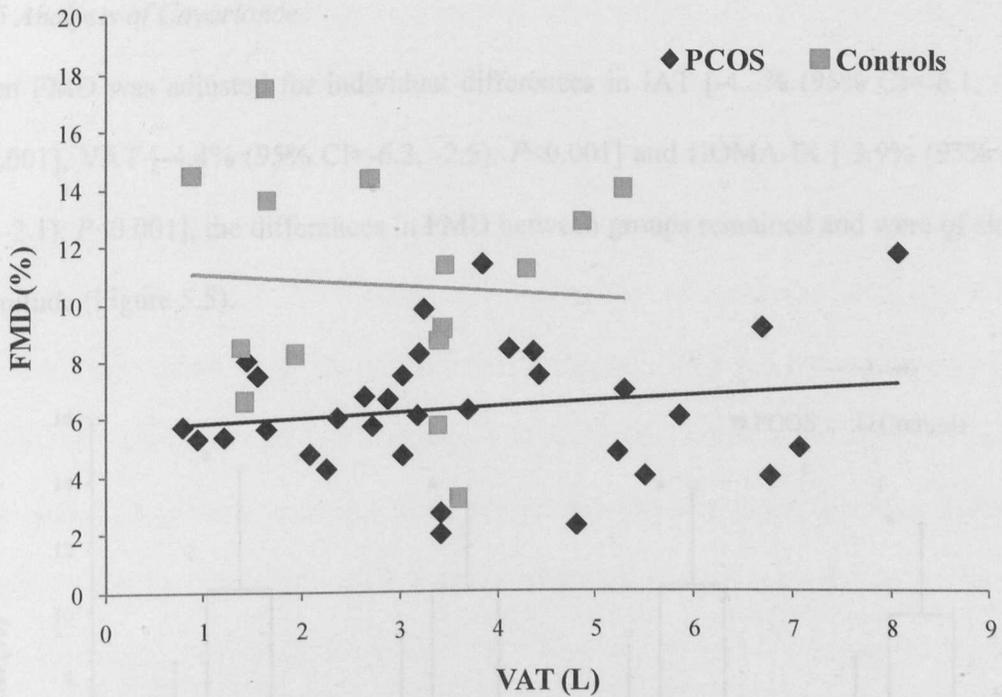
### 5.3.4 FMD Correlations

There was no relationship between brachial artery FMD and MRI-derived IAT ( $r=0.04$ ,  $P=0.85$ ;  $r=-0.08$ ,  $P=0.75$ , Figure 5.2) or VAT ( $r=0.15$ ,  $P=0.40$ ;  $r=-0.01$ ,  $P=0.98$ , Figure 5.3) in either PCOS or control women. Endothelial function did not correlate with traditional

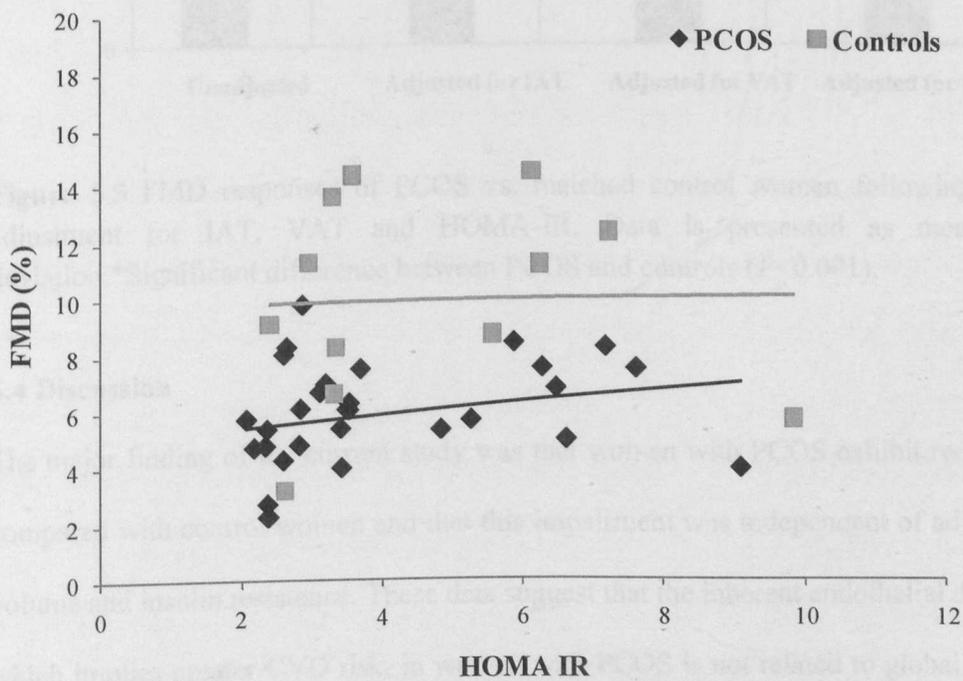
CVD risk factors including HOMA-IR ( $r=0.24$ ,  $P=0.20$ ;  $r=0.02$ ,  $P=0.95$ , Figure 5.4) and insulin ( $r=0.22$ ,  $P=0.25$ ;  $r=0.18$ ,  $P=0.58$ ), in women with PCOS or controls, respectively. Moreover, a comparison of slopes between FMD and IAT ( $P=0.45$ , Figure 5.2), FMD and VAT ( $P=0.54$ , Figure 5.3) and FMD and HOMA-IR ( $P=0.63$ ; Figure 5.4) did not significantly differ between PCOS and control women.



**Figure 5.2** The relationship between brachial artery FMD and IAT in PCOS ( $r=0.04$ ,  $P=0.85$ ) and control women ( $r=-0.08$ ,  $P=0.75$ ).



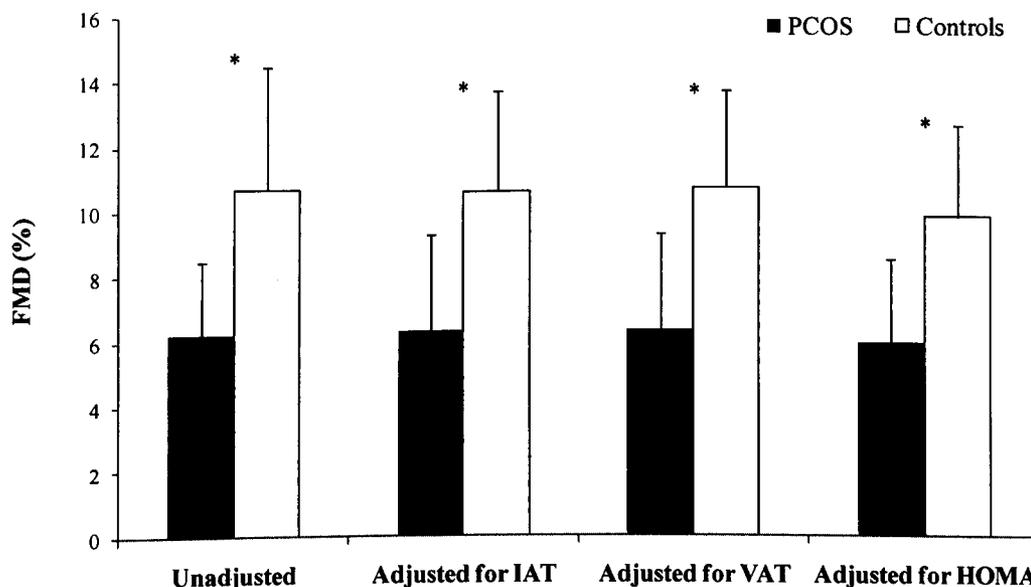
**Figure 5.3** The relationship between brachial artery FMD and VAT in PCOS ( $r=0.15$ ,  $P=0.40$ ) and control women ( $r=-0.01$ ,  $P=0.98$ ).



**Figure 5.4** The relationship between brachial artery FMD and HOMA-IR in PCOS ( $r=0.24$ ,  $P=0.20$ ) and control women ( $r=-0.02$ ,  $P=0.95$ ).

### 5.3.5 Analysis of Covariance

When FMD was adjusted for individual differences in IAT [-4.3% (95% CI=-6.1, -2.4),  $P<0.001$ ], VAT [-4.4% (95% CI=-6.3, -2.5),  $P<0.001$ ] and HOMA-IR [-3.9% (95% CI=-5.6, -2.1),  $P<0.001$ ], the differences in FMD between groups remained and were of similar magnitude (Figure 5.5).



**Figure 5.5** FMD responses of PCOS vs. matched control women following statistical adjustment for IAT, VAT and HOMA-IR. Data is presented as mean±standard deviation.\*Significant difference between PCOS and controls ( $P<0.001$ ).

### 5.4 Discussion

The major finding of the current study was that women with PCOS exhibit reduced FMD compared with control women and that this impairment was independent of adipose tissue volume and insulin resistance. These data suggest that the inherent endothelial dysfunction, which implies greater CVD risk, in women with PCOS is not related to global or regional adiposity or insulin resistance. Therefore, FMD might be a useful independent prognostic tool to assess CVD risk in this population.

Endothelial dysfunction was evident in women with PCOS compared with controls, which

supports the findings from *Chapter 3*, and suggests an inherent impairment in FMD in PCOS, that persists despite statistical adjustment for internal and visceral adipose tissue volume. This finding seems somewhat surprising given that VAT is metabolically active and excess VAT promotes increased circulating FFA and cytokines, (Panagiotakos *et al.*, 2005; Cascella *et al.*, 2008a) which may contribute to the endothelial dysfunction. Moreover, increased visceral fat thickness has been previously associated with endothelial dysfunction in women with PCOS (Cascella *et al.*, 2008a) although, to date, such studies have quantified visceral adiposity using ultrasound which is highly operator dependent (Iacobellis, 2005). Conversely, differences in adipose tissue volume between women with PCOS and controls of similar BMI were not evident in the current study, a finding which supports a previous study using the gold standard non-invasive MRI technique (Barber *et al.*, 2008). Taken together, our data indicate that endothelial dysfunction in PCOS is not explained by obesity.

Despite observing endothelial dysfunction in women with PCOS no differences in 'traditional' cardiovascular disease risk factors, including insulin resistance, were observed between women with PCOS and control women. This is not surprising given that no differences in VAT were evident, which is considered an accurate and independent biomarker for insulin resistance (Lord *et al.*, 2006). This finding supports the extensive work from Green and colleagues (Green *et al.*, 2003; Green *et al.*, 2008; Green, 2009; Green *et al.*, 2011) demonstrating that modification of traditional CVD risk factors does not explain changes in endothelial function. A novel finding of the current study was that time to reach peak arterial diameter following forearm ischemia was significantly greater in women with PCOS. This suggests, for the first time, that a reduced NO bioavailability maybe a mechanism for endothelial dysfunction in women with PCOS as opposed to delayed relaxation at the level of the smooth muscle (Black *et al.*, 2008a).

A possible alternative mechanism influencing FMD in PCOS is hyperandrogenism. In the current study, both testosterone and free androgen index were elevated in women with PCOS. El-Kannishy *et al.* (2010a) observed a negative correlation between brachial artery FMD and testosterone in women with PCOS, but such an adverse impact of androgens on FMD is not a unanimous finding (Empen *et al.*, 2012). A notable limiting factor when evaluating previous studies investigating endothelial function in women with PCOS is the different criteria utilised for diagnosis. In this study, women with PCOS were recruited based on diagnosis using the Rotterdam criteria for which hyperandrogenism is not a fundamental requirement, unlike the 'classical' National Institute of Health (NIH) or Androgen Excess Society (AES) criteria. Although, 28 out of the 35 recruited women fulfilled all three Rotterdam criteria and thus met the 'classical' NIH criteria, it is important to note that only 22 of the 35 exhibited biochemical hyperandrogenism and the remaining 6 exhibited clinical signs of elevated androgens (e.g. acne, hirsutism). Previous studies that have explored the possibility of hyperandrogenism being an important pathological feature of PCOS, have all used specific biochemical parameters including testosterone (El-Kannishy *et al.*, 2010a) and free androgen index (Jones *et al.*, 2012) as markers of hyperandrogenism. Specifically, free testosterone is now considered the most sensitive biomarker supporting the diagnosis of PCOS (Sharquie *et al.*, 2007). Therefore, it is plausible that androgens impose a direct impact on the vessel wall, as steroid receptors exist within the vasculature. Nevertheless, this hypothesis remains unconfirmed and the impact of androgens on vascular reactivity in women with PCOS warrants further specific investigation.

One major advantage of this study is the continuous and simultaneous measurement of arterial diameter and blood flow velocity in combination with edge detection and wall tracking software, which is independent of observer bias and in accordance with the latest guidelines for the assessment of FMD (Thijssen *et al.*, 2011). This technique enables the

continuous measurement of the shear stress stimulus, absolute arterial diameters and time to reach peak diameter. Another noteworthy strength of this study was the utilisation of <sup>1</sup>H-MRS and whole body MRI, which are considered to be the most sensitive, non-invasive methods to quantify liver fat and VAT, respectively. The study incorporated a suitable control group, which were similar in terms of age, BMI and cardiorespiratory fitness.

