REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree *Ph.D.*   Year *2005*   Name of Author   LAKHANI , K.

ARTERIAL WALL MECHANICS IN WOMEN WITH POLYCYSTIC OVARY SYNDROME

Kalpana Lakhani

Submitted for the Degree of Doctor of Philosophy (PhD)

(University of London), March 2005

(Field of study – Obstetrics and Gynaecology)

UMI Number: U592071

UMI U592071

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Around 20% women of reproductive age are found to have polycystic ovaries (PCO) during ultrasound examinations and approximately 10% have symptoms of polycystic ovary syndrome (PCOS), which is associated with multiple risk factors for cardiovascular disease. The aim of this thesis was to investigate arterial mechanical properties and responsiveness to vasoactive stimuli in young women with PCOS, PCO and controls, using non-invasive ultrasound techniques. The influence of PCOS-related endocrine and metabolic perturbations on aortic function was investigated in a mifepristone-treated rat model of PCOS.

The carotid artery pulsatility index was decreased in PCO and PCOS women and there was a paradoxical vasoconstrictor response to a dilator stimulus in these women relative to controls. Vascular compliance was decreased in the internal carotid artery in PCO and PCOS women; PCOS women also exhibited increased intima-media thickness (IMT) of the common carotid and common femoral arteries compared with controls. In the cutaneous microcirculation, the response to the vasodilator acetylcholine (ACh) was depressed in PCOS women, whilst the response to sodium nitroprusside (SNP; nitric oxide donor - NO) was unaffected.

Mifepristone-treated rats exhibited increased serum luteinising hormone, testosterone, and polycystic ovaries. Ultrasound analysis indicated that aortic diameter and blood flow *in vivo* were unaffected in treated rats, but aortic compliance was reduced.

2

In-vitro assessment of endothelial and vascular smooth muscle function of rat aorta demonstrated decreased relaxation with ACh, which was not abolished by L-NAME, however, the effect of SNP was greater, a finding which raises the possibility of an alternative dilator mechanism that may be independent of NO system.

Since increased IMT, endothelial dysfunction and abnormal reactivity are independent risk factors for cardiovascular disease, these results also provide evidence of preclinical atherosclerotic surrogate markers in women with PCOS and PCO. These findings increase the likelihood of an association between PCOS (and probably PCO) and cardiovascular morbidity and mortality.

CONTENTS

STATEMENT OF DECLARATION

I was responsible for all the experimental work conducted in this thesis except where stated in the text. I undertook patient recruitment, ultrasound measurements, rat model work and operation of the wall-tracking system, and laser Doppler equipment. I undertook all data collection and input, and the production of all the manuscripts arising from this thesis. The manuscript and text was written by myself and any errors are my responsibility.

DEDICATION

This thesis is in honour of my late father.

ACKNOWLEDGEMENTS

PUBLICATIONS

1. Lakhani, K & Hardiman P. Sonography in infertility. RAD Magazine 1999; 25: 37 – 38.

2. Lakhani, K., Constantinovici., N, Purcell, W.M., Fernando, R., & Hardiman, P. Internal carotid artery haemodynamics in women with polycystic ovaries. Clin Sci, 2000; 98(6): 661 – 665.

3. Lakhani, K., Constantinovici, N., Purcell, W.M., Fernando, R., & Hardiman, P. Internal carotid artery response to 5% carbon dioxide in women with polycystic ovaries. Lancet 2000; 356: 1166 – 1167.

4. Lakhani, K., Seifalian A.M., Atiomo, W.U. & Hardiman, P. Review Article Polycystic ovaries. The British Journal of Radiology 2002; 75: 9-16.

5. Lakhani, K., Seifalian, A.M. & Hardiman, P. Impaired Carotid Viscoelastic Properties in Women with Polycystic Ovaries. Circulation 2002; 106: 81-85.

6. Prelevic, G.M., Lakhani, K. P. & Hardiman, P.Metabolic Disorders and Cardiovascular Disease in PCOS. In The Polycystic Ovary Syndrome. D. Shah, R.K Bhathena & S. Shroff editors. Orient Longman Private Ltd. Chennai. India. 2004; 49-55.

7. Lakhani, K., Hardiman, P. & Seifalian, A. M. Intima-media thickness in elastic and muscular arteries in young women with polycystic ovaries. Atherosclerosis 2004; 175: 353-359.

8. Lakhani, K., Prelevic, G. M., Seifalian, A. M., Atiomo, W. U. & Hardiman, P. Polycystic ovary syndrome, diabetes and cardiovascular disease: Risk and Risk factors. Journal of Obstetrics and Gynaecology 2004; 24: 613-621.

PRESENTATIONS

1. Lakhai, K., Purcell., W., R. Fernando., Prelvic, G., & Hardiman, P. Carotid artery haemodynamics in young women with polycystic ovaries – preliminary results. Society for Endocrinology, Royal College of Physicians, London. 1997

2. Lakhani, K., Purcell, W.M., Fernando, R & Hardiman P. Ovarian volume and polycystic ovaries. Meeting of the British Medical Ultrasound society, Bournemouth, UK. 1998

3. Lakhani, K., Purcell, W., Fernando, R., & Hardiman, P. Carotid artery haemodynamics in young women with polycystic ovaries. Meeting of the Blair Bell Research society, Birmingham, UK. 1998.

4. Lakhani, K., Purcell, W.M., Fernando, R., Vanderpump, M & Hardiman P. The effect of weight loss on internal carotid artery haemodynamics in women with polycystic ovarian syndrome. European Society for Human Reproduction and Embryology, Tours, France. 1999.

5. Lakhani, K., Purcell, W.M., Fernando, R & Hardiman P. Cerebral responses to carbon dioxide in women with PCO and PCOS. Meeting of the Blair Bell Research Society, Royal College of Obstetrics and Gynaecology, London. 1999.

6. Lakhani, K., Cortes, E, & P Hardiman. Is uterine artery pulsatility index predictive of conception in clomiphene citrate – stimulated cycles? American Society for Reproductive Medicine meeting, San Diego, California. 2000

7. Lakhani, K. Polycystic ovary syndrome – a fatal disease? North East Thames Obstetrics and Gynaecology – Annual General Meeting, North Middlesex Hospital, London. 2000.

8. Lakhani, K., Tai, N., Seifalian, A, & Hardiman, P. Increased arterial stiffness: a risk factor in women with polycystic ovary syndrome? Meeting of the Blair Bell Research Society, Liverpool, UK. 2001

9. Lakhani, K, Carotid artery haemodynamics in women with polycystic ovaries. Meeting of the Ovarian Club, Royal Society of Medicine, London. 2001.

10. Lakhani, K., Tai, N., Seifalian, A & Hardiman P. Elastic properties of arteries in women with polycystic ovaries. European Society for Human Reproduction and Embryology, Luasanne, Switzerland. 2001.

11. Lakhani, K., Seifalian., A.M, & Hardiman P. Evidence of atherosclerosis in young women with polycystic ovary syndrome. European Society for Human Reproduction and Embryology, Madrid, Spain. 2003.

12. Lakhani, K., Yang, W., Dooley, A., El-Mahdi, E., Sundaresan, M., Bruckdorfer, R., Leonard, A., Seifalian, A, & Hardiman, P. *In-vivo* assessment of the effect of acetylcholine and sodium nitroprusside on polycystic ovary induced rat aorta. Meeting of the Blair Bell Research Society, London, UK. 2003.

13. Lakhani, K., Seifalian, A, & Hardiman, P. Microvascular dysfunction in women with polycystic ovary syndrome. European Society for Human Reproduction and Embryology, Berlin, Germany. 2004.

ABBREVIATIONS

A	Androstenedione
ACh	Acetylcholine
β	Stiffness index
BMI	Body mass index
BP	Blood pressure
c	Speed of propogation of sound
Ca^{2+}	Calcium ion
cAMP	Cyclic adenomonophosphate
cGMP	Cyclic guanophosphate
CC	Compliance coefficient
CCA	Common carotid artery
CFA	Common femoral artery
CO_2	Carbon dioxide
COV	Coefficient of variation
CW	Continuous wave
DBP	Diastolic blood pressure
DC	Distensibility coefficient
DHEA	Dihydroepiandrostenedione
ECG	Electrocardiograph
EDHF	Endothelial derived hyperpolarizing factor
EDRF	Endothelial derived relaxing factor
EDTA	Ethylene diamino tetra acetic acid
eNOS	Endothelial nitric oxide synthase
EIA	Enzyme-linked immuno-assay
E_P	Petersen's elastic modulus
Et	Echo tracking

ET	Endothelin
f	Transmitted frequency
f_d	Change in frequency
FAI	Free androgen index
FSH	Follicle stimulating hormone
GTP	Guanosine triphosphate
GLM	General linear model
h	height
HCG	Human chorionic gonadotrophin
HDL	High density lipoprotein
ICA	Internal carotid artery
IDDM	Insulin dependent diabetes mellitus
iNOS	Inducible nitric oxide synthase
IMT	Intima-media thickness
IP_3	Inositol triphosphate
IVUS	Intravascular ultrasound
LDL	Low density lipoprotein
LH	Luteinising hormone
L-NAME	N^G – nitro-L-arginine methyl ester
MCh	Methacholine chloride
MLCK	Myosin light chain kinase
NIDDM	Non-insulin dependent diabetes mellitus
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Not significant
O_2	Oxygen
O_2 -	Super oxide anion

PCO	Polycystic ovary
PCOS	Polycystic ovary syndrome
PDGF	Platelet derived growth factor
PGI_2	Prostacyclin
PE	Phenylephrine
PI	Pulsatility index
PIP_2	Phosphatidyl inositol biphosphate
PW	Pulsed wave
PWV	Pulse wave velocity
RF	Radio frequecny
RI	Resistance index
SBP	Systolic blood pressure
SD	Standard deviation
SHBG	Sex hormone binding globulin
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
t	Time
T	Testosterone
TA	Transabdominal
TOE	Trans-oesophagial echocardiography
TTE	Trans-thoracic echocardiography
TVS	Transvaginal scan
v	Velocity of moving blood
V	Volume
VEGF	Vascular endothelial growth factor
V_{max}	Maximum velocity
W/H	Waist to hip ratio

TABLES AND FIGURES

19

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

The definition of polycystic ovary syndrome (PCOS) is now much wider than that originally described by Stein and Leventhal (1935) of 'oligo/amenorrhoea, hirsutism, obesity and enlarged ovaries with multiple cysts and thickened tunica' (Taylor, 1998). Traditionally, the diagnosis of PCOS was based primarily on the typical appearance on histological examination of bilateral sclerotic ovaries with thickened stroma, i.e. polycystic ovaries (PCO), in women presenting with anovulation, hirsutism or both (Goldzieher and Green, 1962). In the 1970s, the introduction of radioimmunoassay techniques caused a shift away from histological diagnosis, to the use of serum biochemical markers characteristic of PCOS, such as elevated concentrations of luteinising hormone (LH), testosterone (T) and/or androstenedione (A), low or normal levels of follicle stimulating hormone (FSH), and decreased sex hormone binding globulin (SHBG) (Conway et al, 1989; Franks, 1989). The advent of ultrasound technology in the 1980s led to a definition based on non-invasive ovarian imaging (Swanson et al, 1981; Parisi et al, 1982; Adams et al, 1985). Although the ultrasound criteria for the diagnosis of PCO have never been universally agreed until 2004, the initially accepted characteristic features in PCO were an increase in the size (volume) / number of follicles and stromal volume within an ovary relative normal ovaries (Dewailly, 1997).

Until recently, there was no consensus about which criteria (clinical, morphological or endocrine) best characterised PCOS. The predominantly European definition required PCO morphology on ultrasound (Adams et al, 1985), associated with menstrual disturbance (usually oligo- or amenorrhoea) and/or clinical signs of hyperandrogenism (hirsutism, acne or alopecia). No serum hormonal parameters

were required to make the diagnosis. In contrast, the definition proposed by the National Institute of Health of the USA (1990) did not include ultrasound features of PCO, but stipulated that clinical symptoms such as anovulation and/or hyperandrogenism should serve as the selection criteria, with serum hormonal results used to exclude other conditions, such as diseases of the adrenals or pituitary including adult-onset congenital adrenal hyperplasia, hyperprolactinaemia and androgen secreting neoplasms (Zawadzki and Dunaif, 1992).

The lack of universally accepted and used diagnostic criteria for defining PCOS has made the comparison of studies assessing cardiovascular risk difficult. At a recent joint American Society of Reproductive Medicine/European Society of Human Reproduction and Embryology (ASRM/ESHRE) consensus meeting (The Rotterdam ESHRE/ASRM-Sponsored Consensus Workshop Group, 2004), refined diagnostic criteria for PCOS were agreed, which should have worldwide acceptability. The criteria require two out of three of the following to support a diagnosis of PCOS:

1. Symptoms – menstrual disturbance, anovulation
2. Clinical and/or biochemical signs of hyperandrogenism (hirsutism and/or acne)
3. Ultrasound appearance of PCO

Although there have been no specific population-based prevalence studies, the incidence of PCOS has been estimated at 5% to 10%, making it one of the most common endocrine disorder in women of reproductive age (Dunaif, 1997). This estimate was based on the upper limit of the prevalence of around 20% in self

selected normal women with PCO on ultrasound (Adams et al, 1985; Polson et al, 1988; Clayton et al, 1992); the lower estimate was based on the prevalence of androgen excess and chronic anovulation in a random population (Dewailley, 1997).

A spectrum of clinical and endocrine variations in PCOS has not only been reported within a population (Clayton et al, 1992), but there appears to be ethnic diversity as well. For example in the USA, the prevalence of PCOS in Caribbean-Hispanic women is twice that in African-American and Caucasian women (Weiss et al, 1987). In contrast to women in the USA or Italy, Japanese women with PCOS exhibit a lesser degree of hirsutism (Carmina et al, 1997), whilst in the British population hirsutism is present in 14% of women with PCOS (Clayton et al, 1992). A major difference between the Asian, i.e. women from the Indian subcontinent (Rodin et al, 1998), and the Caucasian groups is related to menstrual patterns (Clayton et al 1992). In the Asian population nearly 50% of women with PCOS have menstrual irregularity, whilst menstrual irregularities occur much less commonly in the Caucasian group (24%; Clayton et al, 1992).

PCOS was not considered to affect general health until 1980, when it was found to be associated with insulin resistance (Burghen et al, 1980). Hyperinsulinaemia is frequently present in 18% – 20% of obese PCOS women and has an important influence on symptomatology, affecting menstrual cyclicity and the severity of the clinical signs of hyperandrogenism (Dunaif, 1997). In addition to obesity (Evans et al, 1988; Pasquali et al, 1993) and insulin resistance (Dunaif, 1992), the syndrome is also associated with dyslipidaemia (Wild et al, 1985; Conway et al, 1992, Talbott

23

et al, 1998), impaired glucose tolerance (Dunaif et al, 1987; Conway et al, 1990; Robinson et al, 1993) and hypertension (Conway et al, 1992, Talbott et al, 1995; 1998), suggesting that PCOS women might be at increased risk of cardiovascular disease (Wild et al, 1990; Guzick et al, 1996; Birdsall et al, 1997; Talbott et al, 1998).

Insulin resistance which in turn is associated with risk factors for cardiovascular disease is often seen in association with a cluster of abnormalities in the Metabolic Syndrome such as obesity (body mass index (BMI) > 30 kg/m^2) particularly abdominal obesity ((W/H $\geq$ 0.88), hypertension (systolic blood pressure (SBP) $\geq$ 160 mmHg or diastolic blood pressure (DBP) $\geq$ 95 mmHg or drug treatment for hypertension), elevated fasting serum triglyceride ($\geq$ 1.70 mmol/L), fasting HDL ($\geq$1.20 mmol/L), hyperinsulinaemia (fasting insulin levels $\geq$ 13.0 mU/L) (Reaven, 1995). The fact that women with PCOS exhibit features of the Metabolic Syndrome is therefore suggestive that they be at an increased risk of developing coronary heart disease or stroke. It therefore appears that hyperinsulinaemia could be involved in the pathogenesis of a wider spectrum of diseases than originally anticipated.

Long-term follow-up studies have shown that women with PCOS are at an increased risk (3–7 fold) of developing type II diabetes, relative to the general female population, (Dahlgren et al, 1992a; Pierpoint et al, 1998; Wild et al, 2000, Cibula et al, 2000) and exhibit a greater than 7-fold increased risk of cardiovascular disease (Dahlgren et al, 1992b). However, the relationship between cardiovascular disease and PCOS was challenged by Pierpoint in 1998 in the only mortality study in women with PCOS performed to date, which showed no increase in deaths from

heart disease related to PCOS.