In summary, the current data suggest that impaired brachial artery endothelial function in obese women with PCOS is independent of VAT, IAT or insulin resistance. Further exploration of potential moderators of FMD and endothelial function in PCOS women with a hyperandrogenic phenotype, determined by raised free testosterone, is warranted.

## **CHAPTER 6**

# **EXERCISE TRAINING IMPROVES CONDUIT ARTERY ENDOTHELIAL FUNCTION IN OBESE WOMEN WITH PCOS**

## 6.1 Introduction

Internationally, PCOS affects up to 10% of women according to the 'classical' National Institute of Health (NIH) criteria (Azziz *et al.*, 2004) and up to 20% using the broader Rotterdam criteria (Broekmans *et al.*, 2006) and is therefore the most common endocrinopathy in females of reproductive age. PCOS is associated with several cardio-metabolic pathologies including obesity, insulin resistance, impaired glucose tolerance or type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), dyslipidaemia and hypertension (Goodarzi *et al.*, 2011). Therefore, it might be expected that there would be an increased prevalence of cardiovascular disease (CVD) in this population. A plethora of studies using surrogate markers of CVD risk in women with PCOS, including measures of vascular structure (Arikan *et al.*, 2009) and function (*Chapter 3 and 5*) and biochemical serum concentrations of CVD risk markers (Toulis *et al.*, 2011), have supported this association. The relationship between PCOS and CVD is further evidenced by a recent meta-analysis of follow-up studies in women with PCOS which detailed a ~2 fold increased risk of arterial disease compared with non-PCOS women, specifically coronary heart disease and stroke (de Groot *et al.*, 2011).

Lifestyle modification has been endorsed by the Androgen Excess (AE)-PCOS Society as a first line treatment in the prevention of CVD (Wild *et al.*, 2010). Existing exercise-intervention studies in women with PCOS have reported the beneficial effects of exercise on reproductive function (Palomba *et al.*, 2008), obesity (Moran *et al.*, 2009b) and cardiorespiratory fitness (Vigorito *et al.*, 2007) but the effect of exercise on cardiovascular parameters remains unknown.

Exercise training has been found to improve endothelial function in patients who exhibit similar risk factors to women with PCOS, for example in patients with type 2 diabetes (Maiorana *et al.*, 2001). Indeed, exercise induced improvements in endothelial function

have been reported to occur both prior to, and independent of, changes in traditional markers of CVD risk, such as BMI and blood pressure, in patients with coronary artery disease (Walsh *et al.*, 2003), hypertension (Higashi *et al.*, 1999) and type 2 diabetes (Maiorana *et al.*, 2001). Nevertheless, it is unknown how the inherent endothelial dysfunction in women with PCOS is modified by interventions. For instance, a study assessing the effects of metformin on arterial stiffness, a surrogate measure of vascular function, reported that enhanced vascular function may be independent of changes in insulin resistance (Agarwal *et al.*, 2010). Therefore, the aim of this study was to determine the effects of a 16-week moderate-intensity aerobic exercise-training programme, compared with a control intervention, on endothelial function in obese women with PCOS. It was hypothesised that 16-weeks of supervised exercise training would enhance FMD to a greater degree than conventional care.

## **6.2 Methods**

### *6.2.1 Participants*

Seventeen women with PCOS aged 28y (95% CI=25, 31), of BMI 33kg/m<sup>2</sup> (95% CI=30, 35) were recruited from a reproductive clinic. For details of the inclusion and exclusion criteria please refer to *Chapter 4, General Methods*. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee. All participants were informed of the methods before providing written informed consent.

### *6.2.2 Research Design*

Participants reported to the laboratory on two separate occasions. Visit one included anthropometric measurements, a fasting blood sample, assessment of brachial artery endothelial function and a cardiorespiratory fitness test. Visit two involved whole body magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy (<sup>1</sup>H MRS). For details of the experimental procedures please refer to *Chapter 4, General*

*Methods.* A 30-year Framingham risk score was calculated for general CVD risk in all participants (Pencina *et al.*, 2009). It was not feasible to control for menstrual cycle phase in women with PCOS, due to the erratic nature of their cycles. Participants were asked to fast for 12-hours, abstain from alcohol and caffeine for 24-hours and refrain from exercise for 48-hours prior to testing sessions. All participants were studied at the same time of day to control for the impact of circadian variation (Jones *et al.*, 2010).

Ten [ $n=10$ , 27y (95% CI=23, 32), 31kg/m<sup>2</sup> (95% CI=28, 34)] women with PCOS received a 16-week programme of supervised moderate-intensity aerobic exercise training while seven women [ $n=7$ , 29y (95% CI=24, 35), 35kg/m<sup>2</sup> (95%CI=31, 40)] were used as a control group. All patients underwent the physiological measurements at baseline and after the 16-weeks of intervention. For details regarding the respective interventions please refer to *Chapter 4, General Methods*.

### 6.2.3 Statistical Analyses

Analyses were performed using the Statistics Package for Social Sciences for windows, version 17.0 (SPSS Inc. Chicago, IL, USA). The primary outcome variable for this study was FMD (%) and the primary comparison was the effect of exercise vs. conventional care. Based on the data reported in *Chapter 3* between PCOS and control women, and using the NQUERY (Statistical Solutions, Ireland) software, it was estimated that a sample size of 9 in the exercise and CC groups will have 80% power to detect a difference between groups in mean change of 4%. Firstly, all data were analysed for distribution and transformed appropriately. For the comparison of the exercise vs. conventional care intervention, delta ( $\Delta$ ) change from pre-intervention was calculated (Vickers & Altman, 2001) and analysed using generalised estimating equations (GEE), with pre-exercise data as a covariate. The analysis approach based on GEE is considered a powerful and robust approach to the analysis of repeated measures data (Ballinger, 2004). Logarithmically-transformed data

were back-transformed to the original units and presented in the text as mean (95% CI), unless otherwise stated. Statistical significance was delimited at  $P < 0.05$  and exact  $P$  values are cited (values of  $P$  of “0.000” provided by the statistics package are reported as “ $< 0.001$ ”).

### **6.3 Results**

Women with PCOS that completed 16-weeks of exercise training demonstrated 91% compliance to exercise sessions.

#### *6.3.1 Cardiorespiratory fitness*

There was a greater improvement in cardiorespiratory fitness following exercise training compared with CC [4.7ml/kg/min (95% CI=1.4, 7.9),  $P=0.005$ , Figure 6.1].

#### *6.3.2 Biochemical Characteristics*

Exercise training and conventional care did not affect fasting glucose [-0.02mmol/l (95% CI=-0.09, 0.13),  $P=0.78$ , Table 6.1] or insulin [-4.4 $\mu$ U/ml (95% CI=-11.3, 2.5),  $P=0.21$ , Table 6.1] differently; therefore, there was no difference in HOMA-IR following the respective interventions [-0.6 (95% CI=-2.2, 1.0),  $P=0.47$ , Figure 6.1]. Cholesterol reduced to a greater degree following exercise training compared with the conventional care group [-0.20mmol/l (95% CI=-0.28, 0.04),  $P=0.01$ ,]. LDL also decreased following exercise training [-0.7mmol/l (95% CI=-1.1, -0.3),  $P=0.001$ , Table 6.1].

**Table 6.1** Changes in the characteristics of women with PCOS following supervised exercise training and conventional care (CC).

	Pre Exercise	Post Exercise	Ex Δ Change	Pre CC	Post CC	CC Δ Change	P
<b>Clinical characteristics</b>							
Body mass index (kg/m <sup>2</sup> )	31(28, 34)	31(27, 34)	-0.2(-0.9, 0.6)	35(31, 40)	36(31, 40)	0.2(-0.3, 0.8)	0.42
Weight (kg)	82.1(71.3, 92.8)	81.6(70.2, 93.1)	-0.1(-2.0, 1.9)	98.6(83.5, 113.6)	99.6(83.5, 115.6)	0.5(-1.2, 2.1)	0.72
Waist (cm)	100(90, 101)	97(84, 109)	-3.1(-7.6, 1.4)	109(95, 124)	108(97, 119)	-1.3(-4.9, 2.3)	0.55
VO <sub>2</sub> peak (ml/kg/min)	29.0(25.1, 32.8)	33.7(28.5, 38.8)	4.5(2.7, 6.3)*	23.8(21.5, 26.2)	23.3(19.8, 26.8)	-0.2(-2.9, 2.5)*	<b>0.005</b>
Testosterone (nmol/l)	2.7(2.3, 3.1)	2.5(2.1, 2.9)	-0.2(-0.4, 0.1)	2.5(1.9, 3.2)	2.2(1.4, 2.9)	-0.4(-0.6, 0.1)	0.33
SHBG (nmol/l)	32.2(11.7, 52.6)	28.8(11.6, 46.1)	-3.1(-13.5, 7.3)	31.7(10.6, 52.8)	29.7(6.9, 52.5)	-2.2(-19.0, 14.6)	0.93
FAI†	8.7(5.3, 14.2)	9.8(3.9, 15.8)	1.1(0.9, 1.4)	8.7(3.9, 19.4)	9.3(5.1, 17.3)	0.8(0.5, 1.5)	0.38
FSH(iu/l)	4.5(2.8, 6.1)	5.1(3.4, 6.8)	0.3(-0.9, 1.6)	5.2(2.6, 7.7)	5.5(3.6, 7.4)	0.6(-0.9, 2.0)	0.80
LH (iu/l)	8.1(3.9, 16.7)	8.8(5.6, 13.8)	0.23(-0.08, 0.54)	5.9(2.5, 14.2)	7.0(3.6, 13.9)	0.03(-0.47, 0.53)	0.49
Oestradiol (pmol/l)†	252(170, 334)	214(168, 260)	-123(-181, -65)	423(103, 741)	418(153, 682)	80(-145, 306)	0.13
Progesterone (nmol/l)	4.7(-0.3, 9.8)	2.2(0.9, 3.4)	-3.0(-4.6, -1.5)	5.6(0.5, 10.6)	6.1(-3.2, 15.5)	-1.1(-5.9, 8.0)	0.27
Framingham Risk (CHD 30-year risk)	6.1(3.7, 8.5)	5.7(3.3, 8.1)	-0.4(-1.0, 0.1)	7.6(4.3, 10.8)	7.7(4.7, 10.7)	0.2(-0.5, 0.9)	0.16
<b>Biochemical and metabolic parameters</b>							
ALT (U/l)	22.5(12.9, 32.1)	25.7(12.5, 38.8)	3.4(-4.2, 11.0)	20.8(13.4, 28.2)	20.8(12.7, 28.9)	0.3(-5.8, 5.2)	0.51
Glucose (mmol/l)†	4.6(4.4, 4.8)	4.7(4.5, 4.9)	0.01(-0.05, 0.07)	4.8(4.4, 5.3)	4.8(3.9, 5.9)	-0.01(-1.09, 0.10)	0.78

	Pre Exercise	Post Exercise	Ex Δ Change	Pre CC	Post CC	CC Δ Change	P
Insulin (μU/ml)	16.8(10.1, 23.6)	17.3(10.4, 24.2)	0.6(-2.1, 3.3)	16.1(7.8, 24.3)	21.1(11.1, 31.2)	4.9(-1.4, 11.3)	0.21
HOMA-IR†	3.4(1.1, 5.7)	3.8(1.7, 6.0)	0.5(-0.2, 1.2)	3.4(1.7, 5.2)	4.5(2.4, 6.5)	1.1(-0.4, 2.5)	0.47
Cholesterol (mmol/l)†	5.3(3.7, 7.8)	4.7(4.1, 5.4)	-0.10(-0.19, 0.1)*	4.9(4.5, 5.5)	5.4(4.8, 6.1)	0.06(-0.02, 0.15)*	<b>0.01</b>
Triglycerides (mmol/l)	1.5(6.8, 2.4)	1.6(1.0, 2.2)	0.2 (-0.2, 0.7)	1.1(0.7, 1.6)	1.3(0.8, 1.8)	0.03(-2.7, 0.34)	0.48
HDL (mmol/l)	1.2(1.0, 1.5)	1.2(0.9, 1.5)	-0.03(-0.15, 0.09)	1.4(1.2, 1.5)	1.4(1.2, 1.5)	0.01(-0.07, 0.09)	0.58
LDL (mmol/l)	3.1(2.5, 3.6)	2.8(2.4, 3.2)	-0.3(-0.5, -0.1)*	3.1(2.5, 3.7)	3.5(2.7, 4.2)	0.4 (0.1, 0.8)*	<b>0.001</b>
Chol:HDL ratio	3.9(3.0, 4.7)	4.3(3.7, 4.9)	0.5(0.0, 0.9)	3.7(3.0, 4.4)	4.0(3.2, 4.8)	0.3(-0.9, 0.6)	<b>0.49</b>
<b>MRI-measured Adipose Tissue Volumes</b>							
Visceral fat (l)	3.4(1.5, 5.1)	3.4(2.1, 4.7)	-0.1(-0.5, 0.4)	3.6(1.8, 5.3)	3.4(2.1, 4.6)	-0.2(-0.7, 0.3)	0.73
Abdominal subcutaneous fat (l)	12.5(9.6, 15.4)	11.8(8.7, 15.0)	-0.82(-1.7, 0.03)	15.4(11.7, 19.7)	15.6(11.0, 20.2)	-0.89(-2.74, 0.96)	0.94
<b><sup>1</sup>H MR Spectroscopy</b>							
Liver fat (% CH <sub>2</sub> /H <sub>2</sub> O)†	4.0(0.2, 20.7)	2.1(-0.2, 13.1)	-0.4(-0.6, 0.0)	4.2(1.4, 10.4)	3.2(0.1, 16.2)	-0.2(-0.6, 0.7)	0.56
Soleus IMCL (CH <sub>2</sub> /creatinine)	17.5(9.2, 25.8)	16.0(7.3, 24.8)	0.2(-4.8, 5.2)	11.1(4.5, 17.7)	12.6(1.8, 23.3)	-0.6(-8.4, 7.2)	0.85
Tibialis Anterior IMCL(CH <sub>2</sub> /creatinine)	12.5(6.2, 18.8)	15.7(6.9, 24.5)	2.0(-4.6, 8.7)	16.2(3.2, 29.2)	11.9(2.6, 21.1)	-2.9(-9.4, 3.6)	0.30

† Variables analysed after logarithmic transformation. Data are presented as mean (95% CI). Delta (Δ) change from pre-intervention following adjustment for pre-intervention values (95% CI). \* Significant difference between Δ Ex and Δ CC (P<0.05).

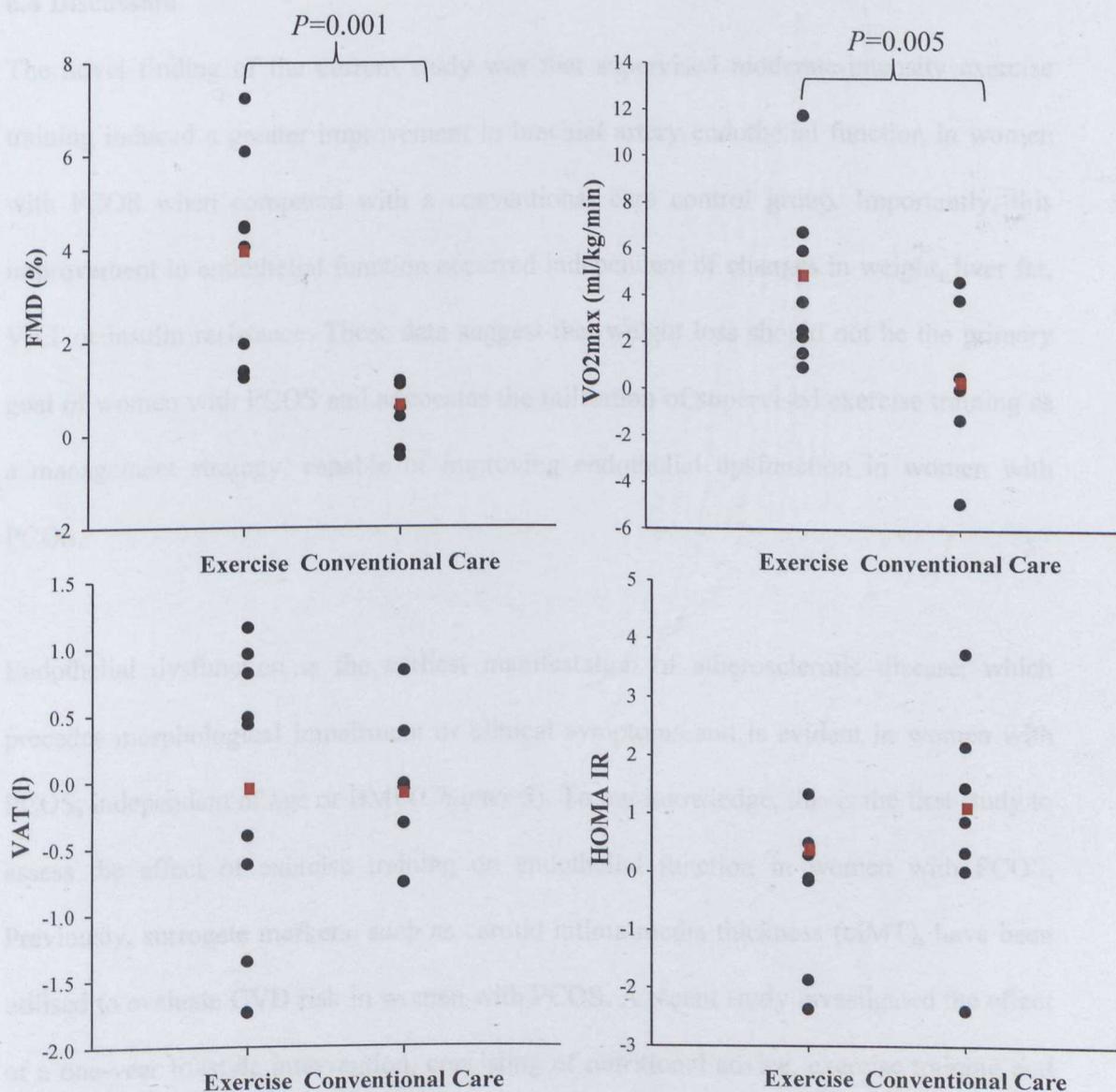
**Table 6.2** Changes in the brachial artery vascular function women with PCOS following supervised exercise training and conventional care (CC).

	Pre Exercise	Post Exercise	Ex $\Delta$ Change	Pre CC	Post CC	CC $\Delta$ Change	P
Flow-Mediated Dilation (%)	5.9(4.0, 7.8)	10.1(7.6, 12.6)	4.0(2.2, 5.8)*	7.1(5.6, 8.5)	7.2(6.1, 8.3)	0.7(0.3, 1.1)*	<0.001
Baseline Diameter (mm)	0.32(0.30, 0.34)	0.32(0.29, 0.35)	0.00(-0.01, 0.01)	0.33(0.28, 0.37)	0.34(0.29, 0.39)	0.00(0.00, 0.01)	0.42
Peak Diameter (mm)	0.34(0.32, 0.36)	0.35(0.32, 0.39)	0.02(0.00, 0.03)	0.35(0.30, 0.40)	0.36(0.31, 0.40)	0.01(0.00, 0.01)	0.51
Shear rate <sub>AUC</sub> (s <sup>-1</sup> × 10 <sup>3</sup> )	21.7(10.0, 33.3)	23.1(12.3, 32.5)	1.7(-6.7, 7.1)	17.2(-0.2, 34.5)	16.3(8.9, 31.0)	-1.1(-7.9, 6.2)	0.32
Time to Peak (s)	63.8(42.4, 85.1)	44.9(37.0, 68.2)	-12.9(-37.4, 1.3)*	45.5(10.7, 80.4)	58.6(30.5, 86.6)	3.9(-15.2, 23.0)*	0.09
GTN-Mediated Dilation (%)	19.3(15.1, 24.5)	19.5(17.2, 22.2)	0.03(-0.05, 0.11)	18.3(14.3, 23.2)	20.1(15.2, 26.7)	0.08(-0.14, 0.29)	0.69

Data are presented as mean (95% CI). Delta ( $\Delta$ ) change from pre-intervention following adjustment for pre-intervention values (95% CI).  
 \* Significant difference between  $\Delta$  Ex and  $\Delta$  CC ( $P < 0.05$ ).

### 6.3.3 Vascular Measurements

Mean FMD demonstrated a greater improvement following exercise training compared with conventional care [3.5% (95% CI=1.7, 5.1),  $P>0.001$ , Figure 6.1]. Time to reach peak diameter was faster following exercise training [-16.8seconds (95% CI=-32.1, 1.5),  $P=0.09$ ]. There was no difference in baseline or peak arterial diameter or shear rate between interventions ( $P>0.05$ ). All vascular data are summarised in Table 6.2.



**Figure 6.1** Individual FMD delta change scores for following exercise training and conventional care. Mean values indicated by red data points.

#### 6.3.4 Hepatic and Abdominal Fat Deposition

There was no significant difference in change in liver fat between exercise-trained and conventional care [-0.5% CH<sub>2</sub>/H<sub>2</sub>O (95% CI=-1.2, 0.6), *P*=0.56, Table 6.1]. Similarly, there was no significant difference in weight, BMI, waist circumference muscle fat, VAT or abdominal SAT between interventions (*P*>0.05; Table 6.1).

### 6.4 Discussion

The novel finding of the current study was that supervised moderate-intensity exercise training induced a greater improvement in brachial artery endothelial function in women with PCOS when compared with a conventional care control group. Importantly, this improvement in endothelial function occurred independent of changes in weight, liver fat, VAT or insulin resistance. These data suggest that weight loss should not be the primary goal of women with PCOS and advocates the utilisation of supervised exercise training as a management strategy, capable of improving endothelial dysfunction in women with PCOS.

Endothelial dysfunction is the earliest manifestation of atherosclerotic disease, which precedes morphological impairment or clinical symptoms and is evident in women with PCOS, independent of age or BMI (*Chapter 3*). To our knowledge, this is the first study to assess the effect of exercise training on endothelial function in women with PCOS. Previously, surrogate markers, such as carotid intima-media thickness (cIMT), have been utilised to evaluate CVD risk in women with PCOS. A recent study investigated the effect of a one-year lifestyle intervention, consisting of nutritional advice, exercise training and behavioural therapy, in obese adolescent PCOS girls and reported a reduced cIMT in those girls who achieved significant weight loss only (Lass *et al.*, 2011). Nevertheless, the authors could not distinguish between the effects of exercise, diet, behavioural therapy and

weight loss, nor did they include a control group for comparison. Importantly, the present findings indicate that moderate-intensity aerobic exercise, that meets the current recommended physical activity guidelines of the American College of Sports Medicine (Gordon, 2009) and the American Heart Association (Pearson *et al.*, 2002), can induce significant improvements in brachial artery FMD, an early and prognostic marker of future cardiovascular risk, in the absence of weight loss.

There were no significant changes in parameters of obesity including BMI, waist circumference, or abdominal adipose tissue volumes following exercise training in these obese women. This finding is in contrast to Hutchinson *et al.* (2011) who reported a significant decrease in BMI, VAT and insulin resistance following 12 weeks of exercise, which consisted of alternated moderate-intensity continuous exercise (~70%  $VO_{2peak}$ ) and high intensity intermittent exercise (~90-100%  $VO_{2peak}$ ) for ~3hr/week, in women with PCOS. It is plausible that an exercise training stimulus of higher intensity may have elicited changes in adipose tissue volume within the current study. Nevertheless, given that the exercise intervention enhanced FMD in women with PCOS to a level comparable to control women of similar age and BMI (*Chapter 3*), and body composition does not mediate the difference in FMD observed in PCOS and control women, as observed in *Chapter 5*, it is unlikely that changes in body composition within the current study would induce a greater increase in FMD.

The exercise intervention did cause significant reductions in total and LDL cholesterol, which provides further evidence for the positive effect of exercise training on CVD risk reduction in PCOS. Nevertheless, insulin resistance and liver fat did not significantly change with exercise training. Intriguingly both insulin resistance and liver fat (liver fat <5.56%) (Szczepaniak *et al.*, 2005) were not deemed elevated in these women prior to the

intervention. One explanation for the data being within normal healthy limits may be related to the criteria employed to diagnose PCOS in these women. National Institute of Health (Zawadzki & Dunaif, 1992) and Androgen Excess Society (Azziz *et al.*, 2006) definitions describe more obese and insulin resistant phenotypes of PCOS, due to hyperandrogenism being an integral criteria for diagnosis (Moran & Teede, 2009). Biochemical hyperandrogenic PCOS represents a distinct metabolic phenotype characterised by an increased risk of hepatic steatosis and insulin resistance (Jones *et al.*, 2012, *Journal of Clinical Endocrinology and Metabolism*, under review). Hyperandrogenism is not an essential feature of the Rotterdam (2004) criteria; as indicated within the current study population, as only 11 of the 17 women demonstrated biochemical hyperandrogenism. Nevertheless, it is unlikely that androgens contributed to the exercise-induced improvement in FMD observed in the present study, as testosterone and free androgen index did not change following either exercise or conventional care.