As previously stated, some there are studies suggest that women with PCOS have extensive cardiovascular disease. Wild et al (1990) reported that both pre- and post-menopausal women with coronary artery stenosis on cardiac catherisation exhibited an increased prevalence of hirsutism, increased waist-to-hip ratio (W/H), diabetes and hypertension than women without coronary artery lesions. Furthermore, Guzick et al (1996) reported increased intima-media thickness (IMT) in the carotid artery in PCOS women relative to controls, this being an early indication of subclinical atherosclerosis. Similarly, in a larger study, Talbott et al (1998) reported increased IMT in PCOS women aged 45 years or more compared to controls, even after adjusting for age and body mass index. However, in women aged less than 45 years, no significant difference in IMT was found between those with PCOS and controls, although a higher proportion of those with PCOS had an increased atherosclerotic index (the mean IMT measured at eight sites) compared to controls. Although the progression of disease from increased IMT, through plaque formation to alteration in blood flow is lacking, increased IMT is viewed as an early indication of atherosclerosis. Further evidence of an association with atherosclerosis was provided by Birdsall et al, (1997), who reported that women with ultrasound detected PCO had more coronary artery segments exhibiting > 50% stenosis, and a trend towards greater severity of ischaemic heart disease, than women with normal ovaries.

There is evidence of circulatory changes in women with PCOS (Battaglia et al, 1995; Aleem and Predanic, 1996), though haemodynamic studies outside the pelvis are limited. Using two dimensional, M-mode and pulsed Doppler echocardiography in 26 women with PCOS (menstrual irregularity, hirsutism – elevated T, raised LH and decreased SHBG concentrations and PCO on ultrasound, as described by Adams, 1985) and 11 healthy non-hirsute age-matched control women with regular ovulatory cycles, Prelevic reported decreased flow velocity over the aortic arch (Prelevic et al, 1995) and higher resting forearm flow (Prelevic et al, 1996) in women with PCOS.

It is generally accepted that the endothelium plays an active role in regulating vascular tone and in modulating vascular smooth muscle cell migration and growth (Cannon, 1998; Quyyumi, 1998). Intact endothelium is required to prevent premature atherosclerosis, and impaired endothelial function has been associated with increased risk of cardiovascular disease (Li and Fostermann, 2000). There is increasing evidence of endothelial dysfunction in women with PCOS (Wild, 2002). In obese PCOS women (exhibiting elevated free serum T levels, hirsutism and amenorrhoea or oligomenorrhoea < 6 periods per year), methacholine chloride (MCh) induced dilatation in leg blood flow (measured using a thermodilution catheter placed in the femoral vein) was impaired relative to obese controls (Steinberg et al, 1996). This was suggestive of endothelial dysfunction since MCh requires active endothelium to mediate its effect. Furthermore, the impairments correlated directly with insulin resistance and hyperandrogenaemia in PCOS women (Steinberg et al, 1996), and returned to normal with insulin sensitising agents (Paradisi et al, 2001), suggesting that PCOS is associated with endothelial

dysfunction may be associated with elevated serum T and/or insulin concentrations.

It is apparent that women with PCOS exhibit a number of hormonal, metabolic and vascular abnormalities that may increase their risk of premature cardiovascular disease. The process of macro-and micro-arterial dysfunction may start at an earlier stage in the reproductive life of women with PCOS, indicating that the vascular function is different in young women with PCOS and that they might be 'older' from the circulatory point of view.

The hypothesis under study in this thesis is that women with PCOS have vascular and haemodynamic abnormalities, similar to those seen in Type II diabetes, which are consequently associated with an increased risk of developing cardiovascular disease.

To test this hypothesis the aims of this thesis are to assess:

1. Vascular resistance and vascular reactivity in the common and internal carotid in women with PCOS, asymptomatic women with PCO and age-matched control women.

2. Viscoelastic properties in the common and internal carotid arteries in women with PCOS, asymptomatic women with PCO and age-matched control women.

3. IMT in muscular and elastic arteries in women with PCOS, asymptomatic women with PCO and age-matched control women.

4. Endothelial function in the skin microcirculation in *young* women with PCOS and age-matched control women.

5. Mechanical vascular properties, and endothelial and vascular smooth muscle function, in a rat model of PCOS.

CHAPTER 2

POLYCYSTIC OVARY SYNDROME

2.1 Introduction

Numerous biochemical parameters and ultrasound appearances together with a combination of clinical features have been reported in the diagnosis of PCOS, but until recently no universally accepted criteria have been adopted for clinical and research purposes (Homburg, 2002). Since the first international conference on PCOS (1990) at the National Institute of Health in Bethesda, Maryland, it has become apparent that the syndrome encompasses a broader spectrum of signs and symptoms of ovarian dysfunction than was defined by the original diagnostic criteria (Zawadski and Dunaif, 1992). The recent 2003 Rotterdam Consensus workshop on the diagnostic criteria for PCOS bridges the gap between biochemical marker-based diagnosis, predominantly used in the US and Canada, and the European reliance on ultrasound detection of PCO morphology. The diagnostic criteria agreed at this workshop may eliminate the selection bias caused by the variable definitions previously used and also contribute much to future work, especially the long-term consequences of this most common endocrinopathy.

2.2 Diagnosis of Polycystic Ovary Syndrome

As described earlier, although the first description of the condition now known as PCOS dates back to the mid 1930s (Stein and Leventhal, 1935), a consensus definition (clinical, morphological and/or endocrine) which best characterises the syndrome was only reached in 2003 (Balen and Michelmore, 2002; Homburg, 2002; Balen et al, 2003). The range of presentation extends from the sole finding of PCO on pelvic ultrasound in the absence of other symptoms or signs of the syndrome (Jacobs, 1987), to women with symptoms of hyperandrogenism, menstrual irregularity and endocrine disturbances which occur either singly or in

combination (Balen et al, 1995).

Biological evidence of hyperandrogenism is generally accepted as a sensitive marker for PCOS (Hopkinson et al, 1998); however, the serum levels of T and A are dependent on the body mass index (BMI), with increased levels in obese individuals (Dewailly, 1997). The use of clinical signs of menstrual disturbance or androgen excess in the diagnosis of PCOS is limited by the findings of PCO on ultrasound in around 20% of normal ovulatory women and in 50–87% of regularly menstruating women with hirsutism (Michelmore et al, 1999). The first descriptions of the syndrome were based on the macroscopic appearance of the ovary (Stein and Leventhal, 1935), but this is now not essential for the diagnosis, since the development of high-resolution ultrasound equipment allows non-invasive diagnosis of polycystic morphology, in a manner well correlated with histopathological diagnosis. On the other hand, as described before, the National Institute of Health (1990) definition did not include ultrasound features, but stipulated that clinical symptoms such as anovulation and/or hyperandrogenism be used as diagnostic criteria for PCOS, with serum hormonal estimates used to exclude conditions such as tumours of the adrenals and/or pituitary, congenital adrenal hyperplasia and hyperprolactinaemia (Zawadzki and Dunaif, 1992).

2.2.1   Clinical presentation

The seven women described by Stein and Leventhal in 1935 in the index study of PCOS had symptoms of hirsutism, obesity, infertility and had enlarged polycystic ovaries. The accepted clinical definition of PCOS is the association of hyperandrogenism with anovulation without any underlying disease of the adrenal

31

or pituitary glands (Zawadzki and Dunaif, 1992). Hyperandrogenism presents as hirsutism, acne, and/or male-pattern alopecia and the degree of hirsutism can be assessed by the Ferriman-Gallwey score (1961), a simple, semi-quantitative method for recording the distribution and severity of excess body hair. Anovulation manifests as menstrual disturbance such as amenorrhoea, oligomenorrhoea and infertility. Obesity is common but not usually a presenting symptom. The variation in clinical presentation is related to the degree of endocrine disturbance (Conway et al, 1989; Balen et al, 1995). The majority of women will however present with only one or two clinical manifestations, and in clinical practice women with PCOS are seen for three major reasons: infertility (mean incidence of 50–75%), menstrual irregularity (mean incidence of amenorrhoea 70%) and androgen excess (mean incidence of hirsutism 66%; Harrington and Balen, 1996).

In. 1989, Franks analysed 300 patients with clinical features i.e. hirsutism, infertility, acne, ultrasound (Adams *et* al, 1985, 1986) and endocrine features (raised serum LH concentrations) of PCOS and 60 volunteers with normal ovarian morphology on ultrasound with no history of menstrual irregularity and clinical signs of hyperandrogenism. Disturbance of menstrual cycle was a common presenting feature, with 52% of women complaining of an irregular cycle or oligomenorrhoea, 28% having amenorrhoea and 42% with infertility. On clinical examination, 64% of women were found to be hirsute (increased free T levels), 27% had acne and 35% were overweight (BMI > 25 kg/m^2).

Conway et al (1989) studied 556 women with PCOS (clinical – hyperandrogenemia, menstrual irregularity, infertility; ultrasound ovarian morphology of Adams, 1985 and endocrine features – raised LH) and 23 volunteers with normal ovaries on ultrasound and with no history of menstrual irregularity or hyperandrogenism. They also reported disturbance of the menstrual cycle to be the most common presenting feature, 45% of women exhibited irregular cycles, 26% amenorrhoea, and 29% complained of infertility. Sixty one percent of women were hirsute (increased free T levels), 24% had acne and 35% were overweight (BMI > 25 kg/m^2).

The similarities in the prevalence of symptoms in these two studies (Franks, 1989; Conway et al, 1989) in which ultrasound was used as the primary method of diagnosis and that of the classic review (Goldzieher and Green, 1962), in which the diagnosis was based on histological features of ovaries following wedge resection, is shown in Table 2.1. The higher prevalence of infertility in the older series almost certainly reflects different criteria for selection, i.e. clinical symptoms at the extreme end of the PCO spectrum, which sufficed to justify surgical intervention.

Table 2.1   Clinical features of the Polycystic Ovary Syndrome (Modified from Franks, 1989).

Feature	Diagnostic Criteria		
	Ultrasonographic (Franks, 1989) (N = 300)	Ultrasonographic (Conway et al, 1989) (N = 556)	Histologic (Goldzieher and Green, 1962) (N = 1009)
	percent frequency		
Hirsutism	64	61	69
Acne	27	24	-
Obesity	35	35	41
Infertility	42	29	74
Amenorrhoea	28	26	51
Oligomenorrhoea §	52	45	29
Regular menstrual cycle	20	25	15

§ Values include women with abnormal pattern of uterine bleeding.

2.2.2   Endocrine profile in PCOS

With the advent of radioimmunoassay in the 1970s, the diagnostic criteria for diagnosis of PCOS were changed to include the use of serum biochemical markers of the syndrome. As described in Chapter 1, numerous endocrine parameters have been reported in the diagnosis of PCOS including increased circulatory levels of LH, LH/FSH ratio, T, A, free androgen index (FAI), normal or increased levels of FSH and decreased SHBG levels (Yen et al, 1970; Franks, 1989; Conway et al, 1989; Pache et al, 1993; Holte et al, 1998). Such endocrine profiles have been reported in women with PCOS that include oligomenorrhoea and amenorrhoea, obese and non-obese women, hirsute and non-hirsute, high and normal LH, high and normal T, after surgery and following treatment. However, controversy was

still present until recently as to which endocrine parameter, single or in combination, best describes the syndrome.

As described before, in Frank's (1989) study of 300 patients with PCOS and 60 volunteers, the overall serum endocrine results of women with PCOS were similar to that described in the classical Stein-Leventhal syndrome: raised concentrations of LH, T and A. The serum concentrations of LH ($\geq$ 10 IU/L), T ($\geq$ 2.6 nmol/L), and A ($\geq$ 2.6 nmol/L) were significantly higher in women with PCOS than in normal subjects in the early to mid-follicular phase of menstrual cycles. Of particular interest was that, although both hirsute and non-hirsute women had raised total serum concentrations of T and A, the differences between these two groups were not statistically significant. Women with PCOS also had lower concentrations of SHGB, and therefore increased FAI compared to control women. Although there was a wider distribution of prolactin concentrations in the PCOS women, no detectable (radiological) abnormalities of the pituitary were noted and the serum concentrations of LH and T were not associated with prolactin concentrations. There were no significant differences in FSH concentrations between the two groups.

Conway et al (1989) studied 556 women with PCOS and 23 volunteers with normal ovaries and reported raised levels of LH, T, A and FAI, and low concentrations of SHBG and normal FSH levels in women with PCOS. This study revealed that 44% (n = 244) of women with serum LH concentrations > 10 IU/l had a higher incidence of infertility than those with serum < 10 IU/l, and that oligomenorrhoea was significantly more common in the high LH group than in the normal LH group. Secondly, 22% of women (n = 110) had serum T concentrations exceeding 3.0

nmol/l; hirsutism was more prevalent in this group than in those with normal serum concentrations. The prevalence of menstrual disturbances was not different between these two groups.

Until recently no consensus had been agreed on the definitive biochemical definition of the syndrome. Most women with PCOS have evidence of hyperandrogenism, and it has also been suggested that circulating androgen levels may represent an inherited marker for androgen excess (Legro et al, 1998). However, there is evidence that a proportion of women with PCOS may be asymptomatic (Balen et al, 1995). The limitations in defining androgen excess include the wide variability in the normal population; age and BMI have not been considered when assessing androgen levels. Multiple androgen definitions have also been reported (Bili et al, 2001), together with the inaccuracy and variable laboratory methods often used (Vermeulen et al, 1999).

With regards to LH, both the absolute level of circulating hormone as well as its relation to FSH levels is significantly elevated in PCOS women compared with controls (Fauser et al, 1991). Increased LH concentrations have been reported in around 60% of women with PCOS (Van Santbrink et al, 1997), whereas LH/FSH ratio may be elevated in as many as 95% women (Taylor et al, 1997). The limitations of using LH as a diagnostic endocrine parameter is that ovulation normalises its concentration, is higher in lean women and is also influenced by the assay system used.

With these limitations, The Rotterdam ESHRE/ASRM-Sponsored Consensus Workshop Group recommended the measurement of free T or FAI as the most sensitive method of assessing PCOS (Cibula et al, 2000) and the measurement of the serum LH was not considered necessary for clinical diagnosis of PCOS. LH

concentration should only be used as a secondary parameter, especially in lean women with amenorrhoea or in research.

2.2.3 Ultrasound in the diagnosis of PCO

Although ultrasound had already been used in therapy and was proposed for diagnosis in 1937, no successful attempt to apply the echo-sounder principle to medical diagnostics was made until the early 1950s. X-ray pelvic pneumography (air introduced in the peritoneal cavity followed by x-ray examination) was first used to visualise the ovaries (Edwards et al, 1961). Although the ovarian outline was well described, pneumography provided no information regarding the internal morphology of the ovary. In the mid 1960s, Prof. Ian Donald developed ultrasonography as a technical aid for gynaecological diagnosis. The static B-scanners of the mid 1960s allowed visualisation of ovarian enlargement and cysts measuring greater than 1 cm in diameter (Fleming et al, 1999). The poor resolution of the ultrasound equipment used in the early 1970s only permitted visualisation of the ovarian outline, and the diagnosis of PCO was based upon increased maximum length (> 4.0 cm).

However, the use of a single dimension may lead to false positive results when the full bladder compresses the ovary or false negative results when the ovaries are spherical rather than ovoid. In fact the PCO tends to be more spherical in shape so that the sphericity index, expressed as ovarian width to ovarian length ratio < 0.7 and decreased uterine width to ovarian length ratio < 1.0, have also been reported in the diagnosis of PCO (Ardaens et al, 1991). Thereafter, the development of grey scale equipment in the 1970s and of real-time sector scanners in the 1980s

improved resolution, and for the first time cysts less than 1 cm could easily be recognised (Swanson et al, 1981).

In 1981, Swanson and colleagues reviewed, over a 12-month period, 863 female patients referred for pelvic ultrasound examination. Among 863 scans performed (3.5 MHz transabdominal (TA) approach), 22 women showed evidence of PCO. The ovaries were symmetrically enlarged with a mean ovarian volume of 12.5 cm^3 (range 6 - 30 cm^3), three times the normal ovarian volume as reported by Sample et al (1977). Swanson et al (1981) described two patterns of follicular distribution in PCO. The characteristic multiple cysts, measuring 2 - 6 mm in diameter, were either uniform in size and arranged in a 'necklace' distribution around the periphery, or were of variable size and scattered throughout the ovarian parenchyma. Although the number of follicles was recorded, the ovarian stromal characteristics were not described.

In a study by Parisi et al (1982) of 78 women who presented with clinical symptoms of the syndrome (amenorrhoea, oligomenorrhoea, hirsutism, obesity or infertility), symmetrically enlarged ovaries on ultrasound examination were apparent in only nine cases and were surgically confirmed in four cases (mean ovarian volume = 17 cm^3). In the four cases in which surgery was carried out, the symmetrical enlargement of the ovaries corresponded to the ultrasound findings. The remaining 68 patients had ovaries of normal size and were affected by other endocrinopathies.

The importance of ovarian size as an ultrasonographic criterion of PCO has decreased as various groups showed that about one-third of patients with PCO had ovaries of normal volume (Orsini et al, 1985; Nicolini et al, 1985). This is because the later studies exhibited a wide range of clinical and endocrine abnormalities, whereas Swanson (1981) and Parisi (1982) included women with enlarged ovaries and classic Stein and Leventhal syndrome, which therefore represented the extreme end of the clinical spectrum. The presence of an increased number of cysts has become an important ultrasound criterion of PCO, particularly when the ovary is of normal size (Franks, 1989). In 1985, Adams and colleagues, in their study of 76 women referred for either menstrual irregularity, infertility, hirsutism or acne and 17 control women with regular ovulatory cycles and normal ovaries on transabdominal (TA) ultrasound, refined the ultrasound diagnosis of PCO to include the presence of 10 or more cysts measuring 2–8 mm in diameter arranged peripherally or scattered around an echo dense stroma. Ovarian volumes were found to be higher in PCO (14.6 ± 1.1 cm^3) than in either multicystic (8.0 ± 0.8 cm^3) or normal ovaries (6.4 ± 0.4 cm^3).

Because of the increased prevalence of PCO in anovulatory women and in those with hirsutism (Adams et al, 1986), Polson and colleagues (1988) performed ultrasound examination on 257 volunteers (clinical and secretarial staff) who considered themselves to be in good health and had not found it necessary to consult a doctor for menstrual disturbance, infertility or acne. These women had previously completed a detailed menstrual history questionnaire and this was checked at the time of presentation for a scan. Ninety-nine women were taking oral contraceptives and were excluded from the study. Of the remaining 158 women, PCO were found in 36 (23%).

The Adams criteria have remained in widespread use even after the introduction of transvaginal scan (TVS) a decade later. TVS has largely superseded TA ultrasound because of its greater resolution and in many cases patient preference. The TVS approach provides a more accurate view of the internal structures of the ovaries, particularly in obese women. TVS allows visualisation of follicles < 5 mm in diameter and the echogenic stroma (Figure 2.1), which corresponds closely to the characteristic histopathological changes (Figures 2.2 and 2.3)

Figure 2.1   Transvaginal image of a polycystic ovary showing peripheral distribution of follicles (arrows)
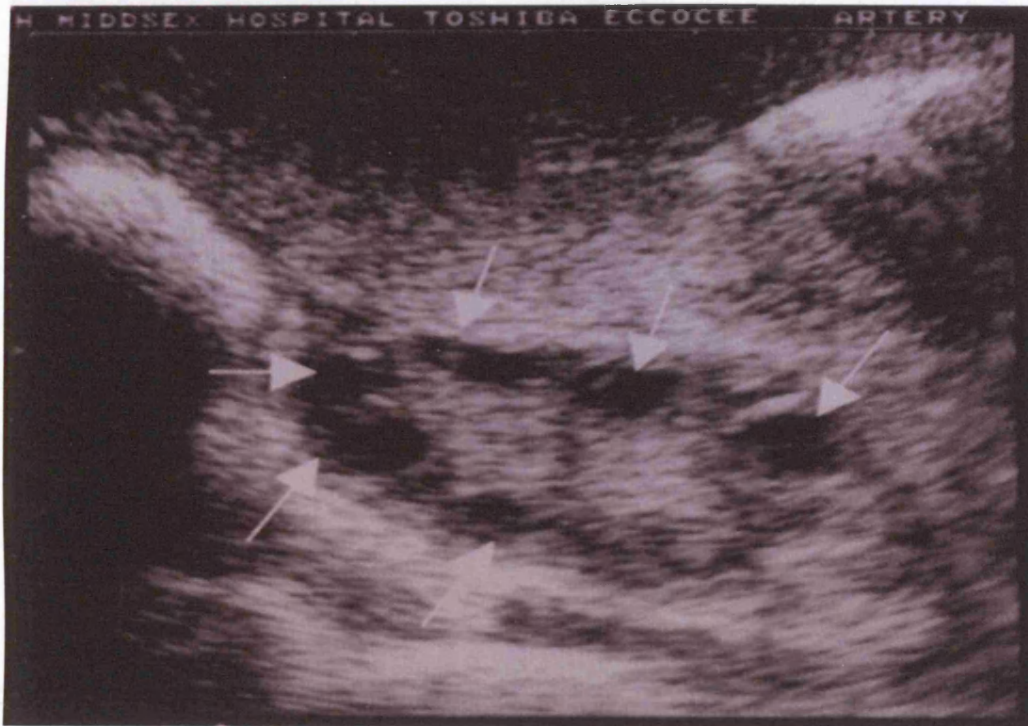
Figure 2.2   Correlation between histological and ultrasonic features of polycystic ovary. This photograph sets artificially side-by-side the ultrasonic (lower part) and the histological (upper part) pictures of polycystic ovary.
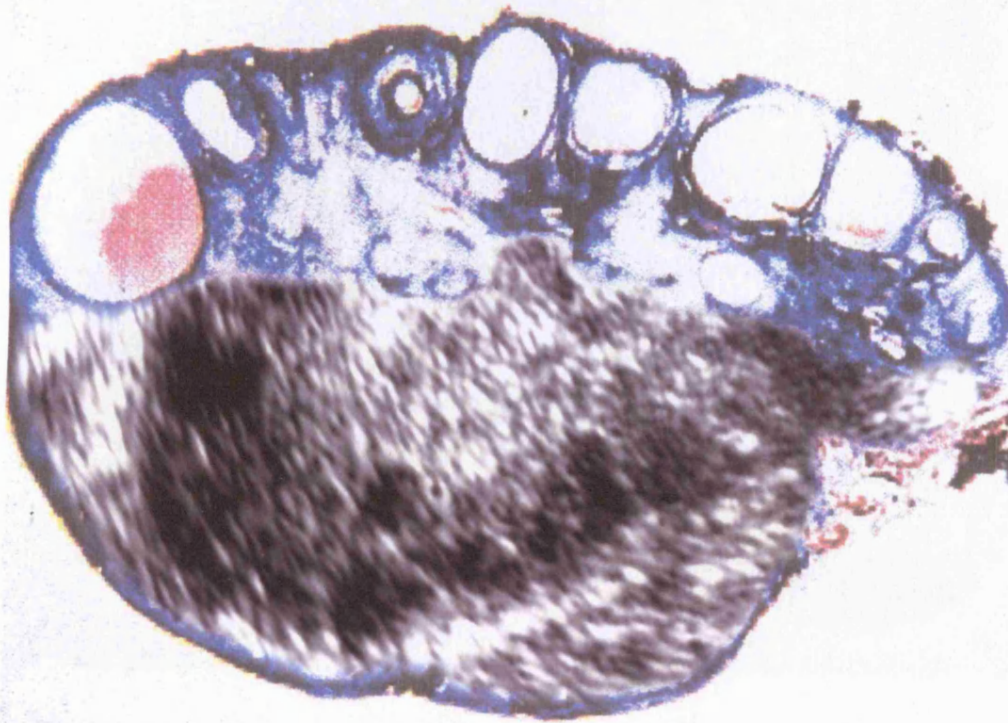
Figure 2.3 Stained longitudinal section of a polycystic ovary showing numerous small peripheral cysts.
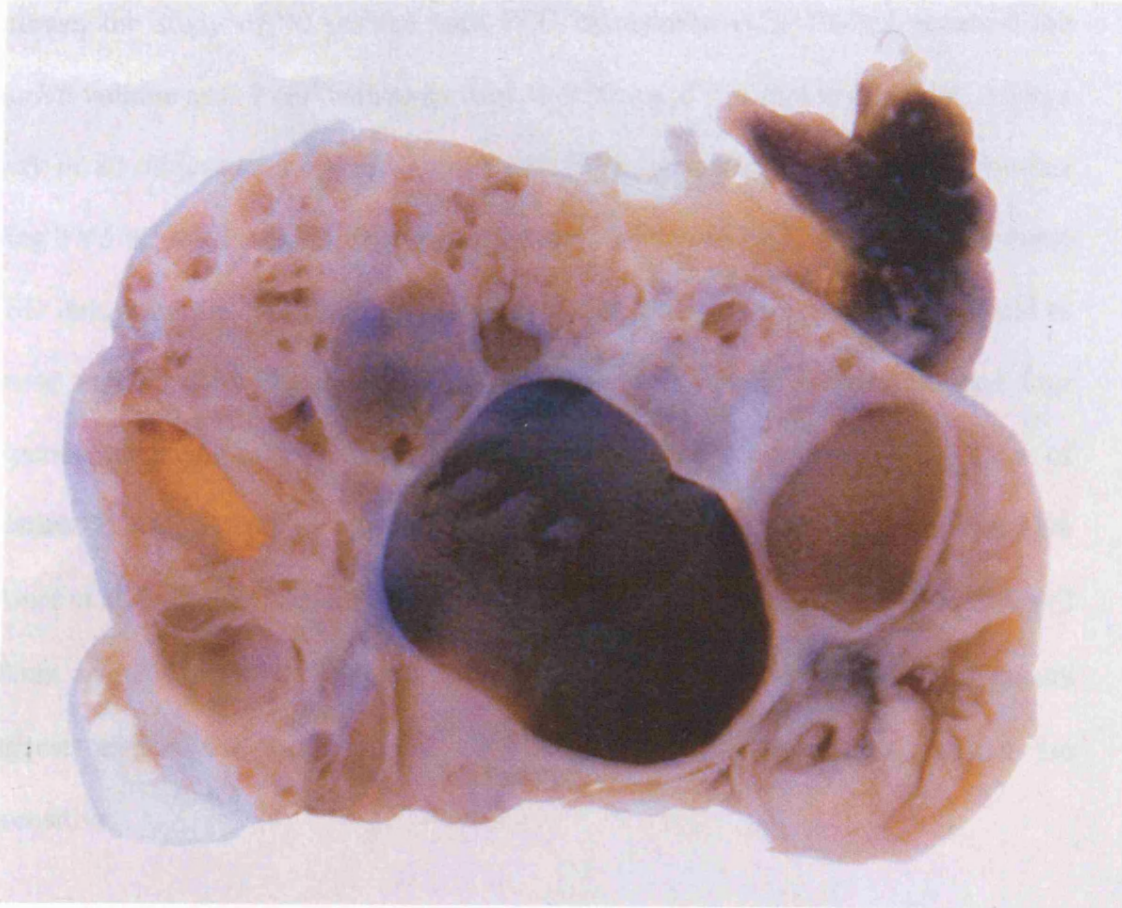
There have been at least four definitions of PCO using TVS, the most recent by Fox

(1999) and Atiomo (2000). In the study by Fox et al (1999) of 60 women with PCO,

the ovarian volume was 17.6 cm^3 with more than 15 follicles of 2–5 mm diameter. In

contrast, the study of 70 women with PCO by Atiomo et al (2000), reported the

ovarian volume as > 9 cm^3 with more than 10 follicles of 2–8 mm in diameter. A large

study of 80 oligo-/amenorrhoeic women with PCO compared with 30 control women

using TVS reported the cut-off values of ovarian volume as 13.21 cm^3, based on mean

± SD data from the control group (Fulgheseu et al, 2001). A recent study designed to

assess variability in the detection of polycystic and normal ovaries utilised four

experienced practitioners, who independently reviewed recordings of 27 pairs of

ovaries. The intra-observer agreement was 69.4% and inter-observer agreement 51%

(Amer et al, 2002). Polycystic ovaries were defined as the presence of ≥ 10 follicles (2

- 8mm diameter), ovarian volume ≥ 12 cm^3 and bright echogenic stroma. These results

suggests either that these criteria are too subjective or their measurement too

insensitive.

Quantification of features of the polycystic ovary

Surface volume

It is necessary to identify each ovary and measure the maximum diameter in each

of the three planes (longitudinal, anteroposterior and transverse). It is appreciated

that, because of the irregular shape of the ovary, any calculation of the volume is an

estimate. Modern ultrasound equipment can calculate the ovarian volume in three

planes, using the ellipsoid formula or by drawing an ellipse round the ovary. The

ultrasound software for this calculation has been shown to be accurate (Balen et al,

2003). In clinical practice ovarian volume is calculated using the simplified ellipse

formula (0.532 x length x width x thickness) (Swanson et al, 1981). It is noteworthy

that numerous parameters of ovarian volumes have been reported in the literature

(Adams et al, 1985; Fox et al, 1999; Atiomo et al, 2000; Fulghesu et al, 2001; Amer

et al, 2002). It is recognised that not all PCO will be enlarged and that there is an

overlap with normal ovaries (Lakhani et al, 1998). The consensus view is that until

more data are available and validated the definition of PCO includes an ovarian

volume $> 10 \, \text{cm}^3$.

<u>Number and size of follicles.</u>

Each ovary should be scanned in longitudinal cross-section from the inner to outer

margins in order to count the total number of follicles. Follicle number should be

estimated in two planes of the ovary in order to estimate their size and position and the

diameter measured in three planes (Balen et al, 2003). Studies using TA and TV

ultrasound have reported numerous prerequisite follicular size and number in defining

PCO (Table 2.2).

Table 2.2 Diversity results of some ultrasound studies in the literature

Keys: TA – Transabdominal, TV – Transvaginal, OS – ovarian stroma, PCO – asymptomatic women with polycystic ovaries, PCOS – Polycystic ovarian syndrome, UER – ultrasound examination route, *total of both ovaries.

Authors	UER	Ultrasonic Variable	Criteria Indicative of PCO	% of patients with clinical PCOS	% of controls having the criteria	No. of patients studied	No. of studied controls
Adams et al. 1985	TA	Ovarian volume	$>15cm^3$	33	0	76	17
		No of follicles 4-10 mm	>10	72	0		
Yeh et al. 1987	TA	Ovarian volume	$>10 cm^3$	70	0	108	25
		No of follicles 5-8mm	>5	74	11	68	18
		Uterine width/ovarian length	>1	7	6	100	24
Pache et al. 1992	TV	Ovarian volume	$>8 cm^3$	About 70	0	52	29
		No of follicles >6mm	>11	About 50	0	52	29
		Mean ovarian size	$>4mm$	About 70	7	52	29
		Increased echogenicity of OS	Present	94	10	52	29
Robert et al. 1995	TV	Increased stromal area	$*. 8 cm^3$	61	4	69	48
		Increased maximal ovarian area	$*>10.8 cm^3$	55	2	69	48
Fox et al. 1999	TV	Ovarian volume	$17.6 cm^3$	60	0	29	35
		No of follicles 2-5mm	>15	46	0	25	0
		Increased echogenicity of OS	Present	60	0	29	2
Atiomo et al. 2000	TV	Ovarian volume	$> 9 cm^3$	Around 70			
		No of follicles 2-8mm	> 10	Around 80	Not mentioned	32	40
		Increased echogenicity of OS	Present	Around 60			

Sample et al (1977) described follicles being < 8 mm in size, whilst Swanson (1981) reported the follicular size to be 2–6 mm in diameter, although the number of follicles was not recorded or defined. In a study of 70 women, Yeh (1987) reported the follicular number as > 5 and the diameter as 5–8 mm for defining PCO. In contrast Adams (1985) described PCO as having, in one plane, at least 10 follicles (4–10 mm in diameter) usually arranged peripherally, although when scattered through the stroma the follicles were usually 2– 4 mm in diameter. Others claimed that the TV definition of PCO should require the presence of at least 15 follicles (2–5 mm in diameter) in a single plane (Fox et al, 1999), Atiomo (2000) suggests the presence of 10 follicles (2–8 mm diameter) and Jonard (2003) gave the threshold of 12 or more follicles of 2–9 mm in diameter.