Given that the exercise-induced improvement in endothelial function was independent of concomitant changes in body composition, insulin resistance or lipid profiles, the current data suggests that exercise has a direct effect on the endothelium in women with PCOS. It is widely accepted that the FMD response in conduit vessels is endothelium-dependent and nitric oxide-mediated (Doshi *et al.*, 2001). Exercise training has been shown to promote NO bioavailability by reducing the number of oxygen free radicals, and up-regulating endothelial NO synthase in CAD patients (Hambrecht *et al.*, 2003). Moreover, it has been hypothesised that episodic increases in arterial shear stress, induced by exercise, act as a key stimulus to these functional adaptations of the endothelium which decrease atherosclerotic risk (Green, 2009). Elevated CVD (hypertension) and event (stroke) risk in this population is vascular in origin and therefore a direct effect on vascular function with exercise is crucial in these women.

One limitation of this study was that the intervention was based on patient choice rather than a more robust randomisation method to allocate the intervention. This limitation is highlighted in the fact that conventional care group had a greater BMI compared to the exercise group. However, given that we employed a robust covariate control analysis technique that incorporate adjustments for differences in baseline measurements and significant changes in BMI did not occur with the intervention, it is unlikely to have an impact on the study outcome. Nevertheless, there are several noteworthy strengths in the methodology of this study. No alteration in diet enabled comprehensive assessment of the impact of exercise training exclusively on endothelial function in PCOS. Finally, the study was appropriately powered to detect a difference of 4% in the primary variable (FMD) between the control and exercise groups.

In summary, the current data suggest that supervised moderate-intensity exercise training enhances endothelial function in women with PCOS. Given that FMD has been found to provide independent prognostic information relating to CVD risk, these data support the utilisation of supervised exercise training as a cardio-protective strategy for vascular dysfunction, and primary prevention for CVD risk independent of weight loss, in women with PCOS.

## **CHAPTER 7**

**NITRIC-OXIDE MEDIATED CUTANEOUS  
MICROVESSEL FUNCTION IS IMPAIRED IN  
POLYCYSTIC OVARIAN SYNDROME AND CAN  
BE IMPROVED WITH EXERCISE TRAINING**

## 7.1 Introduction

Polycystic ovarian syndrome (PCOS) is associated with endothelial dysfunction in conduit arteries (*Chapters 3 & 5*) and a higher prevalence of hypertension in the post-menopausal period (Schmidt *et al.*, 2011). Cutaneous microvessel vasodilator function can be used as a surrogate of generalised microvascular function and provides a translational model to investigate pre-clinical disease (Holowatz *et al.*, 2008). A small number of studies have investigated cutaneous endothelial function in PCOS and have reported cutaneous microvessel dysfunction (Lakhani *et al.*, 2005; Alexandraki *et al.*, 2006). However, the most recent study reported that cutaneous microvessel dysfunction was only apparent during insulin infusion in obese PCOS and control women, but not in lean PCOS or control women, thereby suggesting that obesity, not PCOS *per se*, was associated with microvascular dysfunction (Ketel *et al.*, 2008).

The studies examining microvascular function in women with PCOS have challenged the endothelium of cutaneous microvessels using iontophoresis-mediated infusion of acetylcholine (ACh). Although ACh is a precursor of cutaneous vasodilatation, this technique is limited as the vasodilator response is not exclusive to nitric oxide (NO) (Holowatz *et al.*, 2005). Rather, intradermal infusion of a competitive NO inhibitor is the only method which allows for the accurate assessment of the NO contribution to cutaneous vasodilation (Cracowski *et al.*, 2006). To date, no research study has examined the contribution of NO to cutaneous microvessel dysfunction in obese women with PCOS, which is surprising considering endothelium-derived NO acts as a stimulus to anti-atherogenic adaptations (Green, 2009).

It has been established that NO-mediated vasodilation in the cutaneous microvessels is influenced by fitness in healthy individuals (Black *et al.*, 2008b) and that exercise training

improves NO-mediated cutaneous microvascular function (Black *et al.*, 2008b; Simmons *et al.*, 2011). Despite lifestyle modification being recommended as a primary prevention strategy of CVD in PCOS (*Chapter 6*; Wild *et al.*, 2010), no study to date has investigated the impact of exercise training on cutaneous microvascular function in women with PCOS. Therefore, the aims of the current study were to (i) examine NO-mediated cutaneous microvascular function in obese PCOS and control women using intradermal microdialysis allowing for the infusion of a specific NO antagonist; and (ii) to examine the effect of a 16-week moderate-intensity aerobic exercise training programme on NO-mediated cutaneous microvascular function in women with PCOS. It was hypothesised that (i) the NO-mediated vasodilator response to gradual local heating would be impaired in women with PCOS compared with controls and (ii) that supervised exercise training would induce an improvement.

## **7.2 Methods**

### *7.2.1 Participants*

Eleven women with PCOS [29y (95% CI=25, 34), 34kg/m<sup>2</sup> (95% CI=30, 38)] and six matched controls [29y (95% CI=21, 37), 34kg/m<sup>2</sup> (95% CI=28, 39)] were recruited. For details of the inclusion and exclusion criteria please refer to *Chapter 4, General Methods*. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee. All participants were informed of the methods before providing written informed consent.

### *7.2.2 Research Design*

Participants reported to the laboratory on two separate occasions. Visit one included anthropometric measurements, a fasting blood sample, assessment of cutaneous NO-mediated vasodilator function (described below) and a cardiorespiratory fitness test. Visit

two involved whole body magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy ( $^1\text{H}$  MRS). For details of the experimental procedures please refer to *Chapter 4, General Methods*. In control women, data was collected during the early follicular phase of their menstrual cycle, and confirmed using reproductive hormone profiles. This was not feasible in women with PCOS, due to the erratic nature of their cycles. Participants were asked to fast for 12 hours, abstain from alcohol and caffeine for 24 hours and refrain from exercise for 48 hours prior to testing sessions. All participants were studied at the same time of day to control for the impact of circadian variation (Jones *et al.*, 2010)

Following baseline assessment, six women with PCOS [30y (95% CI=22, 37), 31kg/m<sup>2</sup> (95% CI=25, 37)] enrolled onto a 16-week supervised moderate-intensity aerobic exercise training programme. Upon completion, physiological assessments were repeated. For details regarding the exercise training intervention please refer to *Chapter 4, General Methods*.

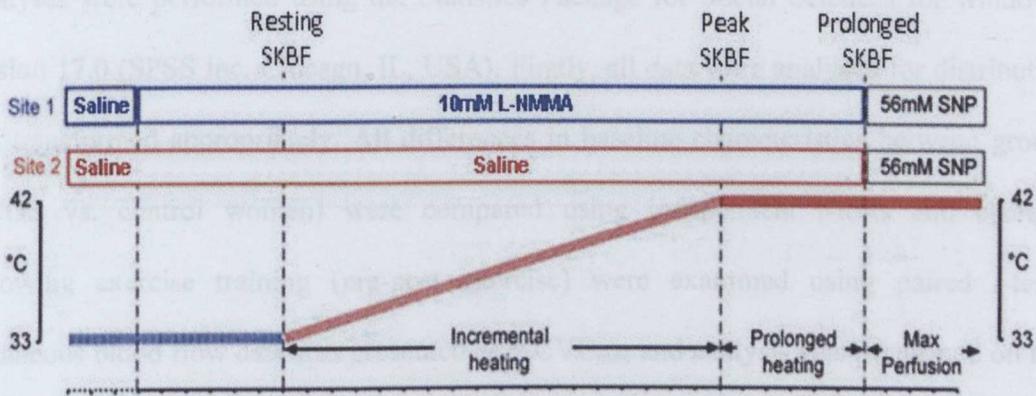
### 7.2.3 Microdialysis fibre instrumentation

All intradermal microdialysis assessments were performed in a quiet, temperature-controlled laboratory. Upon arrival, participants were instrumented and cannulation for microdialysis probe insertion was undertaken (~15 minute). Once the participant was seated comfortably in a custom-designed bed, the right arm was supinated and supported for insertion of microdialysis fibres. The insertion sites were marked on the skin and cold packs were applied as a form of local anaesthesia. Two 21-gauge needles were inserted ~5cm apart from one another and ~0.3-1.0mm beneath the epidermal surface, allowing threading and placement of two microdialysis fibres (Linear 30, CMA Microdialysis Ltd, Stockholm, Sweden), containing 10mm long 6kDa membranes. The needles were then

removed and the embedded fibres were perfused with saline solution at a rate of 5µl/minute using a micro-infusion pump (Model 11 plus, Harvard Apparatus, MA, USA). Following this, integrated laser Doppler probes (Model 413, Periflux 5001 System, Perimed AB, Sweden) combined with local heating disks (Perimed 455, Stockholm, Sweden) set at 33°C were placed above both embedded microdialysis fibres sites. Laser Doppler flowmetry (LDF) uses a laser diode to emit a monochrome light at a penetrative depth of approximately 1mm<sup>3</sup>. The incident monochrome light reflects off moving blood cells causing a shift in the returning wavelength (Doppler effect) from which estimates of cutaneous blood flux (the concentration of red blood cells x their velocity) can be made. Consequently, LDF enables sensitive and quantifiable detection of relative changes in cutaneous blood flow in response to a given stimulus.

#### *7.2.4 Physiological NO-mediated vasodilatation*

Following a ~90 minute equilibration period, the skin surrounding both microdialysis probes was gradually heated, using local heating disks, from 33 to 42°C at a rate of 0.5°C per 2.5 minute (45 minute). Thereafter, both sites were continuously heated at 42°C for a further 30 minutes. This gradual heating protocol was used to minimise the impact of heating on axon reflexes, which are less NO-mediated than slow heating component responses (Minson *et al.*, 2001; Houghton *et al.*, 2006). Saline solution was infused throughout the protocol in one site and L-N<sup>G</sup>-monomethyl arginine (L-NMMA, 10mM, 5µl/minute, Clinalfa, Bachem, Germany) infused through the second, from 30 minutes prior to the onset of heating. Sodium nitroprusside (SNP, 56mM, Mayne Pharma, Warwickshire, UK), a potent NO donor, was infused at the end of the protocol for 30 minutes (Minson *et al.*, 2002; Cracowski *et al.*, 2006) to initiate peak vasodilatation (Figure 2).



**Figure 7.1** Schematic of the physiological (localised heating) NO-mediated vasodilatation protocol.

#### 7.2.5 Assessment of forearm skin blood flow

To obtain an index of cutaneous blood flow, cutaneous red cell flux was measured by placing integrated laser-Doppler probes, each consisting of a seven-laser array, above each microdialysis site. The laser-Doppler probe signals were continuously monitored via an online software chart recorder (PSW, Perimed, Sweden). At each designated study time-point (2.5 minute intervals), cutaneous blood flow was assessed by averaging laser-Doppler flux, measured in perfusion units (PU), over a stable 30-second period. These data were subsequently converted to cutaneous vascular conductance (CVC), calculated as  $LDF/MAP$  (PU mmHg), where MAP was derived from contemporaneous automated blood pressure measures (Dinamap; GE Pro 300V2) in the contralateral arm. Values were then expressed relative to the maximal CVC achieved during infusion of 56mM of SNP at 42 °C, as  $\%CVC_{max}$ ; this is the preferred method of data expression adopted in the literature (Cracowski *et al.*, 2006). Data during the incremental heating were calculated and presented at each temperature (every 0.5°C from 33°C to 42°C) for both the saline and L-NMMA microdialysis sites. The contribution of NO was calculated by subtracting individual L-NMMA data from saline data collected simultaneously.

### 7.2.6 Statistical Analysis

Analyses were performed using the Statistics Package for Social Sciences for windows, version 17.0 (SPSS Inc. Chicago, IL, USA). Firstly, all data were analysed for distribution and transformed appropriately. All differences in baseline characteristics between groups (PCOS vs. control women) were compared using independent *t*-tests and changes following exercise training (pre-post exercise) were examined using paired *t*-tests. Cutaneous blood flow data was presented as %CVC<sub>max</sub> and analysis was performed on this normalised data (Cracowski *et al.*, 2006). Logarithmically transformed data were back-transformed to the original units and presented in the text as mean (95% CI), unless otherwise stated. Statistical significance was delimited at  $P < 0.05$  and exact *P* values are cited (values of *P* of “0.000” provided by the statistics package are reported as “<0.001”).

To ensure successful increase in NO production with the local heat stimulus and successful blockade of NO production, saline and L-NMMA data were individually compared using a two factor (site vs. temperature) analysis of variance (ANOVA). A two factor (group vs. temperature) repeated measures ANOVA was also employed to compare the contribution of NO (saline %CVC<sub>max</sub> minus L-NMMA %CVC<sub>max</sub> at equivalent time points) to %CVC<sub>max</sub> response at baseline (PCOS vs. control) and following exercise training (pre-post exercise). Statistically significant interactions between these two factors were followed-up with least significant difference (LSD) approach to multiple comparisons (Rothman, 1990; Perneger, 1998).