Until recently there was no agreement with regards to the number and size of follicles in defining PCO. In clinical practice the ultrasonographer forms an impression of the ovary from the images obtained in three planes. There is therefore still a degree of subjectivity in this assessment. The recent consensus definition, agreed in 2003, suggests that the ovary should contain 12 or more follicles of 2– 9 mm diameter (Balen et al, 2003).

Stromal volume and echogenicity

Increased ovarian volume and stromal echogenicity in PCOS is not only a key histological characteristic (Hughesdon, 1982) but also an ultrasound feature. However, the later is a subjective assessment, which depends on the setting of the ultrasound machine and patient's body habitus (Pache et al, 1991). In a study by Ardaens et al (1991), subjective assessment of increased stromal echogenicity assessed transvaginally appeared to be

associated with PCOS; normal stromal echogenicity is said to be less than that of the myometrium. Pache (1991) described a semi-quantitative ovarian stromal echogenicity score – normal (1), moderately increased (2) or frankly increased (3) in which the total number of follicles in both ovaries combined correlated with stromal echogenicity, and the follicle number correlated with FAI. In a computer-assisted method used to assess stromal echogenecity in 57 women with hyperandrogenism, 65% had PCO visualised on ultrasound and elevated serum T and LH were found in 50% and 45% respectively. There was a significant correlation between ovarian stromal volume and A, but not T, LH or insulin. Nor were associations present between follicular area and any endocrine parameters (Dewailly et al, 1997), suggesting ovarian stromal volume is a better quantification of PCO than follicles.

Three-dimensional ultrasound has been reported to be more precise technique than 2-D ultrasound for assessing ovarian volume (Kyei-Mensah et al, 1996a, b). The ovarian and stromal values were similar in both PCO and PCOS women compared to those with normal ovaries and the total follicular volume was similar in all three groups of women, indicating that increased stromal volume is the main cause of ovarian enlargement in PCO (Kyei-Mensah et al, 1998). Furthermore, there was a positive correlation between stromal volume and serum A concentration in women with PCOS. In summary, ovarian volume correlates well with ovarian function and is both easily and reliably measured in routine clinical practice than ovarian stroma. The consensus definition for PCO does not require either quantitative or qualitative assessment of the ovarian stroma.

Thus, the international consensus for the diagnosis of PCOS now requires two out of three of the following criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus Workshop Group; 2004):

1. Clinical – menstrual irregularity – oligo-/amenorrhoea, anovulation, infertility

2. Biochemical – free T or FAI

3. Ovarian morphology – increased ovarian volume > 10 cm^3 or 12 or more follicles measuring 2–9 mm in diameter.

Unfortunately, during the time of patient recruitment for this thesis, the international diagnostic criteria were not available. For the purpose of this thesis, the selection criteria for PCOS was that used in the UK and most of Europe, which included clinical (menstrual irregularity – oligo/amenorrhoea, infertility, hirsutism/acne) and/or biochemical (raised LH, T, FAI and reduced SHBG concentration) criteria together with PCO morphology on TVS (Balen, 1999).

2.3 Cardiovascular risk factors, morbidity and mortality in women with PCOS

As described in Chapter 1, women with PCOS not only have a cluster of risk factors for cardiovascular disease but are also at an increased risk of developing type II diabetes, dyslipidaemia, hypertension and an abnormal vascular function in later life. This section summarises cardiovascular risk factors and the associated morbidity and mortality in women with PCOS.

2.4   Vascular risk factors in women with PCOS

2.4.1   Fat distribution

In general, women with PCOS are more obese than their counterparts (Talbott et al, 1995) and, in the original report of PCOS, it was the obese women who tended to have what is now known as PCOS (Stein and Leventhal, 1935). Obesity is seen in 35–50% of women with PCOS (Balen et al, 1995) and is typically 'centripetal' – related to fat accumulation in the centre of the body (truncal abdominal fat) – resulting in an increased waist to hip ratio (Evans et al, 1983; 1988; Pasquali et al, 1993), as opposed to the fat accumulation in the thighs and hips (gluteo femoral fat).

There have been few studies comparing body fat distribution in women with PCOS and in controls. In a small Swedish study comparing 14 women with PCOS, mean age 29.0 ± 3.0 years and 13 normally menstruating control women, mean age 36.7 ± 0.9 years, the mean W/H ratio was significantly higher (W/H = 0.82; a W/H ratio > 0.8 is associated with increased risk of stroke – Bray, 1981) in both obese and non-obese women with PCOS compared to controls. Furthermore, larger abdominal fat cells were observed in women with PCOS (fat cell size measured microscopically from needle biopsies taken from the femoral region – lateral to the trocanter, and abdominal region – lateral to the umbilicus) than controls, indicating preferential abdominal accumulation of lipid within adipose tissue (Rebuffé-Scrive et al, 1989).

Dahlgren et al (1992a) studied a retrospective cohort of 33 women (mean age, 50 years) with ovarian histopathology typical of PCO at wedge resection (22 to 31 years previously), who answered a questionnaire and underwent a physical examination and blood test for biochemical assay (PCOS group). They were compared with 132 age-matched control women (mean age, 51.7 years, invited by questionnaire, and 120 underwent further physical and biochemical tests) recruited from a defined female population in Göteberg, Sweden. No significant difference in mean BMI was noted between women with PCOS and controls. However, the mean W/H was significantly higher in women with PCOS compared to controls (0.82 ± 0.1 $vs.$ 0.79 ± 0.1; p = 0.01). This difference remained in obese subjects (BMI ≥ 25 kg/m^2), the mean W/H being 0.86 ± 0.1 in women with PCOS and 0.81 ± 0.1 in controls.

In a larger case control study (206 women with PCOS and 206 control women) from Pittsburgh, USA, the mean W/H was 0.82 in women with PCOS (clinical diagnosis – history of anovulation with either clinical evidence of androgen excess (hirsutism) or total serum T concentration > 2 nmol/L or LH/FSH > 2) and 0.76 in the controls (selected from voters register and directories of households followed by telephone questionnaire regarding demographic information and medical history; on acceptance a physical examination was performed and blood sample test for biochemical analysis). Although, the average BMI was higher in PCOS women (30.5 ± 8.3 kg/m^2) than the controls (26.3 ± 6.46 kg/m^2), on multivariate analysis, the W/H was associated with PCOS independently of BMI (Talbott et al, 1995).

In a recent population-based study, Korhonen et al (2001) looked at the relationship of metabolic syndrome (characterised by the presence of a cluster of cardiovascular risk factors) and obesity to PCOS, in 204 women (defined by a history of ovulatory disorders, hyperandrogenism, infertility and PCO morphology on TVS ($\geq$ 10 and < 10 mm diameter subcapsular follicles), selected from a random sample of women in 5 age groups (range: 35–54 years) who lived in a defined area. Metabolic syndrome was considered present if three of the eight criteria were present (Korhonen et al, 2001):

(1) First-degree relative with type II diabetes

(2) BMI $\geq$ 30 kg/m^2

(3) Waist to hip ratio $\geq$ 0.88

(4) Blood pressure $\geq$ 160/95 mm Hg or on drug treatment for hypertension

(5) Fasting serum triglyceride level $\geq$ 1.70 mm/L

(6) High density lipoprotein cholesterol level < 1.20 mm/L

(7) Abnormal glucose metabolism

(8) Fasting insulin $\geq$ 13.0 mU/L.

The control group consisted of 62 overweight women without central obesity or metabolic syndrome and 53 healthy lean women (BMI < 27 kg/m^2). The group with the metabolic syndrome differed from the PCOS women according to the selection criteria and also had increased serum free T concentration. Oligomenorrhoea was more common in women with the metabolic syndrome, especially in PCOS women (46.2%), than in obese (25.4%) and lean (15.1%) controls.

2.4.2 *Glucose intolerance*

The link between hyperandrogenaemia and abnormal carbohydrate metabolism was first suggested in the early 1920s by Archard and Thiers, who described the phenomenon as 'le diabetic des femmes á barbe' (the diabetes of the bearded women). Fifty years later, Kahn and co-workers (1976) described the syndrome of insulin resistance in six young women with acanthosis nigricans (a feathering pigmentation considered as a non-specific marker for moderate to severe insulin resistance) – two of these women were found to have PCO morphology, diagnosed by gynaecography in one case and by laparoscopy in the other. These women also had hirsutism, primary amenorrhoea, elevated T levels and virilization in one case, though neither of them was obese. This presentation, is however rare and represents an extreme degree of insulin resistance in PCOS.

As previously described in the cross-sectional, retrospective, cohort follow-up study by Dahlgren et al (1992a, b), 15% of women with PCOS and 2.3% of the control women had diagnosed type II diabetes, a statistically significant difference. This study did not investigate any differences between women other than their PCOS and diabetes status. Therefore, it is unclear whether PCOS alone is the cause of the difference in the prevalence of diabetes between the two groups of women.

In particular, there was no analysis of the relationship between BMI or W/H and diabetes in the PCOS and non-PCOS groups (although these measures of obesity were mentioned elsewhere in the paper).

Ehrmann et al (1999) characterised the prevalence and incidence of glucose intolerance in 122 women with clinical and hormonal evidence of PCOS, recruited from medical, endocrinology, gynaecology and paediatrics clinics. Glucose tolerance (measured by oral glucose tolerance test) was abnormal in 55 of 122 women; 43 had impaired glucose tolerance and 12 had type II diabetes at the time of the study. Women with type II diabetes had a 2.6-fold higher prevalence of first-degree relatives with diabetes and were significantly more obese than those with normal glucose tolerance. The prevalence of impaired glucose tolerance and type II diabetes in PCOS women was significantly higher than expected, when compared with data from studies of age- and weight- matched populations of women without PCOS. The authors suggested that the conversion of impaired glucose intolerance to type II diabetes is accelerated in women with PCOS. This study however is incomplete because it lacked a follow-up of all patients (only 25 from 122 were re-evaluated) and there was no control group within the study. At baseline 45% of women with PCOS had an abnormal glucose tolerance test. While no causality can be inferred, it suggests that the prevalence of abnormal glucose tolerance is higher in women with PCOS than in the general population.

Legro et al (1999) studied 254 women with PCOS (ages 14 – 44 years; unexplained hyperandrogenic chronic anovulation with a history of irregular menses and elevated serum T levels) prospectively at two centres; one urban and ethnically diverse (n = 110), and one rural and ethnically homogenous (n = 144). The control group comprised 80 weight, ethnicity, and age-matched women from the same areas. The prevalence of glucose intolerance (31.1%) and type II diabetes (7.5%) was significantly higher in women

with PCOS. In non-obese PCOS women (BMI < 27 kg/m^2) the prevalence of glucose intolerance and diabetes was 10.3% and 1.5% respectively. Variables that were most associated with post-challenge serum glucose levels were fasting serum glucose (p = 0.0001), PCOS status (p = 0.002), W/H (p = 0.001) and BMI (p = 0.021). The authors suggest that PCOS women are at increased risk of impaired glucose tolerance and type II diabetes at a young age, and that PCOS may be a more important risk factor than ethnicity or race for glucose intolerance in young women.

Cibula et al (2000) selected 28 women with PCOS from a large group who had undergone ovarian wedge resection between 1960 – 1981 (PCOS group – typical appearance of PCO on histology followed by a questionnaire study: menstrual history – oligomenorrhoea > 45 days or amenorrhoea; hirsutism – Ferriman and Gallwey score > 8; anovulatory infertility; biochemical assay) and compared them to 752 control women selected by age (45 – 59 years) from a random female population sample. There was no difference in BMI, waist circumference, or W/H between the two groups. Both groups had comparable family histories with respect to type II diabetes, hypertension, coronary artery disease, and smoking habits. Overall the prevalence of type II diabetes was higher in women with PCOS and was four-fold higher in women with PCOS aged 45 to 54 years (p < 0.05).

In the only study of mortality in relation to PCOS status, Pierpoint et al (1998) studied 786 women with PCOS (histological evidence of PCO – two of the following, thickened capsule, subcapsular follicular cysts, luteinization of theca interna, increase in ovarian stroma; macroscopic evidence of PCO – enlarged ovaries, and fibrotic or pearly white

when examined during culdoscopy, laparoscopy or laparotomy; clinical evidence of ovarian dysfunction – hirsutism, secondary amenorrhoea, oligomenorrhoea) diagnosed between 1930 and 1979 in the UK, who were followed up for an average of 30 years. Though the mortality from all causes in women diagnosed with PCOS was no higher than the national rates for women of the same age group, diabetes mellitus was mentioned as an underlying or contributing cause of death for six of the women with PCOS, when only 1.7 deaths would be expected.

The evaluation of Ehrmann's (1999) study lacks follow-up of all women. At baseline nearly half (45%) of PCOS women had an abnormal glucose tolerance. Although one cannot determine causality, this suggests that the prevalence of abnormal glucose tolerance is high in PCOS women.

Legro (1999) found similar prevalence in two different populations and women with and without PCOS were followed to determine the incidence of carbohydrate deterioration. Whether the risk is equivalent in non-obese PCOS women cannot be determined.

The study by Cibula (2000) included 28 women from a large group who had undergone wedge resection. It is not known whether the 28 women were representative of the main group.

In Pierpoint's study (1998) there were only six diabetes-related deaths recorded and it is not possible to consider factors such as BMI or other measures of obesity, which may

contribute to or cause diabetes mellitus. Furthermore, selection bias could be a problem because women who underwent wedge resection may have had more severe symptoms and for many of the patients potentially available, many records were not retrieved.

2.4.3 Dyslipidaemia

In addition to hirsutism, anovulation, infertility, and gonadotrophin secretion abnormalities, PCOS is also associated with metabolic disturbances. Wild et al (1985) evaluated lipid concentrations in 29 caucasian women (mean age 29 years, range 18 – 37 years) with PCOS (clinical history of menstrual irregularity – oligomenorrhoea or hyperandrogenaemia – hirsutism/acne and biochemical abnormality determined by raised serum levels of free T and/or dihydroepiandrosterone sulphate) and 30 regularly menstruating, healthy caucasian control women (mean age 32 years, range 19 – 40 years) with no acne or hirsutism. They reported that women with PCOS had higher mean serum triglycerides (122 $\pm$ 11 mg/dl vs. 63 $\pm$ 3 mg/dl; p < 0.05) and higher very low-density lipoprotein cholesterol levels (24 $\pm$ 2 mg/dl vs. 13 $\pm$ 1 mg/dl; p < 0.05) but lower high-density lipoprotein cholesterol (HDL) levels (43 $\pm$ 2 mg/dl vs. 58 $\pm$ 2 mg/dl; p < 0.05), compared to control women.

Although women with PCOS were heavier and had higher BP, were more sedentary, and had diets higher in saturated fat and lower in fibre, the differences in lipid concentrations were not solely due to differences in body weight/BMI.

The largest study of dyslipidaemia investigated in 206 PCOS women (clinical diagnosis – history of chronic anovulation in association with either clinical evidence of androgen excess (hirsutism) or increased total T level or LH/FSH > 2) were recruited using records from a large reproductive endocrine clinic, and compared them with the same number of

age and race-matched control women (no history of menstrual irregularity or hyperandrogenemia) recruited from a combination of voter's registration tapes and directories of households (Talbott et al, 1995). PCOS women had increased concentrations of triglycerides, total cholesterol and LDL, and decreased concentrations of HDL compared to control women. After controlling for age, BMI and BP these differences were still significant between case and control subjects. This study was extended with repeat lipid concentration measurements to assess changes over time (Talbott et al, 1998). The results of this subsequent analysis showed that pre- and perimenopausal PCOS women ($\geq$ 40 years) had similar LDL cholesterol and total cholesterol levels as their age-matched controls, reflecting the LDL cholesterol increases with age among controls.

Conway et al (1992) defined two groups, one lean (lean BMI < 25 kg/m^2, n = 48) and one obese (obese BMI > 25 kg/m^2 n = 54), of women with PCO confirmed on ultrasound and with clinical features of PCOS. Nineteen lean women with normal ovaries on ultrasound and with regular menstrual cycles were recruited from hospital staff as controls; however, obese women with normal ovaries were not included. A significant decrease in serum HDL cholesterol, but no change in total cholesterol and triglyceride concentrations, was noted in lean women with PCOS compared with lean controls. Comparison of obese PCOS women and lean controls indicated decreased serum HDL and increased triglyceride concentrations in the former but no significant difference in total cholesterol.

Norman et al (1995) compared 97 women with PCOS (clinical and/or biochemical), 21 women with ultrasonically detected PCO but no clinical or endocrine characteristics of PCOS, and 26 women with neither PCOS nor PCO, who acted as controls. The authors further subdivided the PCO and PCOS groups into those with regular (PCO, n = 15; PCOS, n = 43) and those with irregular cycles (PCO, n = 6; PCOS, n = 54). Their control

group contained 19 lean women without PCO/PCOS. In their initial results, Norman et al (1995) reported decreased serum HDL cholesterol concentrations but no significant differences in triglyceride, LDL and total cholesterol in serum from women with PCO or PCOS compared to control women. When subdivided on the basis of menstrual cycle, Norman et al (1995) found triglyceride levels to be higher in the group of women with PCO and irregular menstrual cycles than in any other group. However, as this group only consisted of six women, the clinical significance of this finding remains open to question.

Most studies have shown a degree of dyslipidaemia in PCOS women. There is however less agreement as to which lipid subfractions are affected. Most studies report higher levels of triglycerides and LDL cholesterol, and reduced HDL cholesterols, in PCOS women compared with controls. However, the dyslipidaemic pattern associated with insulin resistance includes intermediate lipoproteins, which play a role in the development of lipid disturbances (McKeigue, 1996). Dyslipidaemia is present in different populations of women with PCOS throughout the world, though no lipid parameters were distributed to an extreme extent. Cases and controls were age-matched only in Talbott's study (1995) and weight-matched in Conway's study (1992). It is not clear whether the differences in lipids between PCOS women and controls persists upto the peri-menopausal age and beyond the menopause.

2.4.4 Hypertension

Studies examining blood pressure in women with PCOS have produced discrepant results. In the previously described study of Conway et al (1992) of 102 PCOS women and 19 lean women with normal ovaries, obese PCOS women had a higher mean systolic blood pressure (SBP) compared to lean women with normal ovaries (122.3 vs. 108.2 mmHg, respectively) This study did not include controls matched for BMI.

As previously described by Dahlgren et al (1992b) in a study of 33 PCOS women and 132 age-matched controls, there was a higher prevalence of hypertension in both age groups in PCOS women compared to controls (27.8 % in 40 − 49 years and 53.3 % in 50 − 61 years vs. 3.5% and 17.3 %, respectively).

The aim of Holte's (1996) study was to evaluate office and 24-hour blood pressure in 36 women with PCOS (mean age 28.2 years [26.6 − 29.8 years]) and 55 control women (mean age 25.3 years [23.9 − 26.7 years]) matched for BMI (26.3 kg/m^2 [24.6 − 28.2 kg/m^2] vs. 25.1 kg/m^2 [24.0 − 26.9 kg/m^2]). The diagnosis of PCOS was based on menstrual irregularity, hyperandrogenism and PCO morphology on ultrasound. Control women were volunteers, hospital staff or students with regular menstruation, no hirsutism and normal ovaries on ultrasound. Compared with the controls, women with PCOS had higher mean daytime systolic blood pressure (126 ± 11 vs. 119 ± 12 mmHg, p < 0.05) and higher mean ambulatory arterial blood pressures (92 ± 7 vs. 86 ± 7 mmHg; p < 0.05); the groups did not differ significantly in daytime diastolic blood pressure or in night time

recordings. The increased daytime blood pressure in women with PCOS persisted after adjusting for BMI, body fat distribution and insulin resistance, which may indicate a prehypertensive state in these women.

Elting et al (2001), evaluated 346 women registered as having PCOS (menstrual irregularity and/or hirsutism and/or infertility), who were traced and interviewed by telephone. The mean age was 38.7 years (range 30.3 – 55.7 years) and the mean BMI was 24.4 kg/m^2 (range 17.5 – 55.8 kg/m^2). In women aged 45 54 years, the prevalence of hypertension (assessed by a series of questions and if on relevant medication) was 2.5 times higher ($p < 0.01$) than in the corresponding age group of the Dutch female population. This difference in prevalence was even greater in the younger women (35 – 44 years).

Zimmermann et al (1992) measured 24-hour ambulatory systolic and diastolic blood pressure in 14 women with PCOS (defined hyperandrogenaemia in conjunction with oligomenorrhoea, day 21 progesterone < 6.2 nmol/L; mean age 30 ± 1 years) and 18 normal control women (no menstrual and/or fertility history and day 21 progesterone > 16 nmol/L; mean age 31 ± 1) of comparable BMI (31 ± 2 vs. 30 ± 2 kg/m^2) and race. In contrast to the results of many studies, they reported that the ambulatory systolic (121 ± 2 vs. 118 ± 2 mmHg) and diastolic (76 ± 2 vs. 73 ± 2 mmHg) blood pressures were similar in women with PCOS and control subjects.

Sampson et al (1996) performed a cross-sectional study of 24 non-obese PCOS women with irregular menses and classic PCO morphology on ultrasound examination (mean age 31.7 ± 6.6 years, BMI 23.4 ± 2.8 kg/m^2), matched with 26 women with normal menses and PCO morphology on ultrasound (mean age 30.3 ± 6.9 years, BMI 22.0 ± 2.7 kg/m^2) and 10 controls with normal menstrual cycles and ovarian morphology on ultrasound scan (mean age 31.4 ± 7.2 years, BMI 22.9 ± 2.1 kg/m^2). Again no differences in 24-hour, daytime, or night time ambulatory blood pressure were noted between groups.

The results of the studies described are conflicting and several potential causes are apparent. In Dahlgren's study, patients were evaluated by wedge-resection, but the histological diagnosis of PCOS was controversial, unstandardised, and not uniformly recognised among pathologists 22 to 31 years ago. Questionnaire data can be unreliable unless the questions are well designed. Blood pressure is commonly not measured with strict attention to detail and accuracy, including calibration of the instruments used. Numerous factors including genetic causes, inactivity, stress and salt loading affect blood pressure, yet none of these factors were controlled for in any of the studies that evaluated the small number of PCOS women. Thus further evidence is required to clarify whether PCOS is independently associated with hypertension or the increased blood pressures found in some studies are related to other contributing factors such as BMI.

2.5 Polycystic ovary syndrome and the Metabolic Syndrome

Reduced sensitivity to insulin and compensatory hyperinsulinaemia noted in women with PCOS was first reported over 25 years ago (Burghen et al, 1980; Dunaif et al, 1987). As discussed previously, the metabolic abnormality in women with PCOS are similar to that in Metabolic Syndrome, a clustering within an individual of hyperinsulinaemia, glucose intolerance, dyslipidaemia, and hypertension (Reaven, 1988) all of which have been associated with an increased risk of cardiovascular disease.

When women with Metabolic Syndrome were studied for characteristic reproductive features of PCOS, they were no more likely to have PCO than other segments of the population; while only about half of them had a history of oligomenorrhoea (Korhonen et al, 2001). Nor do all women with PCOS have the Metabolic Syndrome.

In some countries obesity appears to be less common in women with PCOS. For instance, in the United Kingdom, a large study of 1700 women reported a high percentage of non-obese women with PCOS (Balen et al, 1995). Similarly, in the Netherlands, a follow-up study of over 300 women with PCOS showed that over half of these women were not over weight (Elting et al, 2001). Although these studies are not a representative population sample, they suggest a less obese PCOS phenotype is found in the UK and the Netherlands than is encountered in the United States (Legro et al, 1999; Ehrmann et al, 1999).

In a recent retrospective study of 106 women with PCOS, the prevalence of PCOS and Metabolic Syndrome (n = 46) was 43%, nearly 2-fold higher than reported for age

matched women in the general population; these women also presented with increased hyperandrogenism (increased free serum T levels and decreased levels of serum SHBG) and acanthosis nigricans, all features which reflect increased insulin resistance (Apridonidze et al, 2004). Therefore, it is probable that the existence of women with PCOS but without symptoms of the Metabolic Syndrome suggest it is more likely that the two syndromes are closely interwoven pathophysiologically to a variable degree, such that PCOS and Metabolic Syndrome often co-segregate, but can also be seen in isolation.

2.6 Polycystic ovary syndrome and the link to coronary vascular disease

2.6.1 Coronary artery

Wild *et al* (1990) surveyed 102 women undergoing cardiac catheterisation for evaluation of chest pain, 52 with coronary artery disease (mean age 63.6 years) and 50 with normal coronary arteries (mean age 60.5 years). In women with an abnormal angiogram, the incidence of hirsutism, diabetes mellitus and hypertension was significantly increased; these women also had a higher W/H than women with normal coronary arteries, despite similar BMI. The strongest associations were in women ≥ 60 years.

Dahlgren et al (1992b) compared the distribution of risk factors for vascular disease in 33 women with PCOS (based on histopathological evidence in ovarian biopsies) with 132 age-matched controls. They estimated that myocardial infarction would be seven times more common in women with PCOS than in the general population. This estimate was based on applying a logistic regression model, derived in an earlier follow-up study

(Dahlgren et al, 1992a), in which myocardial infarction was correlated with earlier results for serum triglycerides, W/H ratio, diabetes and hypertension.

Birdsall et al (1997) reported the association between PCO on TAS or TVS and the extent of coronary artery disease in 143 women aged 60 years or younger, undergoing cardiac catheterisation for the assessment of chest pain. Women with bilateral oophrectomy were excluded. The quantitative extent of the disease assessed by blind evaluation of angiograms and compared with the presence or absence of PCO. PCO were present in 42% of the 143 women. Women with PCO had more coronary arteries stenosed > 50%, and exhibited a greater prevalence of hirsutism, previous history of hysterectomy, lower HDL cholesterol concentrations, and higher free T, triglyceride and C-peptide levels, than women with normal ovaries.

The findings of the study of Wild et al, (1990) suggest that the results were significant in older women (> 60 years), probably because of the increased incidence of coronary artery disease in general. Asymptomatic women were not included for ethical reasons (radiation and invasive nature of the procedure). Although the data collected was based on recall ability and from a pre-selected population (i.e. chest pain), the result is evenly distributed in those with and without confirmed disease. Some post-menopausal women reported hirsutism, but it is not clear whether this was related to PCOS. Secondly, ultrasound was not performed to assess ovarian morphology and it is unknown if those without chest pain would have the same findings.

The results of Dahlgren's study (1992b) may be biased due to its retrospective design and the inclusion of both premenopausal (n = 18; mean age 45.9 years) and postmenopausal women (n = 12; mean age 55.6 years). A questionnaire was used with no mention on types of questions asked, in particular smoking, which is a known risk factor for heart disease, at least in the younger women (Rosenberg, 1991). Age constitutes an important risk factor for myocardial infarction. Despite age matching, the control subjects were somewhat older than the PCOS subjects (mean age 45.9 and 46.5 years between 40 – 49 years; mean age 55.1 and 55.6 years between 50 – 61 years, respectively), albeit the differences were not significant.

The study of Birdsall (1997) has the advantage of ultrasound scanning (albeit PCO morphology alone does not support diagnosis of PCOS). Oophrectomy is associated with premature heart disease in women with or without PCOS; this was a useful and valid exclusion criterion. All the three studies discussed above establish the prevalence of PCOS in women having angiography; however, the disadvantage of angiography is that it is a surrogate endpoint for events.

2.6.2 Carotid artery

Guzick et al (1996) studied carotid artery intimal media thickness by carotid ultrasonography in 16 premenopausal women with PCOS (mean age 44.4 years; history of chronic anovulation in association with clinical evidence of androgen excess – hirsutism or total T concentration > 2 nmol/L or LH/FSH ratio > 2) and 16 age-matched control women (mean age 43.9 years) with normal menstrual cycle. Carotid artery IMT was

increased in patients with PCOS compared with healthy control subjects, but there was no difference in the prevalence of atherosclerotic plaques. Although evidence for the progression of vascular disease from increased IMT through plaque formation to disturbance in blood flow is lacking, increased IMT is viewed as an early indication of subclinical atherosclerosis.

In a larger retrospective study Talbott et al (1998) recruited 46 women (age 42 ± 8 years) with PCOS (chronic anovulation in association with clinical evidence of androgen excess (hirsutism) or elevated total serum T > 2 nmol/l or LH/FSH ratio greater than 2.0) and 59 controls (age 43 ± 7 years) with normal menstrual cycle. Subjects underwent carotid artery scanning at eight carotid artery sites using B-mode ultrasound. The carotid atherosclerotic index was calculated as the overall mean of the IMT measurements at the eight sites. The atherosclerotic index was significantly greater in PCOS women than in controls, and this difference was associated with age, BMI, diastolic blood pressure, and serum LDL cholesterol concentration. Some of these correlation factors were however collected 5 − 7 years before the IMT measurement, and total cholesterol was not controlled for in the analysis.

In 2000, Talbott et al extended their study having recruited 125 women (mean age 37.5 ± 6.2 years) with PCOS (chronic anovulation in association with clinical evidence of androgen excess (hirsutism) or elevated total T > 2 nmol/l or LH/FSH ratio > 2.0) and 142 control women (mean age 39.0 ± 6.2 years) with normal menstrual cycle. Baseline sociodemographic data, reproductive endocrine hormone levels and cardiovascular risk

factors were collected between 1992 and 1994. During follow-up, four years later, B-mode ultrasound of the carotid arteries was performed to evaluate IMT and the prevalence of plaque. IMT was assessed as an average measure at each location (across 1.0 cm segments of the near and far walls of the distal common carotid artery (CCA), the far wall of the carotid bulb, and the internal carotid artery (ICA) on the left and right sides) to produce an overall measure of the IMT. The prevalence of plaque was defined as a distinct area protruding into the vessel lumen with at least 50% greater thickness than the surrounding areas and for each segment the degree of plaque was graded (0 = no plaque; 1 = one small plaque < 30% of vessel diameter; 2 = one medium plaque between 30 – 50% of vessel diameter or multiple small plaque; 3 = one large plaque > 50% of vessel diameter or multiple plaques with at least 1 medium plaque). The grades were summed across the right and left carotid arteries for an overall focal plaque index. The IMT and the overall plaque index were significantly greater in the PCOS women compared with controls and the difference remained significant after adjusting for age and BMI. Although there was this significant difference between the two groups, plasma lipids were not analysed in the second study (data from the previous study were used).

The study by Guzick (1996) has several flaws. Although PCOS women and controls were matched for age, race and neighbourhood, they were not matched for smoking, a known risk factor for cardiovascular disease (Cheng et al, 2002). The authors used a new tool to assess the extent of vascular damage in the carotid vessels but did not mention if they included the presence of plaque(s) in the bulb or in the first centimetre of the internal carotid artery. Were the ultrasonographers blinded? Although the sample size in this study

is limited, the results do suggest that women with PCOS have an increased risk of subclinical atherosclerosis in their 40s.

In Talbott's studies (1998, 2000) there was a 5 – 7 year lapse between the collection of biochemical and biometric data, and the IMT estimates, and in addition total cholesterol was not assessed. There is evidence, both clinical and experimental, to suggest that elevated total cholesterol is associated with abnormal endothelial function in patients with and without atherosclerotic disease detected both by angiography and ultrasound (Vogel, 1999).

Increased IMT is a surrogate marker for coronary artery disease and stroke. Atherosclerotic changes do not necessarily translate into more events and progression of disease can be halted with preventative efforts. The significance in both the Guzick and Talbott studies lies in the fact that increased IMT is associated with increase risk of heart disease and stroke. As summarised in Table 2.3, studies have shown increased morbidity in women with PCOS, suggesting these women may be at an increased risk of developing cardiovascular disease and may be 'older' from the circulatory point of view.

Table 2.3   Summary of studies looking at morbidity in women with Polycystic Ovary Syndrome

Researcher	Study design	Subjects (no)	Method	Outcome
Wild et al, 1990	Cross-sectional; questionnaire	102	Questionnaire	Hirsutism was common in PCOS women with positive coronary angiography. Android fat distribution was associated with androgen excess and may be an indicator for greater risk for ischaemic heart disease.
Dahlgren et al, 1992a	Retrospective study	33 PCOS 132 Controls	Questionnaire, interview and blood samples	Infertility, hirsutism, and oligomenorrhoea were common among women diagnosed clinically and histologically with PCOS. These women had increased central obesity and basal serum insulin concentrations and a higher (15%) prevalence of diabetes and hypertension (39%).
Dahlgren et al, 1992b	Retrospective follow-up	33 PCOS 132 Controls	Statistical	By risk-model analysis, PCOS women had a 7.4-fold greater risk of myocardial infarction than in age-matched controls.
Guzick et al, 1996	Prospective cross-sectional	16 PCOS 16 Controls	Ultrasound	Increased intima-media thickness of the common carotid bulb and internal carotid arteries in PCOS women may be associated with sub-clinical atherosclerosis.
Birdsall et al, 1997	Cross-sectional study	143	Questionnaire	PCOS women had greater than 50% coronary artery stenosis during cardiac catheterisation than women with normal ovaries.
Cibula et al, 2000	Retrospective follow-up study	28 PCOS 752 Controls	Evaluation of histological, clinical and biochemical data	The prevalence of non-insulin dependent diabetes mellitus (32%) and coronary artery disease (21%) was higher in PCOS women compared with controls.