## 7.3 Results

### 7.3.1 PCOS vs. controls: Clinical Characteristics

PCOS and control women were similar in terms of age, BMI and cardiorespiratory fitness (Table 7.1). Women with PCOS displayed elevated testosterone [0.8mmol/l (95% CI=0.3, 1.4),  $P=0.006$ ] and free androgen index [4.0mmol/l (95% CI=0.1, 7.9),  $P=0.05$ ] compared

with control women.

**Table 7.1** Baseline characteristics of PCOS ( $n=11$ ) and control women ( $n=6$ ).

	PCOS	Controls	<i>P</i>
<b>Clinical Characteristics</b>			
Age (y)	29(25, 34)	29(21, 37)	0.98
BMI(kg/m <sup>2</sup> )	34(30, 38)	34(28, 39)	0.84
Weight (kg)	91.2(79.3, 103.0)	91.0(75.7, 106.3)	0.98
Waist circumference (cm)	106(97, 115)	106(89, 123)	0.98
VO <sub>2peak</sub> (ml/kg/min)	26.1(23.0, 29.2)	25.3(17.4, 36.3)	0.44
FSH (iu/l)	4.9(3.8, 6.0)	5.7(4.0, 7.3)	0.37
LH (iu/l)	11.0(6.2, 15.8)	7.6(3.5, 11.7)	0.31
Progesterone (nmol/l)†	3.0(2.1, 4.2)	2.2(0.8, 6.1)	0.41
Oestradiol (pmol/l)†	253(188, 340)	244(127, 467)	0.89
Testosterone(nmol/l)	2.8(2.4, 3.2)	1.9(1.5, 2.4)	<b>0.006</b>
Free Androgen Index (%)	9.7(7.0, 12.5)	5.7(3.8, 7.7)	<b>0.05</b>
SHBG (nmol/l)†	31.3(21.5, 45.7)	40.0(26.6, 48.7)	0.59
<b>Biochemical and metabolic parameters</b>			
ALT (iu/l)†	22.3(14.5, 34.5)	24.5(10.4, 57.7)	0.80
Glucose (mmol/l)	4.6(4.4, 4.8)	4.6(4.4, 4.9)	0.97
Insulin (pmol/l)†	17.5(13.2, 23.2)	18.8(12.0, 29.5)	0.75
HOMA-IR†	3.6(2.6, 4.9)	3.9(2.5, 6.3)	0.71
Cholesterol (mmol/l)†	5.0(4.5, 5.5)	5.1(4.5, 5.7)	0.89
Triglyceride (mmol/l)†	2.0(1.2, 3.2)	2.2(1.3, 3.7)	0.74
HDL (mmol/l)†	1.4(1.2, 1.7)	1.4(1.1, 1.7)	0.79
LDL (mmol/l)	3.0(2.6, 3.4)	3.1(2.6, 3.6)	0.74
Chol:HDL ratio	3.5(2.9, 4.2)	3.7(2.4, 4.9)	0.82
<b>MRI measured adipose tissue volumes</b>			
IAT (l)	6.0(4.5, 7.4)	7.1(5.9, 8.4)	0.25
SAT (l)	39.8(31.7, 47.9)	41.5(33.7, 49.3)	0.77
VAT (l)	3.2(2.3, 4.1)	3.8(2.9, 4.7)	0.35
Abdominal SAT (l)	14.2(11.1, 17.2)	13.7(10.3, 17.1)	0.83
Total body fat (l)	45.8(36.9, 57.7)	48.6(41.5, 55.8)	0.64
<b><sup>1</sup>H MR Spectroscopy</b>			
Liver fat (% CH <sub>2</sub> /H <sub>2</sub> O)†	3.7(0.6, 12.8)	4.0(1.6, 8.6)	0.93
Soleus IMCL (CH <sub>2</sub> /creatine)†	11.1(7.1, 17.5)	15.5(8.3, 29.0)	0.33
Tibialis Anterior IMCL(CH <sub>2</sub> /creatine)†	13.2(8.1, 21.5)	9.8(3.3, 29.2)	0.50

†Variables analysed following logarithmic transformation.

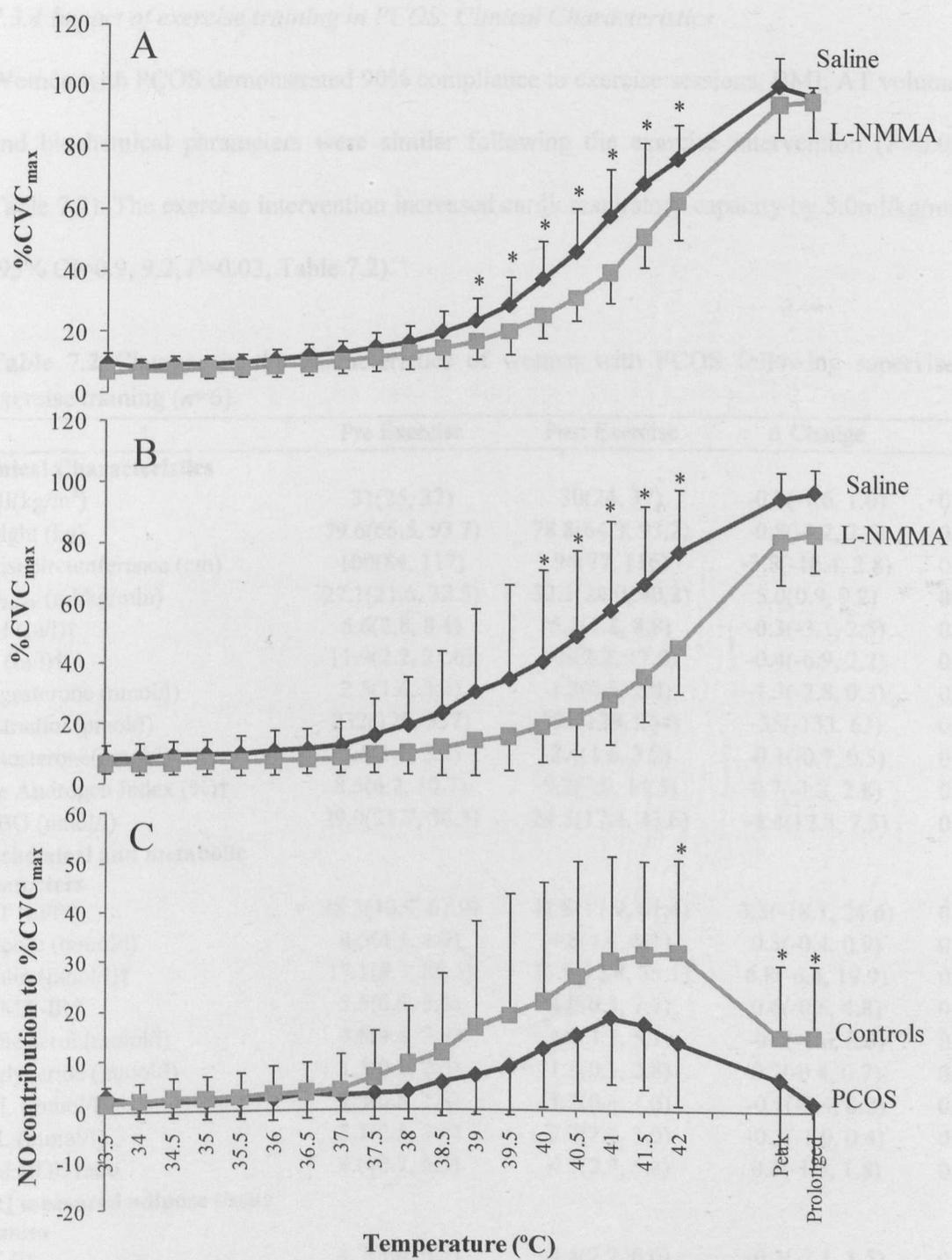
### 7.3.2 PCOS vs. Controls: Incremental Heating

In response to local heating, %CVC<sub>max</sub> increased steadily at microdialysis sites infused with either saline or L-NMMA in all participants at baseline ( $P<0.001$ , Figure 7.2).

However, the site infused with L-NMMA displayed a reduced %CVC<sub>max</sub> in both PCOS and control women ( $P=0.001$ , Figure 7.2); suggesting that the cutaneous response to local heating in both groups was, at least in part, mediated by the NO dilator system. A significant microdialysis site x temperature interaction was evident ( $P<0.001$ , Figure 7.2) and pairwise comparisons revealed significant differences between the saline and L-NMMA site from 39-42°C in PCOS and 40-42°C in control women ( $P<0.05$ ).

### 7.3.3 PCOS vs. Controls: Nitric Oxide Contribution to Incremental Heating

There was a significant group by temperature interaction between PCOS and control women ( $P=0.01$ , Figure 7.2). Subsequent pairwise comparisons revealed that there was a significant difference in NO contribution between PCOS and control women when the incremental heating protocol reached 42°C [-17.5%CVC<sub>max</sub> (95% CI=-33.3, -1.7),  $P=0.03$ ] and subsequently when the heating stimulus remained at 42°C for both 5 (peak) [-16.0%CVC<sub>max</sub> (95% CI=-32.5, 0.6),  $P=0.05$ ] and 30 minutes (prolonged) [-15.4%CVC<sub>max</sub> (95% CI=-29.6, -1.3),  $P=0.04$ ] (Figure 7.2).



**Figure 7.2** The effect of incremental and prolonged heating on cutaneous blood flow in the saline and L-NMMA microdialysis site in (A) PCOS and (B) control women and (C) the contribution of NO (saline %CVC<sub>max</sub> minus L-NMMA %CVC<sub>max</sub>) in women with PCOS and matched controls. Data is presented as mean±standard deviation. \*Significant difference between L-NMMA and saline sites ( $P < 0.05$ ).

### 7.3.4 Impact of exercise training in PCOS: Clinical Characteristics

Women with PCOS demonstrated 90% compliance to exercise sessions. BMI, AT volumes and biochemical parameters were similar following the exercise intervention ( $P>0.05$ , Table 7.2). The exercise intervention increased cardiorespiratory capacity by 5.0ml/kg/min (95% CI=0.9, 9.2,  $P=0.03$ , Table 7.2).

**Table 7.2** Changes in the characteristics of women with PCOS following supervised exercise training ( $n=6$ ).

	Pre Exercise	Post Exercise	$\Delta$ Change	<i>P</i>
<b>Clinical Characteristics</b>				
BMI(kg/m <sup>2</sup> )	31(25, 37)	30(24, 37)	-0.3(-1.6, 1.0)	0.54
Weight (kg)	79.6(65.5, 93.7)	78.8(64.3, 93.2)	-0.8(-4.2, 2.6)	0.56
Waist circumference (cm)	100(84, 117)	96(77, 116)	-3.8(-10.4, 2.8)	0.20
VO <sub>2peak</sub> (ml/kg/min)	27.1(21.6, 32.5)	32.1(24.0, 40.2)	5.0(0.9, 9.2)	<b>0.03</b>
FSH (iu/l)†	5.6(2.8, 8.4)	5.3(1.8, 8.8)	-0.3(-3.1, 2.5)	0.75
LH (iu/l)†	11.9(2.2, 21.6)	9.6(2.2, 17.0)	-0.4(-6.9, 2.2)	0.22
Progesterone (nmol/l)	2.5(1.6, 3.4)	1.3(0.5, 2.0)	-1.3(-2.8, 0.3)	0.08
Oestradiol (pmol/l)	232(126, 337)	197(129, 264)	-35(-133, 63)	0.34
Testosterone(nmol/l)	2.5(1.7, 3.3)	2.4(1.6, 3.2)	-0.1(-0.7, 0.5)	0.64
Free Androgen Index (%)†	8.5(6.2, 10.7)	9.2(7.0, 11.5)	0.7(-1.2, 2.8)	0.15
SHBG (nmol/l)	29.0(21.7, 36.3)	24.5(17.4, 33.6)	-4.4(-12.3, 7.5)	0.51
<b>Biochemical and metabolic parameters</b>				
ALT (iu/l)†	38.5(10.9, 67.9)	41.8(17.9, 61.4)	3.3(-18.1, 24.6)	0.66
Glucose (mmol/l)	4.5(4.1, 4.9)	4.8(4.4, 5.1)	0.3(-0.4, 0.9)	0.32
Insulin (pmol/l)†	17.1(8.7, 25.5)	23.9(12.8, 35.1)	6.8(-6.4, 19.9)	0.24
HOMA-IR†	3.5(0.6, 5.5)	4.0(0.3, 7.7)	0.6(-0.6, 4.8)	0.80
Cholesterol (mmol/l)	4.8(4.4, 5.3)	4.6(4.1, 5.1)	-0.2(-1.0, 0.6)	0.43
Triglyceride (mmol/l)	1.3(0.1, 2.5)	1.5(0.1, 2.8)	0.2(-0.4, 0.7)	0.44
HDL (mmol/l)	1.3(0.8, 1.8)	1.2(0.4, 1.0)	-0.1(-0.4, 0.2)	0.35
LDL (mmol/l)	3.1(2.5, 3.6)	2.7(2.4, 3.0)	-0.3(-1.0, 0.4)	0.23
Chol:HDL ratio	4.0(2.7, 5.3)	4.3(2.7, 5.8)	0.3(-1.3, 1.8)	0.64
<b>MRI measured adipose tissue volumes</b>				
IAT (l)	4.7(3.0, 6.3)	4.4(2.7, 6.0)	-0.3(-2.1, 1.5)	0.70
SAT (l)	30.7(20.9, 40.5)	29.8(19.0, 40.5)	-1.0(-3.6, 1.7)	0.38
VAT (l)	2.4(1.5, 3.2)	2.5(1.4, 3.7)	0.2(-0.8, 1.1)	0.65
Abdominal SAT (l)	11.7(7.3, 16.0)	11.3(6.4, 16.1)	-0.4(-1.4, 0.6)	0.36
Total body fat (l)	35.2(24.3, 46.2)	34.3(21.5, 47.0)	-1.0(-5.2, 3.3)	0.56
<b><sup>1</sup>H MR Spectroscopy</b>				
Liver fat (% CH <sub>2</sub> /H <sub>2</sub> O)†	1.5(0.2, 2.3)	0.4(0.1, 1.1)	-0.4(-0.8, 0.6)	0.21
Soleus IMCL (CH <sub>2</sub> /creatine)†	17.1(5.6, 28.6)	16.2(4.5, 27.9)	-0.9(-15.4, 13.6)	0.88
Tibialis Anterior IMCL(CH <sub>2</sub> /creatine)†	8.6(5.1, 14.5)	13.4(7.4, 24.3)	4.8(0.6, 9.8)	0.28