2.7 Cardiovascular mortality in PCOS

In 1998, Pierpoint and colleagues published the first finding which has addressed this important issue. In this study, with a mean follow-up of 30 years, Pierpoint reported that mortality from all causes in a group of women with PCOS was no higher than the national mortality rates for women of the same age. Of the total of 59 deaths, 15 were from circulatory diseases (standardised mortality ratio 0.83; 95% CI 0.46 – 1.36); 13 from ischemic heart disease (standardised mortality ratio 1.40; 95% CI 0.75 – 2.40) and two from other circulatory diseases. Deaths from type II diabetes were higher than expected in PCOS women (odds ratio 3.6, range 1.5 – 8.4). The authors concluded that women with PCOS do not have increased mortality rates from circulatory disease, although the condition is associated with type II diabetes, lipid abnormalities, hypertension and obesity.

The results of this study have been widely discussed because they appear to conflict with the anticipated results. Diagnosis of PCO was based primarily on ovarian wedge resection. Clinical indices, androgen excess and abnormal menses were not assessed. Polycystic ovary is a marker of PCOS, and is seen in 5 – 10% of women and the indication for wedge resection is not mentioned. The authors suggest that because these women underwent wedge resection, metabolic risk factors should be more severe; however, the main indication for wedge resection historically was for fertility, not for severity. Of all women who were potentially available and traceable, many records were not complete or retrieved. Selection bias was an issue and one cannot be sure if the excluded data were similar to the retrieved data. Regional differences for cardiovascular

disease rates are known. Standardised mortality rates came from a national database, not taking into account regional variations. The hazards of using death certificate data and the problems with their accuracy are well known. In spite of these limitations, there was no increase in cardiovascular deaths.

In light of this scenario, the global problems associated with PCO transcend the younger women's problems of amenorrhoea, irregular menses, infertility, and hirsutism; and may include potential long-term, metabolic and cardiovascular problems. Paediatricians, endocrinologists, dermatologists, gynaecologists and cardiologists may see these women from puberty through reproductive age to menopausal years. It is clear that PCOS is a multifaceted clinical entity with both short-and long-term consequences. Resistance to insulin is a key element of the syndrome, including upper body obesity, glucose intolerance, hypertension and dyslipidaemia; all factors leading to increased risk for cardiovascular diseases.

CHAPTER 3

MECHANICAL AND PHYSICAL PROPERTIES OF

BLOOD VESSELS

3.1 Introduction

Atherosclerosis is a primary cause of heart disease and stroke (Lusis, 2000), characterised by progressive accumulation of lipids and fibrous elements in the large arteries. Cardiovascular disease represents the leading cause of morbidity and mortality in Western society (Peterson et al, 1999). The underlying pathological process of atherosclerosis progresses with age and is accelerated by the presence of cardiovascular risk factors such as hypertension, smoking, diabetes mellitus and hypercholesterolaemia.

A number of expert panels have published classification schemes and guidelines designed to aid diagnosis, assess severity and determine prognosis in patients with risk factors for cardiovascular disease (Anonymous - British Cardiac Society, 1998). The recommendations emphasize the role of high BP, cholesterol and glucose as examples of major risk factors that exhibit strong, positive, and continuous relationships with the risk of cardiovascular disease, even within defined normal ranges.

There are currently no progressive criteria in the UK for the detection of cardiovascular risk factors. There is however good evidence that treatment of hypertension and hypercholesterolaemia, as well as cessation of cigarette smoking and increasing exercise reduces the risk of cardiovascular incidents (CAPRIE Steering Committee, 1996; Zanchetti et al, 1998; Peterson et al, 1999; Robeer et al, 1998; Frishman, 2000).

Atherosclerosis is a geometrically focused disease affecting well-defined areas of the cardiovascular tree. The importance of the biophysical properties of blood vessels and

flow on the development and localisation of cardiovascular disease is now being appreciated. Arterial dysfunction is reflected by changes in vascular elastic properties i.e. increased IMT and stiffness index and decreased vascular compliance, to the extent that these parameters are now recognised risk markers, as well as potentially modifiable risk factors in cardiovascular disease.

Changes in the structure (intrinsic properties) of the arterial wall occur to alter the physical characteristics of blood vessel walls and impair the pulsatile nature of arteries before the clinical manifestations of cardiovascular disease (Hickler, 1990; Geroulakos et al, 1994; Glasser et al, 1997; Bots et al, 1997; Aminbaksh and Mancini, 1999; Simons et al, 1999; Balbarini et al, 2000). Physical properties which are modified include arterial elastic properties and increased IMT, both of which are regarded as markers of early atherosclerosis (Burke et al, 1995; Glasser et al, 1997; Reneman and Hoeks, 2000; Van Popel et al, 2000).

3.2   Structure of normal artery

Blood vessels are responsible for the circulation of blood throughout the body. Whilst arteries carry blood at high pressure away from the heart, veins predominantly carry blood at low pressure back into the heart. Structurally the two vessels vary in order to carry out their functional roles efficiently.

In order to understand arterial biomechanical parameters, the structure and function of the arterial wall must be defined. The arterial wall is composed of three main layers, these

being, from within outward; the tunica intima, the tunica media and the tunica adventitia (Figure 3.1). The tunica intima consists of a layer of endothelial cells resting on a basal lamina closely abutting upon the internal elastic lamina. This layer has an important role in the maintenance of the integrity of the intravascular function. The tunica media consists of non-striated myocytes with varying amounts of collagen and elastin fibres. The larger elastic arteries are distributed predominantly near the heart and organs, e.g. the aorta and its major branches including the common carotid artery, and their key functional role involves the pulsatile movement of large volumes of blood. Structurally, these vessels are composed of a high density of layered, elastic membrane in the tunica media. The smaller muscular arteries, such as the femoral artery, have the same basic structures as the elastic arteries, except that the elastin fibres within the tunica media are reduced to two well defined sheets, the internal and external laminae. Also in these arteries, the tunica media contains an increased abundance of smooth muscle cells relative to the elastic fibres. The tunica adventitia, the outermost layer of the blood vessel is entirely composed of collagen, the branches of blood vessels, together with lymphatics and nerve plexi terminate. Functionally these arteries are responsible for the rapid and complete distribution of blood to all organs and tissues within the body.

Figure 3.1   Composition of the arterial wall (Reproduced with permission from The Basis of Medicine by Pocock and Richards, 1999)
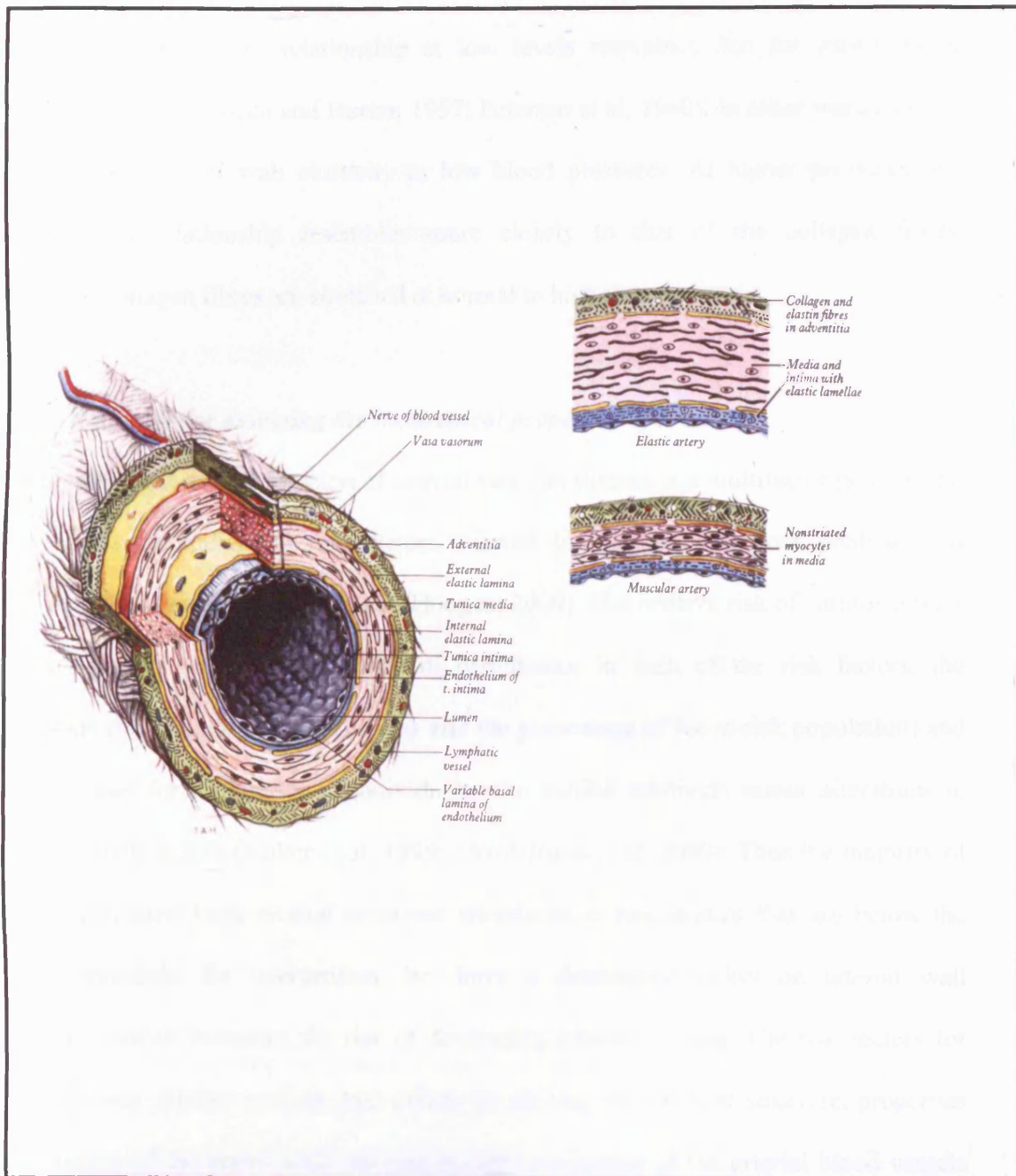
The vascular smooth muscle cells within the tunica media have an important role in maintaining the active tension of the vessel wall (Burton, 1954; Roach and Burton 1957; Petersen et al, 1960). Passive tension is attributed to the elastin and collagen fibres, and the vessel tension-strain relationship at low levels resembles that for elastin fibres (Burton AC, 1954; Roach and Burton 1957; Petersen et al, 1960). In other words, elastin fibres provide vessel wall elasticity at low blood pressures. At higher pressures, the tension-strain relationship resembles more closely to that of the collagen fibres, indicating collagen fibres are stretched at normal to high temperatures.

3.2.1 *Rationale for assessing the mechanical properties of arteries*

The development and progression of arterial vascular disease is a multifactor process and abnormalities in individual risk factors, viewed in isolation, are poor predictors of increased risk (Kannel, 1996; Van den Hoogen, 2000). The relative risk of cardiovascular events increases with the magnitude of disturbance in each of the risk factors, the population risk (function of relative risk and the percentage of the at-risk population) and also increases for a majority of individuals who exhibit relatively minor alterations in traditional risk factors (Kuller et al, 1999; Lloyd-Jones et al, 2000). Thus the majority of at-risk population have normal or minor alterations in risk factors that are below the present threshold for intervention, but have a detrimental effect on arterial wall properties, which increases the risk of developing arterial disease. The risk factors for cardiovascular disease mediate their effects by altering the vascular structure, properties and function of the vessel wall and endothelial components of the arterial blood vessels (Ross, 1993). The ability to detect sub-clinical vascular damage, representing the

cumulative and integrated influence of risk factors in impairing arterial wall integrity, holds the potential to monitor disease progression and assess the impact of pharmacological intervention in this process (St. John, 2000).

The importance of assessing arterial wall integrity is demonstrated by the observation that a reduction in the pulsatile function of large arteries represents an independent risk factor for cardiovascular events (Franklin et al, 1999; Chae et al, 1999; de Simone et al, 1999; Vaccarino et al, 2000). There is increasing evidence that the pulsatile characteristics of arteries are modified early in the disease process (Yamashita et al; 1998; Arnett et al, 1999; Leeson et al, 2000) and impaired arterial function is recognised as an independent predictor for risk for vascular events including coronary heart disease (Benetos et al, 1993; Franklin et al; 1999) and hypertension (Benetos et al; 1998) as well as for diabetes mellitus (Emoto et al, 1998; Van Popel et al, 2000).

Changes in the mechanical behaviour of the blood vessels provides direct information on the arterial wall integrity and progression of arterial wall disease, and holds predictive potential for therapeutic intervention (Cohn; 1999). The consequences of impaired pulsatile characteristics of the arterial circulation are outlined in Figure 3.2.

Figure 3.2   Reduced compliance promotes blood vessel damage and predisposes to premature clinical events (Reproduced with permission from McVeigh et al, 2002)

```
                    ┌──────────────────────────────────┐
                    │   Reduced Arterial Compliance     │
                    └──────────────────────────────────┘
                                     │
                                     ▼
          ┌────────────────────────────────────────────────────┐
          │   Impaired Ventricular – Vascular Interaction       │
          └────────────────────────────────────────────────────┘
                                     │
                                     ▼
                    ┌──────────────────────────────┐
                    │      Blood Vessel Damage      │
                    └──────────────────────────────┘
                                     │
              ┌──────────────────────┴──────────────────────┐
              ▼                                              ▼
┌──────────────────────────────┐           ┌──────────────────────────────┐
│          Intima              │ ─ ─ ─ ─ ─▶│          Media               │
│  (Endothelial dysfunction)   │           │  (Impaired pulsatile function)│
└──────────────────────────────┘           └──────────────────────────────┘
              │                                              │
              ▼                                              ▼
┌──────────────────────────────┐           ┌──────────────────────────────┐
│ • Accelerates atherogenesis  │           │ • Systolic blood pressure     │
│ • Promotes plaque rupture    │           │                               │
│ • Enhances vasoconstriction  │           │ • ↑ Pulse pressure            │
│ • Decreases dilator reserve  │           │                               │
│ • Promotes thrombogenesis    │           │ • ↓ Diastolic blood pressure  │
└──────────────────────────────┘           └──────────────────────────────┘
              │         ┌──────────────────┐         │
              ▼         │  Cardiovascular  │         ▼
     ┌─────────────┐    │     Events       │    ┌─────────────────┐
     │  Ischaemic  │    └──────────────────┘    │ Impedance load  │
     └─────────────┘             ▲              └─────────────────┘
              │      ┌──────────────────────────────┐      │
              └─────▶│ • Left ventricular hypertrophy│◀─────┘
                     │ • Congestive cardiac failure  │
                     └──────────────────────────────┘
```

3.3 Arterial haemodynamics

There is no universally established standard for the characterisation of arterial wall mechanics. This is largely due to the complex physical and mechanical nature of the arterial tree. At any point the mechanical properties of arteries are different depending on the arterial wall structure (elastin and collagen) and the pressure of the blood flow. The arterial system is composed of a branching network of elastic conduits and large distributing arteries, which offer little resistance to blood flow. For maximal efficiency, blood flow should be achieved with the least possible energy expenditure (McVeigh et al, 2002).

The fibrous proteins, elastin and collagen largely determine the mechanical behaviour of blood vessels. As discussed previously, the distribution of elastin, collagen and smooth muscle fibres differs between central and peripheral arteries. Looking at each component separately, elastin fibres are up to 10 times more distensible compared with rubber and determine the mechanical strength of blood vessels at low pressures. The collagen fibres on the other hand are less extensible and function to protect the blood vessel against structural damage at normal to high blood pressures. Characteristically, the vascular smooth muscle, whether relaxed or contracted, contributes very little to the overall elasticity of the blood vessel wall. Rather, it produces active tension; on extension vascular smooth muscle exhibits deformation and maintains its length for a period of time, but then has the ability to contract when activated and return to its original length.

When blood vessels are subjected to pressure, they distend in the circumferential and longitudinal directions and undergo radial thinning of the vessel wall. These changes occur in three directions at 90° to each other. Movement of blood through the vessels is governed by Newtonian fluid mechanics which, in turn, is influenced by the physical properties of the vessel wall and how the vessel wall responds when subjected to pressure.

Blood pressure can be divided into two main components: the mean arterial pressure and the pulse pressure. The mean arterial pressure reflects the steady state component of the blood pressure and is dependent on the cardiac output and total peripheral resistance. The local pulse pressure (systolic minus end-diastolic blood pressure) is dependent on a combination of the forward pressure wave produced by blood being ejected from the left ventricle into the vascular system, and the reflected waves (wave reflections) returning from the periphery. Hence, a simple circuit cannot be used to describe the complexity of the vascular network.

Due to the complexity of the vascular system, there are no universally established methods for the characterisation of vascular mechanical properties. The concepts related to the direct assessment of mechanical wall properties are however gaining recognition in the research community but barriers remain to be overcome before acceptance is gained in the clinical arena. The lack of a 'gold standard' method has fostered the growth of a variety of techniques and methodologies for the estimation of mechanical wall properties. The relationship between the different descriptive terms, representative of the mechanical

properties of the arteries and the circulation is therefore difficult to grasp. For example, arterial stiffness has been employed as an all encompassing term to include arterial compliance, arterial distensibility (change in diameter or area for a given change in pressure), elastic modulus (the change in stress for a given change in strain of the wall material) and stiffness index, a measure representative of the mechanical properties of arteries (O'Rouke and Mancia, 1999; Table 3.1).

In attempting to understand the complex biophysics of blood vessels *in vivo*, a variety of indices have evolved. Different methods have also been developed to measure these indices (Table 3.1) (Bramwell and Hill, 1922; Petersen et al, 1960; O'Rouke, 1982; Hickler, 1990; Lehman et al, 1996, Glasser et al, 1997). This had led to debate over the best indices for measuring these arterial properties, as well as to confusion in the comparison of results between studies. Techniques that can provide quantitative estimates of arterial wall properties are outlined in Table 3.2.

Table 3.1   Indices defining the arterial wall elastic properties.

Arterial Index	Definition
Petersen's elastic modulus (Ep) (mmHg)	$= \dfrac{(P_s - P_d)}{(D_s - D_d)/D_d}$ Modified changes in the volume to changes in diameter as an artery is usually circular in cross-section and the change in diameter (Ds) is small with respect to the initial end-diastolic diameter (Dd)
Stiffness index (β) (Dimensionless)	$\beta = \dfrac{[\log_e (P_s / P_d)]D_d}{(D_s - D_d)}$ Thought to be less dependent on blood pressure as it is expressed in the natural logarithm of the ratio of the systolic to diastolic blood pressure
Pulse wave velocity (PWV) (cm/sec)	$= \dfrac{d}{t} = 1/\sqrt{P \times DC}$ The distance travelled by the pulse wave for a given period of time. Relies on high fidelity equipment and problematic if the pulse wave travels over segments with opposite wave directions. PWV is related to the elastic modulus or the inverse of the distensibility
Compliance (cm x mmHg^{-1})	$= \dfrac{(A(D)_s - A(D)_d)}{(P_s - P_d)}$ Defined as the change in volume for a given change in pressure. Assuming a linear volume pressure relationship, the change in volume could be replaced by the difference between the systolic and diastolic volume and the change in pressure by the pulse pressure. For a circular arterial segment in cross-section, the volume change could be replaced by area change or diameter change.
Diametrical compliance or Distensibility (C) (%mmHg^{-1})	$= \dfrac{(A(D)_s - A(D)_d) \times 100}{(P_s - Pd) \times A(D)_d}$ Defines the relative change in the dimension of an artery for a given change in pressure. Allows vessels of different diameters to be compared.
Compliance coefficient (CC) (m^2 x Pa^{-1})	$= \dfrac{(A_s - A_d)}{(P_s - P_d)}$ Claimed to be less pressure dependent
Distensability coefficient (DC) (Pa^{-1})	$= \dfrac{(A_s - A_d)/A_d}{(P_s - P_d)}$ Claimed to be less pressure dependent

Abbreviations: s – systolic pressure; d – diastolic pressure; P – pressure; D – diameter; A – cross-sectional area; V – volume; and h – height; t – time

Table 3.2   **Methods used to estimate the mechanical properties of arteries**
(Reproduced from McVeigh et al, 2002)

Methods	Advantages	Limitations	Information
Direct			
Angiography	Evaluation of different aortic segments	Expensive, invasive	Regional wall properties
Magnetic resonance imaging	Non-invasive, not limited by acoustic window, can examine multiple arterial segments	Claustrophobia, expensive, limited availability	Regional wall properties
TTE / TOE	TTE non-invasive, reasonable availability	Expensive, TTE limited by acoustic window, operator-dependent techniques, TOE invasive, remote site of BP measurement	Regional wall properties
Transcutaneous Et / IVUS techniques	Transcutaneous technique is non-invasive; both techniques reproducible	Operator-dependent, IVUS invasive, remote site of BP measurement with ET, clinical research application	Local wall properties
Indirect			
Fourier analysis of pressure and flow waveforms	Reference technique, pulsatile and steady-state information	Expensive, invasive, limited to research arena	Total arterial impedance
Stroke - volume to pulse - pressure ratio	Non-invasive, reasonable availability	Non-invasive estimate of stroke volume required, brachial BP measurement	Total arterial compliance
Pulse - wave velocity	Non-invasive, reproducible, potential for wider clinical application	Limited to aorta, errors estimating path length and waveform distortion with pulse propagation	Segmental arterial stiffness
Pulse - contour analysis	Non-invasive, reproducible, potential for wider clinical application	Non-invasive measurement of stroke volume, assumed model of the arterial system	Compliance estimates derived from Windkessel modelling

Abbreviations: BP – blood pressure; TTE – trans-thoracic echocardiography; TOE –trans-oesophageal echocardiography; ET – echo tracking; IVUS – intravascular ultrasound.

3.4 Assessment of vascular haemodynamics and mechanical properties of blood vessels

Methods developed for measurement of peripheral blood flow can be quantitative or qualitative, or inferred from other parameters such as tissue temperature. They differ as to whether they measure blood flow in a single vessel or organ. The various techniques differ in their degree of invasiveness, accuracy and reproducibility.

3.4.1 Doppler Ultrasound

Christian Johann Doppler, an Austrian physicist, first observed the Doppler effect in 1842, noting that the frequency of a wave decreases if the observer is moving away from the source. This observation referred to light emitted from stars, but the implications extend beyond astronomy and form the basis of an important technique for circulatory assessment. For over 40 years, ultrasound scanners have exploited the Doppler effect where analysis of echoes scattered by moving blood provides estimate of blood flow velocity. The clinical use of blood flow imaging systems has expanded immensely since Satumura performed the first measurement of flow in the heart in 1956. Many disease processes in the body result from alterations in the circulatory system (Hoskins, 1999). There may be gross tissue changes such as proliferation of new vessels, deposition of atheroma, arterial plaque development and regions of infarction. Many of these changes alter the overall blood flow and also local blood flow patterns in the vessels.

When an ultrasound beam from a stationary source is transmitted to the blood vessel, it becomes scattered by the moving blood cells. The change in frequency (f_d) of the reflected beam is related to the transmitted frequency (f), the velocity of the moving blood, (v), and the angle the beam subtends to the vessel (φ), as well as the speed of

propagation of ultrasound through the medium, (c). The Doppler shift frequency is given
by the equation;

$$f_d = \frac{2fv \cos \varphi}{c}$$

Over the past 20 years, successive generations of equipment have evolved rapidly to
assess vascular dynamics. In brief:

1. **Spectral Doppler.** Referring to the display of Doppler shift frequency in the form
   of a time-frequency plot. Spectral analysis yields various parameters such as peak
   systolic and end diastolic velocities, pulsatility index (PI) and resistance index (RI)
   that aid in the recognition and quantification of disease (Hoskins, 1999).

2. **Continuous wave (CW) system.** Device in which ultrasound is transmitted and
   received continuously. Continuous wave transducers are light and easy to
   manipulate, and the signals can be obtained quickly. Signals from the vessel are
   recognised by the shape of the flow-velocity waveform. A disadvantage of this
   method is that signals from several vessels in the path of the beam are superimposed
   on one another (Forsberg, 1997).

3. **Pulsed Doppler (PW) system.** With pulsed-wave Doppler, ultrasound is
   transmitted in short bursts and the reflected signals are detected at timed intervals
   after the signal is sent out. This ensures that the detected scattered echoes received
   originate from a fixed depth, which is determined by time delay between the
   transmission and detection. The advantage of pulsed Doppler is that it is depth
   selective, and if this is combined with ultrasound imaging the precise area of
   insonation can be visualised (Forsberg, 1997).

4. **Duplex system**. Device consisting of a real-time B-scanner and a pulsed wave or continuous waves Doppler. The advantage of the duplex system is that the angle of incidence can be estimated from the B-scan allowing Doppler frequency shifts to be transformed to flow velocities. Assessment of the angle of incidence rests on a number of assumptions, such as the flow is parallel to the vessel walls and there is no curvature of the vessel in the scan plane. The velocity estimate error is dependent on the angle of incidence, and in modern machines the angle cursor increments every 1 or $2°$ ensuring smaller velocity estimate errors and thus greater accuracy (Hoskins, 1999).

5. **Colour flow imaging**. Detection of blood flow within the scan plane of an ultrasound scanner and the colour display of haemodynamic parameters, such as flow velocity (magnitude and direction), turbulence or simply the presence of flow, is known as colour Doppler imaging (Fish, 1999). Colour Doppler flow mapping provides information on the direction of flow, towards or away from the probe being represented by different colours. Colour Doppler machines incorporate a computer, which enables the system to provide a real-time image of an area and also to sample simultaneously multiple, tiny volumes within that area for Doppler information. Colour Doppler imaging extracts and displays two parameters of the Doppler signal: mean velocity and variance (width) of the spectrum. This information is processed, and the mean frequency shift in each sample is colour coded on a real-time image. Colour flow mapping affords the possibility of repeated measurements on the same vessel over a period of time (Allan, 1992).

Quantitation of Doppler waveforms

Analysis of the waveform can provide information on disease (Hoskins, 1999). The simplest quantitative approach is to use indices, which are related to the degree of waveform pulsatility i.e. the degree of diastolic flow. The two common indices used are the PI and RI (Hoskins, 1999). PI and RI are calculated from the ratios of systolic and diastolic Doppler shift frequencies rather than from absolute velocimetry of blood flow. PI expresses the difference between peak systolic Doppler shift frequency (A) and the diastolic Doppler shift frequency (B) relative to the mean Doppler shift frequency (mean), and this is defined as:

$$PI = (A-B)/mean$$

The RI is defined as the ratio of the difference between peak systole and end diastole Doppler shift frequencies, relative to the peak systolic Doppler shift frequency (Pourcelot 1974) and this is defined as (Figure 3.3):

$$RI = (A - B)/A$$

Figure 3.3   **Parameters of the maximum velocity waveform used to calculate PI and RI.**
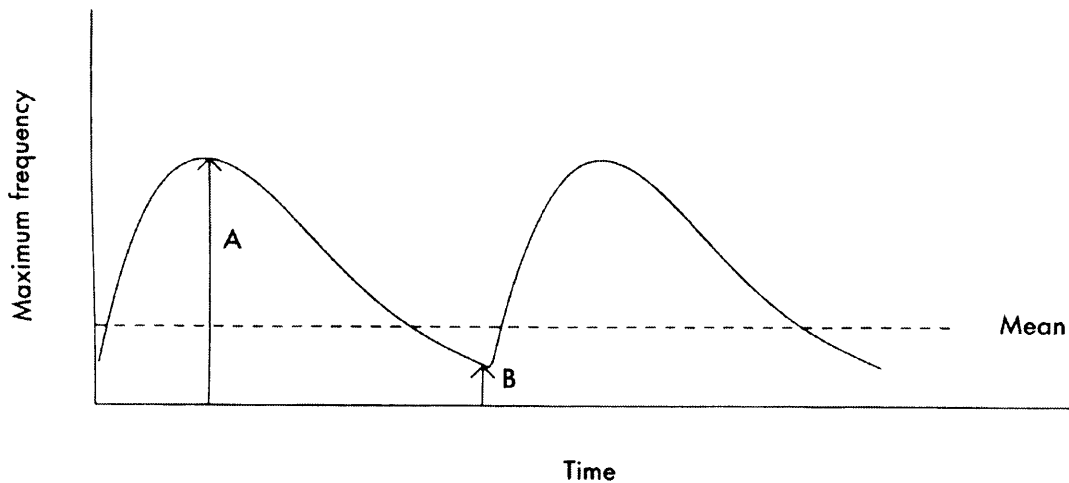
PI and RI provide a semi-quantitative assessment of vascular resistance in the vessels interrogated. For smaller vessels, both PI and RI are independent of the angle of insonation and do not require the vessel diameter. The advantages of PI are that abnormal waveforms can be identified with a high sensitivity and that small and tortuous vessels can be evaluated. Unlike RI, PI requires collection of the complete spectral waveform because it requires an estimate of the Mean Doppler shift frequency i.e. the averaging of all frequency shifts throughout the cardiac cycle. This is an advantage of the PI measurement, but it requires a computerised spectrum analyser to calculate the mean; modern systems have this facility incorporated in the equipment. It is important to use the same machine if repeated measurements are to be used for comparison and this requirement was met in the studies described in this thesis.

The movements of the Doppler shift frequency-time waveform correspond directly to measures of flow velocities that are of physiologic interest. In particular, the time averaged mean velocity, peak systolic velocity and the end diastolic velocity can be extracted from the velocity waveform (Fish, 1999). The peak systolic velocity (V_{max}) or the end diastolic velocity (V_{min}) are both useful for the diagnosis of carotid artery stenosis (Taylor et al, 1990). The ratio of velocities in different vessels provides a quantitative measure which allows for the wide differences in arterial velocities between individuals, which depends on factors such as ventricular contraction and arterial compliance, and can be used to compare flow between individuals (Taylor et al, 1990).

Limitations and sources of errors with conventional Doppler ultrasound

Although Duplex (spectral and colour) instrumentation is widely accepted as valid for most vascular applications, this method has disadvantages (Gill, 1985):

1) Causes of errors in estimating blood vessel resistance/velocity:

o Imaging not perpendicular to the longitudinal axis of the vessel.

o Pulsatility of blood flow, causing variation in vessel diameter with time.

o Only short length of vessel being available for imaging.

Thus high bifurcations and anatomic variations may result in sub-optimal results.

2) Sampling problems: All pulse mode Doppler machines provide centre-line measurements of peak velocity (Langlois et al, 1984). Abnormalities may not be detected due to failure to sample nearer the vessel wall where flow disturbances

are most likely to occur (Rittgers et al, 1983).

3) Operator errors:

o Excessive pressure of the transducer against the skin results in deformation of the vessel wall; superficial vessels are more prone than deeper vessels.

o Angle of insonation – referring to the equation in Section 3.4.1, it is evident that the angle of insonation affects the Doppler shift. When the ultrasound beam is at right angle to the vessel (cos 90° = 0), the Doppler shift frequency is theoretically zero and no signal is obtained. When the transducer is aligned with the vessel (cos 0° = 1), the largest Doppler shift is obtained. However, there is difficulty in obtaining signals at such low angles because of total reflection of sound waves at the vessel walls. For these reasons, angles between 30° and 60° are recommended (Stacey-Clear and Fish, 1984).

4) Patient-related factors:

o Any patient movement during measurement results in a blurred image, yielding inaccurate results.

o Carotid artery blood flow is influenced by noise (Gangar et al, 1991).

Doppler ultrasound has gained wide acceptance as a method of circulatory measurement in clinical practice and research. Ultrasound examinations, both grey scale and colour Doppler, can adequately detect the internal morphology and circulatory changes within the organ or vessel can be assessed. Although Doppler ultrasound is not a measure of

blood flow, it provides a measure of downstream vascular resistance, and is of great importance, both in the display of normal haemodynamics and in the recognition of vascular disease.