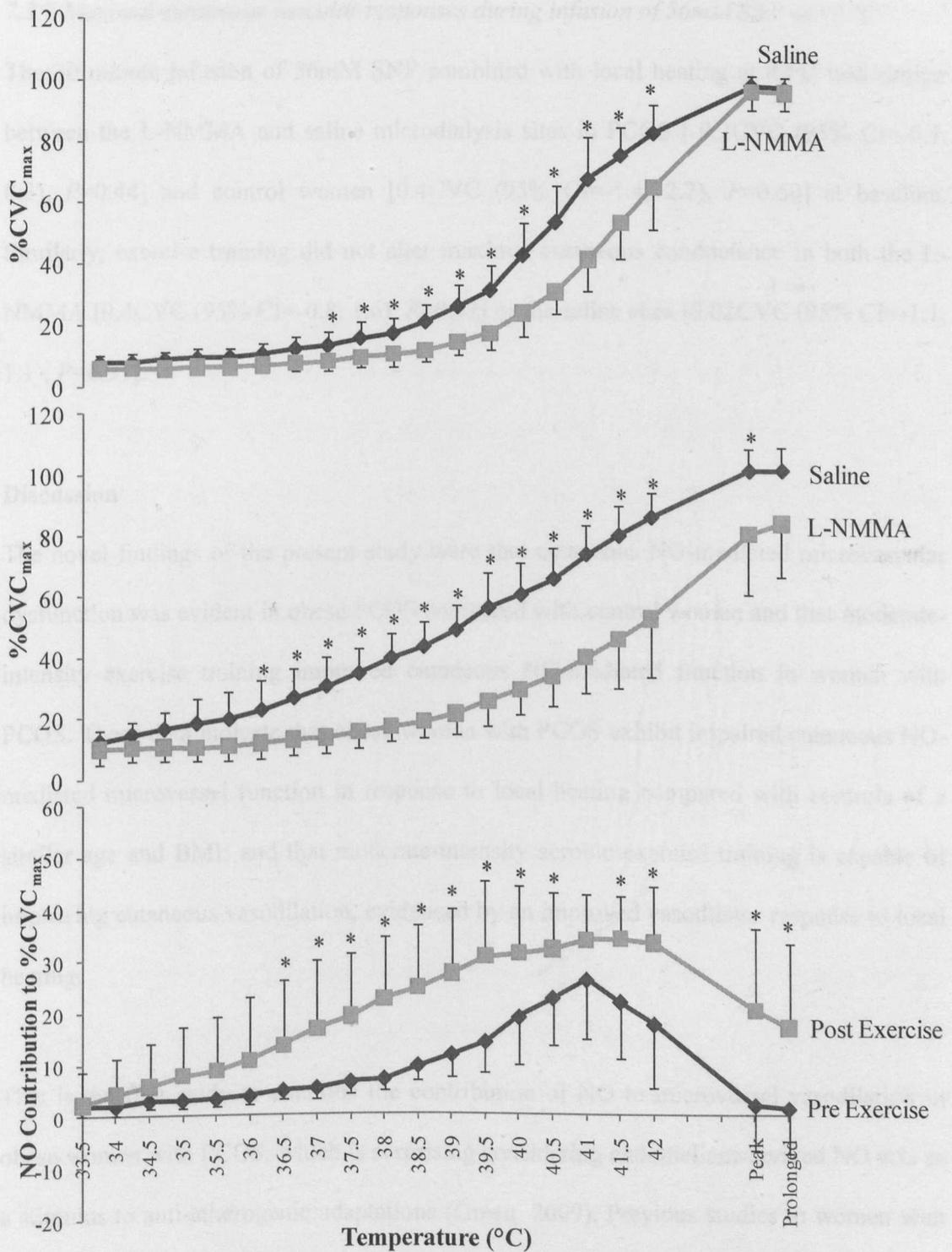
† Variables analysed following logarithmic transformation.

### *7.3.5 Impact of exercise training in PCOS: Incremental heating*

Prior to and following exercise training, cutaneous blood flow increased in response to local heating in both the microdialysis sites infused with saline and L-NMMA ( $P < 0.001$ , Figure 7.3), suggesting that the cutaneous response to local heating was at least in part, mediated by the NO dilator system. There was a significant interaction (microdialysis site x temperature) pre and post exercise training ( $P < 0.001$ , Figure 7.3). Pairwise comparisons revealed differences between sites from 36-42°C pre exercise and 36.5-42°C post exercise ( $P < 0.05$ ).

### *7.3.6 Impact of exercise training in PCOS: Nitric Oxide Contribution to Incremental Heating*

A significant intervention x temperature interaction was evident in NO contribution to %CVC<sub>max</sub> with exercise training ( $P < 0.001$ , Figure 7.3). NO contribution to incremental heating was greater post-exercise when incremental heating reached 36.5°C up to 42°C [-19.4%CVC<sub>max</sub> (95% CI=-38.4, -0.4),  $P=0.05$ ] and subsequently when the heating stimulus remained at 42°C for both 5 (peak) [-19.6%CVC<sub>max</sub> (95% CI=-34.9, -4.3),  $P=0.02$ ] and 30 minutes (prolonged) [-17.1%CVC<sub>max</sub> (95% CI=-32.1, -2.2),  $P=0.03$ ] (Figure 7.3).



**Figure 7.3** The effect of incremental and prolonged heating on cutaneous blood flow in the saline and L-NMMA microdialysis site in women with PCOS (A) pre and (B) post exercise training and (C) the contribution of NO (saline %CVC<sub>max</sub> minus L-NMMA %CVC<sub>max</sub>) pre and post exercise training. Data is presented as mean±standard deviation. \*Significant difference between L-NMMA and saline sites ( $P < 0.05$ ).

### 7.3.7 Maximal cutaneous vascular responses during infusion of 56mM SNP at 42 °C

The 30 minute infusion of 56mM SNP combined with local heating at 42°C was similar between the L-NMMA and saline microdialysis sites in PCOS [-0.2CVC (95% CI=-0.7, 0.3),  $P=0.44$ ] and control women [0.4CVC (95% CI=-1.4, 2.2),  $P=0.60$ ] at baseline. Similarly, exercise training did not alter maximal cutaneous conductance in both the L-NMMA [0.4CVC (95% CI=-0.8, 1.6),  $P=0.42$ ] or the saline sites [0.02CVC (95% CI=-1.1, 1.1),  $P=0.97$ ].

## Discussion

The novel findings of the present study were that cutaneous NO-mediated microvascular dysfunction was evident in obese PCOS compared with control women and that moderate-intensity exercise training improved cutaneous NO-mediated function in women with PCOS. These data indicate that obese women with PCOS exhibit impaired cutaneous NO-mediated microvessel function in response to local heating compared with controls of a similar age and BMI; and that moderate-intensity aerobic exercise training is capable of improving cutaneous vasodilation, evidenced by an improved vasodilator response to local heating.

This is the first study to examine the contribution of NO to microvessel vasodilation in obese women with PCOS, which is surprising considering endothelium-derived NO acts as a stimulus to anti-atherogenic adaptations (Green, 2009). Previous studies in women with PCOS have investigated cutaneous vasodilation in response to iontophoresis-mediated infusion of ACh which is primarily mediated by prostanioids (Khan *et al.*, 1997; Noon *et al.*, 1998) and does not elicit a significant contribution from NO (Holowatz *et al.*, 2005). The NO-mediated vasodilator pathway plays a crucial role in the control of cutaneous endothelial function (Giles *et al.*, 2012), and the stimulus of localised heating at 42°C has

been reported to elicit the greatest contribution of NO (up to 70%) to the vasodilator response (Kellogg *et al.*, 1999; Minson *et al.*, 2001). Moreover, intradermal infusion of L-NMMA, a potent NO blocker (Goldsmith *et al.*, 1996) during gradual heating is the optimal technique to evaluate the cutaneous NO system (Cracowski *et al.*, 2006). Therefore, whilst the previous data suggest cutaneous microvessel dysfunction is evident in PCOS (Lakhani *et al.*, 2005; Alexandraki *et al.*, 2006), and this impairment may be related to obesity (Ketel *et al.*, 2008). The data from the current study, utilising the most up-to-date and appropriate technique, indicate that obese women with PCOS display a reduced bioavailability of NO resulting in impaired cutaneous microvessel vasodilation compared with obese control women. It is plausible that such NO-mediated microvessel dysfunction in PCOS may contribute to increased CVD, specifically the higher prevalence of hypertension in women with PCOS observed during the menopausal years (Schmidt *et al.*, 2011).

Testosterone and free androgen index were elevated in women with PCOS within the current study and could be postulated as a potential explanation for microvessel dysfunction. Previous research studies have suggested that testosterone impairs endothelium-dependent vasodilation in conduit vessels (Herman *et al.*, 1997; Karakitsos *et al.*, 2006). In contrast, the impact of testosterone on the cutaneous vessels is unclear. Lakhani *et al.* (2005) observed microvessel dysfunction in hyperandrogenic women with PCOS, but the impairment remained following statistical adjustment for testosterone. Furthermore, Sokolnicki *et al.* (2007a) reported that the administration of exogenous testosterone did not alter cutaneous microvessel response to gradual local heating in older men. Nevertheless, the impact of clinical and biochemical hyperandrogenism on cutaneous microvessel function, specifically sex hormone binding globulin (SHBG) and the active aspect of testosterone in PCOS (i.e. free testosterone), warrants further investigation.

This is also the first study to demonstrate that exercise training in PCOS is capable of improving cutaneous NO-mediated microvessel vasodilation in response to local heating. The data demonstrates that 16-weeks of moderate-intensity aerobic exercise induced an up-regulation of endogenous NO contribution to local heating, indicative of an improvement in cutaneous endothelial-dependent function. Since the endothelial-independent pathway was unaltered with exercise training, as indexed by maximal cutaneous responses following infusion of a NO donor, the current data indicates that the exercise-induced up-regulation of NO reflects changes in cutaneous microvascular function, independent of changes in maximal vasodilator capacity. These findings may be of clinical relevance given that NO-mediated microvessel vasodilation improved to levels similar to those observed in non-PCOS controls following exercise training. Specifically, this finding provides support to the utilisation of lifestyle modification (exercise) as the first-line prevention strategy in reducing CVD risk in PCOS.

Exercise has previously been reported as an effective intervention to improve cutaneous microvessel function in aged sedentary individuals, in that exercise training induced an increase in NO-mediated cutaneous vasodilation measured using intradermal microdialysis of *N*-nitro-L-arginine methyl ester (L-NAME) during local heating (Black *et al.*, 2008b). The authors postulated that the up-regulation of NO bioavailability may be a function of enhanced cardiorespiratory fitness. Similarly, within the current study the improved NO-mediated cutaneous vasodilation observed in women with PCOS was coupled with an improvement in cardiorespiratory fitness. An alternative but not mutually exclusive mechanistic explanation could be related to the increases in microvascular shear stress associated with repeated bouts of exercise (Green *et al.*, 2010). Indeed, shear stress has been shown to up-regulate eNOS production in conduit arteries (Hambrecht *et al.*, 2003), and limiting the increase in shear stress during exercise (using cuff inflation on one arm),

has been shown to prevent increases in cutaneous microvascular function when compared to normal patterns of shear stress in an uncuffed arm (Green *et al.*, 2010).

The exercise stimulus in the current study did not elicit any changes in clinical characteristics, biochemical parameters or adipose tissue volumes of women with PCOS, supporting the hypothesis that exercise training induced a direct therapeutic effect on the microvascular endothelium. It is plausible that an exercise training stimulus of higher intensity may have elicited changes in adipose tissue volume and thus elicited further improvements in microvascular function. However, Middlebrooke *et al.* (2006) administered 6 months of relatively high intensity exercise training (70-80% HRmax) in type 2 diabetic patients and observed no changes in cutaneous microvessel function in response to either iontophoretically-applied ACh or localised heating to 42°C. Notwithstanding the technique utilised and the different population (which included males) in that study, high-intensity exercise training may not be a suitable stimulus to enhance cutaneous microvessel function. Indeed, the authors postulated that the inefficacy of high-intensity exercise to induce changes in microvascular function may be due to increased oxidative stress during higher intensity exercise resulting in a reduction in NO bioavailability (Goto *et al.*, 2003).

There are several methodological issues that warrant consideration. Previous studies using intra-dermal microdialysis have infused L-NAME, a more specific NO blocker (Black *et al.*, 2008b) rather than L-NMMA. Nevertheless, L-NMMA has been reported to elicit a comparable inhibitory effect of NO-mediated dilation in a study employing whole body heating (Dietz *et al.*, 1994). Furthermore, the inclusion of a non-exercising control group, ideally via a randomised controlled allocation method for comparison, would have enabled a direct comparison to conventional clinical care.

In summary, these data indicate that women with PCOS exhibit a reduced bioavailability of NO, evidenced by a reduced NO-mediated cutaneous vasodilator response to local heating, and that cutaneous vasodilator function can be improved via the up-regulation of the anti-atherogenic molecule NO. These findings advocate the utilisation of exercise training as a preventative strategy for microvascular dysfunction and primary prevention for hypertension in women with PCOS.

**CHAPTER 8**  
**SYNTHESIS OF FINDINGS**

## **8.1 Aims and Objectives**

The research work described in the present thesis was designed to investigate cardiovascular health in women with polycystic ovarian syndrome (PCOS) and to establish whether supervised exercise training could ameliorate the increased risk of cardiovascular disease (CVD) observed in this patient group. Specifically, the studies examined nitric oxide (NO)-mediated endothelial function in both the conduit arteries and cutaneous microvessels of women with PCOS and explored, for the first time, the impact of internal adipose tissue volume, insulin resistance and fitness on conduit artery endothelial function. Additionally, this thesis investigated whether a supervised moderate-intensity aerobic exercise-training programme induced a therapeutic effect on NO-mediated conduit artery and cutaneous microvessel vasodilator function.

## **8.2 Major Findings**

### *8.2.1 PCOS and conduit artery endothelial function*

The systematic review and meta-analysis detailed in *Chapter 3* found that brachial artery flow-mediated dilation (FMD), a strong and independent predictor of future cardiovascular events, is impaired in women with PCOS compared with matched controls. The analysis suggested that BMI may be a contributing factor to the endothelial dysfunction observed in this clinical population; however, given that BMI cannot distinguish between adipose and lean tissue, a regionalised effect of fat deposition and/or distribution could not be disregarded. *Chapter 5* went on to demonstrate that conduit artery endothelial dysfunction in women with PCOS was independent of adipose tissue volume and insulin resistance. Taken together, these studies provide compelling evidence that endothelial dysfunction is an inherent aspect of PCOS and advocates the utilisation of FMD as a valuable prognostic tool for CVD in this patient group.

### 8.2.2 PCOS and cutaneous microvessel endothelial function

In line with the findings in conduit vessels, *Chapter 7* found that obese women with PCOS demonstrate impaired cutaneous NO-mediated microvessel function in response to local heating compared with controls of a similar age and BMI. The infusion of a specific NO blocker via intradermal microdialysis enabled accurate quantification of the precise contribution of NO to the vasodilator response of cutaneous microvessels. This study therefore confirmed that obese women with PCOS display a reduced bioavailability of the anti-atherogenic molecule NO compared with obese control women and therein exhibit a reduced endogenous defence against CVD.

### 8.2.3 Exercise training and endothelial function in PCOS

*Chapter 6* demonstrated that following 16-weeks of supervised moderate-intensity aerobic exercise training, brachial artery FMD improved in women with PCOS. Importantly, this improvement in conduit artery endothelial function was greater than that observed following current UK conventional care guidelines. *Chapter 7* illustrated that the same exercise intervention enhanced the NO contribution to cutaneous microvessel vasodilation in obese women with PCOS. A noteworthy observation of these studies was that exercise training *normalised* the PCOS-induced deficits in both conduit artery and cutaneous microvessel endothelium-dependent vasodilation to values observed in healthy control women of a similar age and BMI. Furthermore, these improvements in endothelial function occurred independent of weight loss and without any change in tradition CVD risk factors including insulin resistance, blood lipids and obesity. Taken together, these studies suggest that exercise training ameliorates the NO-mediated endothelial dysfunction consistently observed in the PCOS population. Given that direct measures of vascular endothelial function have been found to provide independent prognostic information relating to CVD (Green *et al.*, 2011), these data support the utilisation of supervised exercise training as a

cardio-protective strategy for endothelial dysfunction and primary prevention of CVD in this high-risk patient group.

### **8.3 General Discussion**

Findings from *Chapter 3* and *Chapter 5* demonstrate that endothelium-dependent vasodilation, evidenced by brachial artery FMD, is impaired in women with PCOS compared with control women of a similar age and BMI. Endothelium-derived NO is a vital component in the endogenous defence against CVD (Vallance *et al.*, 1989) and has been reported to be the primary mediator of the FMD response (Joannides *et al.*, 1995; Doshi *et al.*, 2001). Therefore, the impaired FMD that appears to be inherent in women with PCOS is indicative of diminished NO vasodilator function and thus an increased risk of CVD. Critically, *Chapter 5* demonstrated that conduit artery endothelial dysfunction in women with PCOS is also independent of visceral and internal adiposity (Figure 8.1). This finding was somewhat surprising given that visceral adipose tissue (VAT), and associated insulin resistance, has been found to be an accurate and independent predictor of CVD (Despres, 2007). Together, these data demonstrate that PCOS may provoke a detrimental impact on conduit artery endothelial function, which exceeds the adverse effect of global obesity, adipose tissue volume and sedentary behaviour (Figure 8.1). Therefore, reduced endothelial function in conduit vessels may contribute to the ~2 fold increased risk of coronary heart disease and stroke exhibited in women with PCOS, which is not explained by an increased prevalence of obesity (de Groot *et al.*, 2011).

Assessment of the cutaneous microvasculature in women with PCOS is logical given that dysfunction of the microvessels is an earlier sentinel of CVD risk than conduit artery dysfunction (Roustit & Cracowski, 2012). The skin is a large, readily accessible organ that provides a suitable site for the assessment of peripheral microvascular reactivity. To date,

the skin has been used as a model of microcirculation to investigate vascular mechanisms in a variety of diseased states (Sax *et al.*, 1987; Levy *et al.*, 2001; Sokolnicki *et al.*, 2007b; Levy *et al.*, 2008) and is now regarded as a valid model of generalised microvascular function (Holowatz *et al.*, 2008). Specifically, the use of gradual localised heating and manipulation of NO, through the use of specific and potent NO blockers, enables researchers to mechanistically assess cutaneous microvascular function. The data presented in *Chapter 7* revealed that obese women with PCOS exhibit a reduced bioavailability of NO compared with obese controls and thus confirm the indirect assessment of the NO system in conduit vessels employed in *Chapters 5* and *6*. These findings indicate that PCOS mediates an adverse effect on NO bioavailability that exceeds the detrimental impact caused by obesity and sedentary behaviour. A recent review in *Microcirculation* suggests that impaired microvascular function causes an increase in peripheral resistance, which may potentially initiate the pathogenic progression of hypertension (De Boer *et al.*, 2012). Therefore, cutaneous microvessel dysfunction may, at least in part, account for the increased prevalence of hypertension observed in women with PCOS during post-menopausal years, which is not explained by traditional CVD risk factors (Schmidt *et al.*, 2011).

*Chapters 6* and *7* demonstrate that exercise training improves conduit vessel and cutaneous microvessel function to a similar level to that exhibited by age- and BMI-matched control women (*Chapters 3* and *5*), thus *normalising* the intrinsic CVD risk observed in women with PCOS. This up regulation of endogenous NO bioavailability at different levels of the arterial tree is indicative of an improvement in systemic endothelium-dependent vasodilator function. Crucially, the endothelium-independent pathway was unaltered with exercise training, as indexed by maximal conduit artery and cutaneous microvessel vasodilation in response to a potent NO donor. Therefore, these data confirm that exercise

training promotes an up-regulation of NO bioavailability rather than an increased sensitivity to NO within vascular smooth muscle. In addition, it is interesting to note that the exercise-induced improvement in brachial artery FMD was ~58%, whereas the improvement in NO contribution to cutaneous microvessel vasodilation at 42°C was over 100%. This suggests that the microcirculation is more *plastic* and therefore amenable to exercise-mediated enhancement compared with conduit vessels. Previous studies have demonstrated that interventions in clinical populations who display similar symptoms to women with PCOS, which improve endothelial-dependent vasodilator function, are associated with improved morbidity and mortality (Green *et al.*, 2003).

Previous studies have postulated that the up-regulation of NO bioavailability may be a function of enhanced cardiorespiratory fitness (Black *et al.*, 2008b; Black *et al.*, 2009). *Chapters 6* and *7* found that improved NO-mediated vasodilation observed in obese women with PCOS was coupled with an improvement in cardiorespiratory fitness. However, there was no statistical correlation between the exercise-induced change in cardiorespiratory fitness and endothelial function suggesting that these adaptations occurred independent of one another. An alternative, but not mutually exclusive mechanistic explanation could be related to the exercise-induced episodic increases in vessel shear stress which act as a key stimulus to functional adaptations of the endothelium and consequently decrease atherosclerotic risk (Green, 2009). Indeed shear stress has been shown to up-regulate eNOS production in conduit arteries (Hambrecht *et al.*, 2003), and limiting the increase in shear stress during exercise (using cuff inflation on one arm) has been shown to prevent increases in cutaneous microvascular function when compared to normal patterns of shear stress in an uncuffed arm (Green *et al.*, 2010).

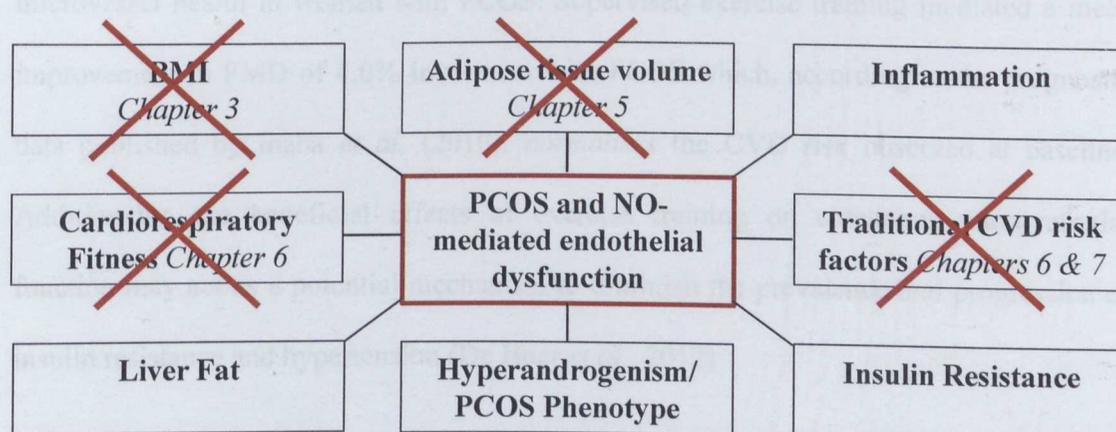
Enhanced FMD and cutaneous microvessel vasodilation was evident independent of

statistically significant reductions in BMI, waist circumference, VAT and insulin resistance. A previous study that employed 12 weeks of alternated training which comprised of both moderate and high intensity exercise reported a significant reduction in VAT (~11%) and improved insulin regulation (Hutchison *et al.*, 2011). Therefore, it is plausible that exercise training interventions of higher intensity may have mediated an improvement in traditional risk factors associated with the metabolic syndrome and thereby prompt further cardiovascular health benefits. Nevertheless, the current studies demonstrate that exercise training of moderate-intensity is capable of improving conduit and microvessel endothelial function independent of changes in obesity status, body composition and insulin resistance. Therefore, this data suggests that exercise training exerts a direct effect on the endothelium in this high-risk patient group.

The fact that exercise training has induced a cardioprotective effect in the absence of a reduction in traditional cardiovascular risk factors is not a novel phenomenon. Cumulatively, the findings detailed within this thesis corroborate the extensive work of Green and colleagues who have shown that NO-mediated endothelium dysfunction can be directly targeted by exercise training in several populations, despite minimal changes in traditional cardiovascular risk factors including blood lipids, insulin regulation and hypertension (Maiorana *et al.*, 2001; Green *et al.*, 2003; Walsh *et al.*, 2003; Green *et al.*, 2004; Watts *et al.*, 2004; Green *et al.*, 2008; Green, 2009). Based on these studies and the work of others, Joyner and Green (2009) have postulated that exercise results in ~40% more “*protection*” from CVD than that which is predicted based on exercise-mediated changes in traditional CVD risk factors.

The work contained within this thesis therefore provides a powerful health message. Although epidemiological studies in women with PCOS relating to cardiovascular

mortality are limited, the literature relating to CVD risk in these women is irrefutable. The experimental studies described have added to the understanding of underlying mechanisms pertaining to the inherent endothelial dysfunction exhibited by women with PCOS, as shown in Figure 8.1. Nevertheless, given that PCOS is the most common endocrine disorder in pre-menopausal women coupled with the fact that current conventional care guidelines fail to induce a reduction in CVD risk, these findings have the potential to impact clinical practice and subsequently improve cardiovascular health this high-risk group.



**Figure 8.1** A schematic of potential influencing factors on endothelial function in women with PCOS, specifically highlighting those that have been ruled out by the work contained within this thesis.

#### 8.4 Implications

PCOS is unique in that it is not a product of Westernised society and, unlike obesity, the metabolic syndrome, non-alcoholic fatty liver disease and type 2 diabetes, PCOS is not reversible. Whilst the phenotypic expression of PCOS can be exacerbated by poor lifestyle, the prevalence of PCOS is approximately ~10% worldwide and is independent of race or environmental factors (Goodarzi *et al.*, 2011). Therefore, the management of risk factors is the sole treatment option to improve CVD prognosis in women with PCOS.

The research work undertaken in this thesis contextualises the extent of CVD risk that is

evident in women with PCOS. Recently published robust risk ratios indicate that the risk of a cardiovascular event increases by 21% for every 1 standard deviation (=3.5%) decrease in FMD (Inaba *et al.*, 2010). Although this previous meta-analysis did not specifically involve women with PCOS, it is interesting to note that *Chapters 3* and *5* evidenced a mean FMD reduction of 3.5-4.5% in PCOS compared with control women. Indeed, these data suggest that the risk of cardiovascular events is ~21% higher in women with PCOS compared with controls. Moreover, *Chapters 6* and *7* are the first studies to demonstrate the therapeutic effects of supervised exercise training on conduit artery and cutaneous microvessel health in women with PCOS. Supervised exercise training mediated a mean improvement in FMD of 4.0% in women with PCOS which, according to the prognostic data published by Inaba *et al.* (2010), *normalises* the CVD risk observed at baseline. Additionally, the beneficial effects of exercise training on cutaneous microvascular function may act as a potential mechanism to diminish the prevalence and progression of insulin resistance and hypertension (De Boer *et al.*, 2012).

Taken together, given that higher levels of NO confer anti-atherogenic benefit, the findings of this thesis have potentially important implications for the prevention of atherosclerosis, coronary heart disease and stroke in women with PCOS and imply that supervised exercise prescription should be recommended as a cardioprotective management strategy.

### **8.5 Methodological considerations and limitations**

There are several noteworthy strengths in the methodology of this thesis. Firstly, the utilisation of the most current peer-reviewed guidelines and technology to assess FMD (Thijssen *et al.*, 2011) coupled with the use of custom designed edge detection and wall tracking analysis software in *Chapters 5* and *6*, maximised the accuracy, validity and prognostic index of this measurement tool. Secondly, application of <sup>1</sup>H-MRS and whole

body MRI, throughout *Chapters 5-7*, is considered to be the most sensitive, non-invasive method to quantify liver fat and adipose tissue volumes. Third, the use of intradermal microdialysis enabled the delivery of a specific NO blockade and vasoactive agonist to directly interrogate the microcirculation, which allowed for accurate evaluation of cutaneous NO-mediated microvascular function. Additionally, a rigorous inclusion and exclusion criteria was adhered to throughout the experimental chapters and importantly all women with PCOS were recruited from the same clinic following in-depth diagnosis. Finally, the absence of a significant change in dietary intake during the interventions employed in *Chapters 6 and 7* enabled the assessment of exercise training effects exclusively.

Nevertheless, limitations within this thesis are apparent. One limitation of this thesis was the methods employed to assess insulin resistance in *Chapters 5-7*; the use of an oral glucose tolerance test (Abdul-Ghani *et al.*, 2007) or a two-stage hyperinsulinaemic-euglycaemic clamp, with infusion of deuterated glucose (Shojaee-Moradie *et al.*, 2007), would have facilitated a more comprehensive assessment of hepatic and peripheral (skeletal muscle) insulin sensitivity. *Chapter 6* was limited with the intervention being based on patient choice rather than a randomised allocation method to the interventions; however, a robust covariate control analysis technique that incorporated adjustments for differences in baseline measurements was utilised. The positive within-groups result reported in *Chapter 7* regarding the effect of exercise training on NO-mediated microvascular function requires confirmation from future studies incorporating a no-exercise control group. Menstrual phase cannot be determined in women with PCOS which is potentially problematic when assessing cardiovascular parameters. In order to control for this we assessed reproductive hormone profiles during all testing sessions in order to determine menstrual phase biochemically.

## 8.6 Future Direction

There are several potential areas of future research which have emerged from the studies detailed within this thesis. Firstly, the heterogeneous nature of PCOS and lack of a judicious definition is problematic when interpreting data and generalising findings. The most recent consensus statement regarding the diagnosis of PCOS was released from the Androgen Excess Society (Azziz *et al.*, 2009). The authors defined PCOS first and foremost as a hyperandrogenic disorder and, indeed, the effect of excess androgens on CVD risk and endothelial function in women with PCOS is currently unknown (Figure 8.1). Nevertheless, unpublished data derived from the work contained within this thesis indicate that biochemical hyperandrogenism worsens the metabolic phenotype of PCOS and thus may negatively influence endothelial function (Jones *et al.*, 2012, *Journal of Clinical Endocrinology and Metabolism*, under review). Examination of phenotypic expression and endothelial function in women with PCOS based on the presence of biochemical hyperandrogenism is therefore warranted in order to accurately determine the adverse effects caused by increased endogenous androgen availability. Specifically, the effect of free testosterone on endothelial function in this population would be an interesting area for future study.

Future research should endeavour to explore the effect of potential modifying variables on endothelial function in women with PCOS (Figure 8.1). Insulin resistance cannot be disregarded as a potential contributor to endothelial dysfunction in women with PCOS as the sole measure of insulin resistance employed within this thesis was HOMA-IR. Therefore further scrutiny of the relationship between insulin resistance and endothelial function in women with PCOS is warranted. Specifically, the utilisation of the “gold standard” two-stage hyperinsulinaemic-euglycaemic clamp, with infusion of deuterated glucose (Shojaee-Moradie *et al.*, 2007), would provide the most in-depth assessment of

hepatic and peripheral insulin sensitivity. Although, endothelial dysfunction in women with PCOS is not attributable to VAT *per se*, excess accumulation of VAT has been reported in exacerbated phenotypes of PCOS (Yildirim *et al.*, 2003). Elevated VAT contributes to increased adipocyte lipolysis that results in augmented secretion of circulating free fatty acids (FFA) and adipokines (Fujioka *et al.*, 1987). VAT is unique in that it is drained via the portal vein and thus excess circulating FFA derived from VAT are absorbed by the liver which leads to increased secretion of inflammatory cytokines and, consequently, a state of chronic inflammation (Matsuzawa *et al.*, 1995). Therefore, the relationship between endothelial function and less overt pathological features such as inflammation may be warranted (Figure 8.1).

Finally, lifestyle modification is difficult to maintain in the absence of supervision; yet, for exercise training to be clinically endorsed as a credible intervention to reduce the risk of CVD, it is essential that the beneficial effects are evident long-term. Thus, research investigating whether exercise-induced improvements in endothelial function are sustainable and long-term in women with PCOS is vital.

**CHAPTER 9**  
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## **APPENDICIES**

**Liverpool (Adult) Research Ethics Committee**

Bishop Goss Complex  
Victoria Building  
Rose Place  
Liverpool  
L3 3AN

Telephone: 0151 330 2071  
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31 October 2008

Dr Daniel Cuthbertson  
Clinical Senior Lecturer and Honorary Consultant Physician  
University of Liverpool  
Clinical Sciences Centre  
University Hospital Aintree  
Liverpool  
L9 7AL

Dear Dr Cuthbertson

**Full title of study:**            **Insulin resistance and polycystic ovarian syndrome (PCOS)**  
**REC reference number:**    **08/H1005/72**

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

The further information has been considered on behalf of the Committee by the Chair

**Confirmation of ethical opinion**

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

**Ethical review of research sites**

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

**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 at NHS sites (“R&D approval”) should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

### Approved documents

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

<i>Document</i>	<i>Version</i>	<i>Date</i>	
Participant Consent Form: Insulin resistance and Polycystic Ovarian Syndrome (PCOS) Part Fat	1	01 July 2008	
Participant Information Sheet: Insulin Resistance, Visceral fat and Endothelial Dysfunction in Polycystic Ovarian Syndrome (PCOS) Part 2 Vascular Assessment	1	01 July 2008	
Participant Information Sheet: Insulin Resistance and Polycystic Ovarian Syndrome (PCOS) Part 3a: The Effects of Exercise Intervention on Insulin Resistance and Visceral Fat	1	01 July 2008	
Participant Information Sheet: Insulin Resistance, Visceral fat and Endothelial Dysfunction in Polycystic Ovarian Syndrome (PCOS): Part 3b Effect of Exercise Intervention on Vascular Function	1	01 July 2008	
Participant Information Sheet: Insulin resistance and Polycystic Ovarian Syndrome (PCOS) Part 1: Quantifying Visceral Fat	1	01 July 2008	
Letter of invitation to participant			
Covering Letter		15 July 2008	
Protocol	1	01 July 2008	
Investigator CV			
Application	5.6	16 July 2008	
letter from dependant review			
letter for pcos			
Schematic of Protocol			
Response to Request for Further Information			
Participant Consent Form		01 October 2008	
Participant Information Sheet	2	01 October 2008	
Participant Information Sheet	2	01 October 2008	
Advertisement			
Statistician Comments			
Covering Letter			
Protocol			

### Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

### After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

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

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
- Progress and safety reports
- Notifying the end of the study

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

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email [referencegroup@nres.npsa.nhs.uk](mailto:referencegroup@nres.npsa.nhs.uk).

**08/H1005/72**

**Please quote this number on all correspondence**

With the Committee’s best wishes for the success of this project

Yours sincerely

**Dr Tej Purewal**  
**Chair**

Email: [adam.lewis@liverpoolpct.nhs.uk](mailto:adam.lewis@liverpoolpct.nhs.uk)

*Enclosures:* “After ethical review – guidance for researchers”  
*Site approval form*

*Copy to:* *Ms Adele Maggs*  
*Faculty of Medicine Support*  
*University of Liverpool*

Systematic Appraisal of Quality for Observational Research (SAQOR)

*Adapted from Ross et al. (2011)*

**Sample**

Is the sample recruited using an established diagnostic criterion? (NIH/AES/Rotterdam)

Yes                      No

Are the patients recruited from the same clinic? (Specific mention required)

Yes                      No

Is a power calculation reported?

Yes                      No

Are the entry criteria and exclusions clearly stated?

Yes No

**Sample:** /4

**Control Group**

Are the control group easily identified? (i.e. clear distinction- non-PCOS)

Yes No

1. Are the control group matched for age and BMI?

Yes No

2. If groups are unmatched, have statistical differences been controlled for? (covariate)

Yes No

**Control group:** /2

**Quality of Assessment**

Does the FMD technique utilise 5 minutes of cuff occlusion?

Yes No

Does the study utilise an ultrasound imaging technique?

Yes No

Does FMD analysis utilise callipers (averaging more than one measure) or edge detection software?

Yes No

**FMD technique:** /3

**Confounding Variables**

Were specific medications excluded? (Notably OCP or other synthetic hormone)

Yes No

Was existing CVD excluded? (specific mention required, hypertension, history of MI, CVD etc.)

Yes No

Were smokers excluded? (specific mention required)

Yes No

**Confounding variables:** /3

**Reporting Data**

Is an explanation for missing data is given?

Yes                      No

Data are clearly and accurately presented? (i.e. mean $\pm$ SD/SE/CI, exact P values cited).

Yes                      No

**Data:**    /2

**Study Quality**    /14

**0-3 Very low quality**

**4-7 Low quality**

**8-11 Adequate quality**

**11+ High quality**