3.4.2 Assessment of mechanical properties of blood vessels

Knowledge of the elastic properties of the arterial wall is essential when investigating cardiovascular dysfunction since the function of the arterial wall is influenced by a combination of elastic and viscous components inherent to arterial tissue. All biological material exhibits a level of elasticity and in the case of blood vessels, the distension is not only influenced by the force applied by the blood flowing through the vessel but also by the elasticity of the constituents of the wall. Similarly, this elasticity also influences the rate at which the vessel regains its original shape. During systole, the increase in blood pressure causes the arterial wall circumference to increase and returns to its previous dimension during diastole. When intraluminal pressure changes are plotted against the changes in vessel diameter, the resultant curve forms a hysteresis loop.

Having identified the dynamic properties of blood vessels, it is important to identify potential indices which define the essential mechanical properties of blood vessels which influence their dynamic performance. Many indices have been derived to describe and quantify the physical behaviour of blood vessels in response to an intra-luminal force. This has lead to discussion as to the best indices for measuring arterial vascular properties leading to discrepancies in comparing results between studies. (Lehmann et al, 1996). The functional behaviour of the arterial system can be described in terms of compliance and distensibility, representative of the dynamic artery wall properties.

In 1960, Petersen and colleagues defined the elastic modulus E_p as an index of arterial stiffness, which describes the relationship of strain to intra-luminal pressure in an open-ended vessel. The original description referred to the change in vessel volume, but as the arterial lumen is generally circular in cross-section, the equation can be simplified to:

$$E_p(mmHg) = \left(P_s - P_d\right) \Big/ Strain$$

where strain is defined as the fractional pulsatile diameter change that occurs in an artery exposed to a given change in intra-luminal pressure:

$$Strain = \left(D_s - D_d\right) \Big/ D_d$$

Where D and P are vessel diameter and blood pressure and s and d denote systole and diastole respectively.

The inverse of Petersen's elastic modulus is known as cross-sectional or diametrical compliance (C) which is a fundamental property of the vessel wall, defined as the change in blood vessel diameter in response to change in pressure, and given by:

$$C(\% mmHg^{-1} x10^{-2}) = \frac{\left(D_s - D_d\right)}{D_d\left(P_s - P_d\right)} x10^4$$

Both E_p and C are useful indices of vascular distensibility and describe the response of vessel wall diameter to changes in blood pressure.

Kawasaki and co-workers (1987), recommended the stiffness index (β) as a useful parameter when comparing physiological changes in vessel wall between individuals. This is because it is less dependent on quantitative pressure change when describing the elasticity of an artery within physiological pressure ranges (Sonesson et al, 1993). Thus β may be the same for two individuals even though their respective blood pressures may be different. Conversely, their measured compliance values would not be similar, as compliance is a pressure dependent variable. β can be calculated as:

$$\beta = \frac{\left[\log_e\left(P_s \,/\, P_d\right)\right]D_d}{\left(D_s - D_d\right)}$$

Whilst mathematical definitions have been derived to describe the arterial physical properties, no consensus has been reached with regards to the definition and terminology best describing the mechanical vessel wall property (Lehmann et al, 1996). The diametrical compliance and stiffness index have been used to assess the vascular properties in this thesis, as diametrical compliance measures the percentage change rather than the absolute change in the viscoelastic properties of the artery and also provides a means by which arteries of varying diameters can be compared. The stiffness index is a calculation of distensibilty whose formula minimizes the influence of the systolic-diastolic pressure differential by logarithmic treatment of pressure factors and provides a more reliable measurement of stiffness of the arterial wall (Hirai et al, 1989).

Assessment of compliance using ultrasound

Over the past two decades, ultrasound has been the favoured method for assessing vascular diametrical compliance. This method requires assessment of the diameter of the

artery under investigation at the onset of the cardiac cycle, and the distension induced during the cardiac cycle by the local pulse pressure. In short, during vascular examination the artery of interest is imaged in B-mode in longitudinal section and the M-line positioned perpendicular to the vessel wall. The ultrasound system is then switched to M-mode. A radio frequency (RF) data acquisition system is linked to a computer onto which the M-mode echo images of the artery under investigation are displayed. The anterior and posterior walls of the vessel are identified either manually or automatically. The RF signals from the ultrasound M-mode output over a cardiac cycle are digitised and relayed to a wall tracking system (Hoeks et al, 1990; Figure 3.4). From these signals end-diastolic and end-systolic intraluminal diameters can be easily determined, and the maximum changes in diameter or distension for each beat. Blood pressure is measured non-invasively and the vascular compliance can be calculated according to the formula described previously.

Ultrasound has inherent inaccuracies related mainly to the recognition of the precise positions of the inner or outer walls of the blood vessel (Beach et al, 1989), producing an inter- and intraobserver variability error of 5% in measuring static diameter, and 10–15% when measuring pulsatile diameter changes (Hansen et al, 1993). Increased angle of insonation by the ultrasound transducer in the longitudinal axis also falsely increases vessel diameter and its changes. Due to the tortuous nature of some vessels, it is difficult to insonate exactly perpendicular to the vessel axis, which is a further source of error when measuring diameter and compliance in small calibre vessels. This uncertainty is

partially overcome by the use of intravascular ultrasound; however this technique has the

major drawback of being invasive (Hansen et al, 1993).

Figure 3.4   B-mode and M-mode image of the common carotid artery (A); typical radiofrequency signal acquired from the artery is analysed to locate and mark anterior (A) and posterior (P) luminal surfaces (B). Vessel distension over 4 cardiac cycles is displayed (C).
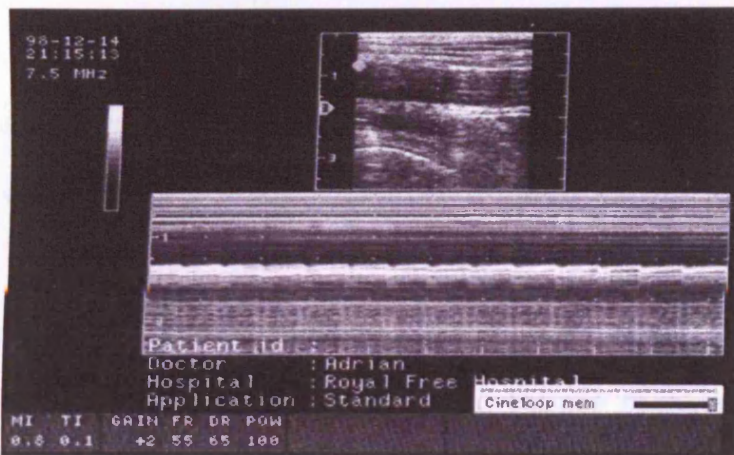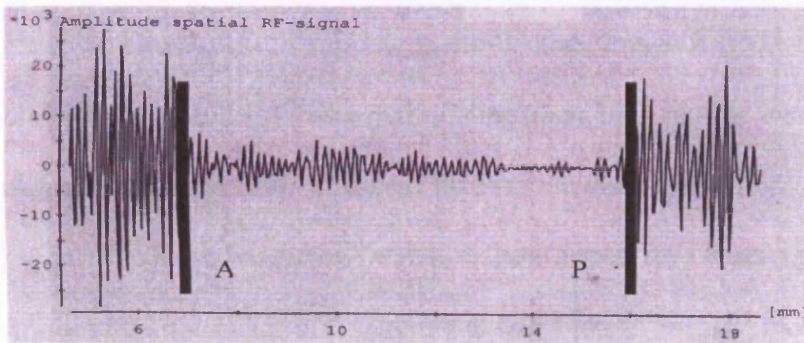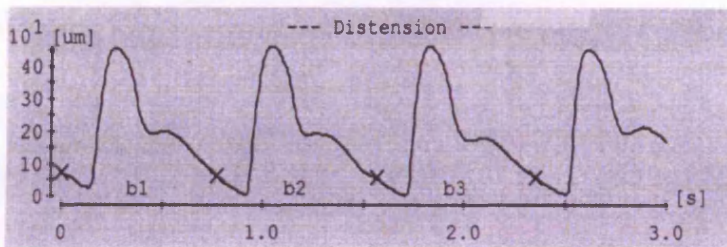
3.4.3 Principles of intima-media thickness measurement

Arterial wall structural changes are seen on ultrasound either as atherosclerotic plaques, found predominately at arterial bifurcations, or as diffuse thickening of the arterial wall. Both are measurable as increases in intima-media thickness (IMT) i.e. the distance between the lumen-intima interface and the media-adventitia interface. Ultrasound allows precise measurement of the IMT in large and medium sized peripheral arteries such as the femoral, carotid or radial arteries (Salonen et al, 1991). Due to the principles of diagnostic ultrasound, the measurement of the IMT thickness is most reliable at the far arterial wall, since the near field is often poorly resolved.

With improved resolution of ultrasound systems, assessment of the dimensions of small structures such as arterial wall thickness has become possible (Pignoli et al, 1986). Unfortunately however, the software which is essential to the running of commercial ultrasound equipment is poorly suited to the detection of small distances with high accuracy. To circumvent this problem, the B-mode image is digitised, transferred onto a personal computer and IMT assessed off-line using high quality image analysis software. Assessment of arterial wall thickness by B-mode ultrasound perpendicular to the vessel wall is possible since the lumen-intimal induces a relatively weak signal, while the media adventitia transition induces a relatively strong signal. Thus the media is seen as a dark, relatively hypoechoic layer between the intima and the adventitia. The transition between the adventitia and the surrounding tissue is unclear, as the connective tissues in which arteries are embedded are hyperechoic and exhibit high ultrasound signals. Therefore, not wall thickness, but only IMT can be obtained.

IMT is normally obtained only on the posterior wall, because the trailing end of the adventitia signal from the anterior wall partly obscures the media, preventing accurate measurement of the anterior wall IMT. The lumen-intima boundary has low reflectivity, but higher reflectivity than red blood cells. The media itself has a very low echogenicity, bounded by weak reflections from the intima and strong reflections from the media-adventitia transition, correlating to the double-line pattern seen on ultrasound. This appearance and IMT determined by B-mode imaging has been correlated with histological and pathologic measurements in the far wall of the artery (Pignoli et al, 1986; Linhart et al, 2000).

Intima-media measurement technique

The most common method for measuring IMT is based on high-resolution B-mode imaging. Repeated and averaged manual measurements are easy to perform (Tardy et al, 1992; Ferrara et al, 1994) but are operator dependent and have poor reproducibility. Modern analysis software assists automatic detection of wall thickness based on RF signals received along the M-line, analysing the grey scale of digitised images to allow accurate measurements with excellent reproducibility (Hoeks et al, 1997). This system requires horizontal visualization of the artery in longitudinal view, producing a well-defined double line pattern corresponding to the IMT (Figures 3.5 and 3.6). The operator defines the region of interest by placing the M-line accordingly, and IMT detection and measurement. Greyscale analysis is performed for each column of pixels along the M-line perpendicular to the vessel wall.

Figure 3.5   Longitudinal ultrasound image of the common carotid artery with the double-line pattern of the far wall corresponding to IMT
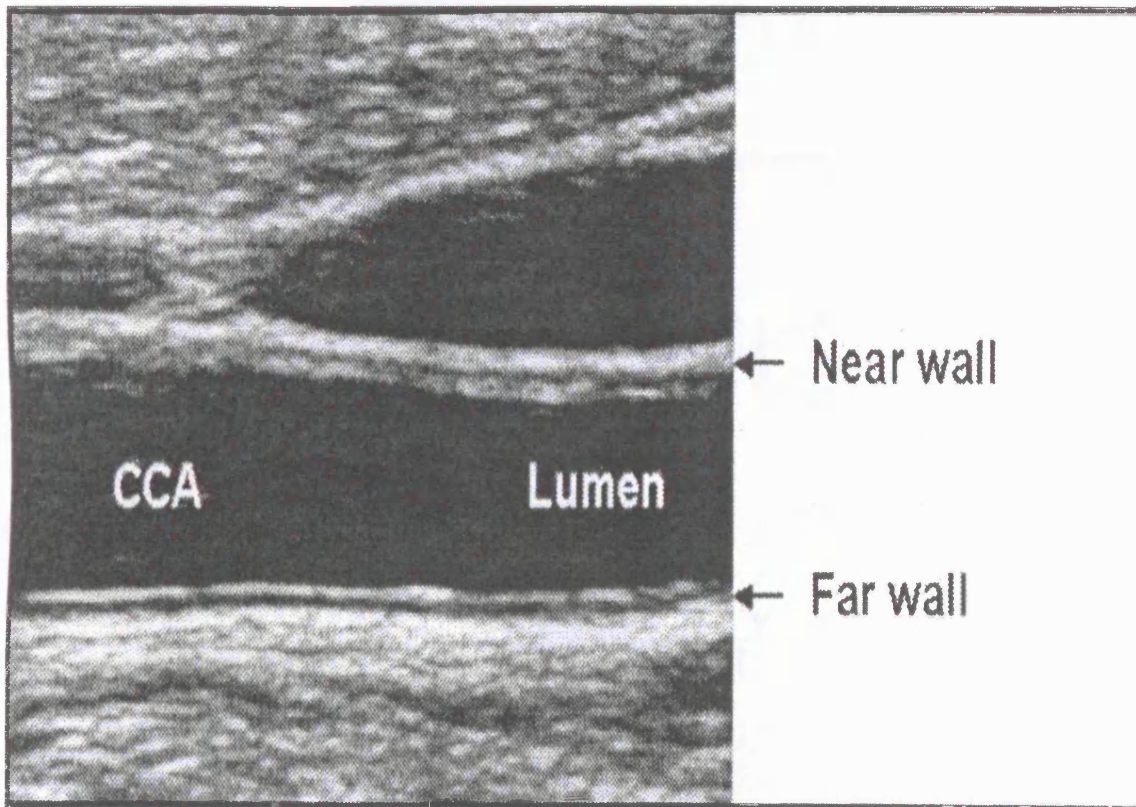
Figure 3.6   Anatomy of the carotid intima-media thickness (IMT). (Reproduced with permission from Linhart et al, 2000).

Anatomy   Sonography   Measurement

Valid   *Invalid*

Adventitia → ①
Media ②
Intima → ③
A
B
C
IMT

Lumen

D   D

Intima → ④
Media ⑤
Adventitia → ⑥
D
E
F
IMT
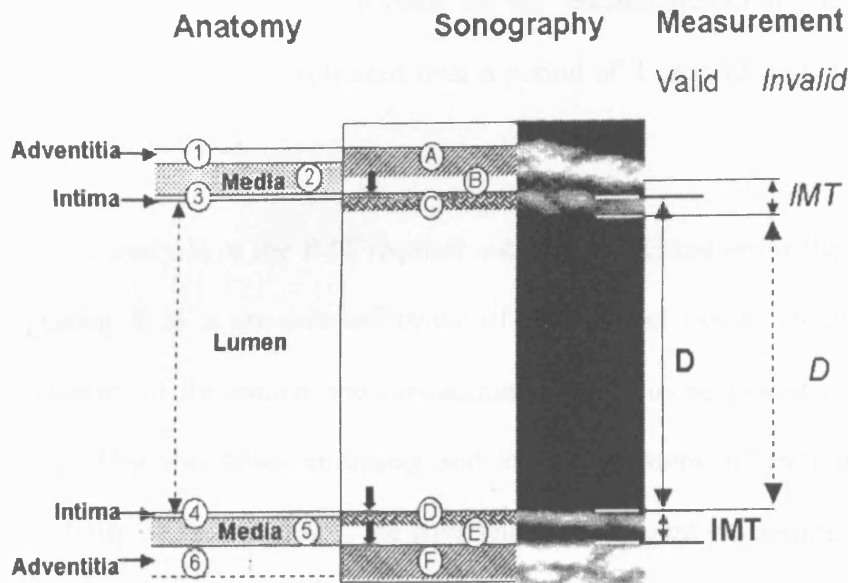
Near wall: The thickness of anatomical adventitia [1] is less than its ultrasound correlate [A]. In contrast, anatomical media [2] is thicker than its ultrasound image [B]. Ultrasound interface between media [B] and intima [C] corresponds to the anatomic boundary between [2 and 3]. Anatomic intima [3] is thinner than its ultrasound correlate [C] which is gain dependent.

Far wall: Ultrasound interface between the lumen and the intima [D on sonogram] corresponds to its anatomic correlate. The intima appears thicker on ultrasound [D] than anatomically. In contrast, the media appears thinner on ultrasound image [E]. The interface between the media [5] and adventitia [6] corresponds to [E and F] on sonogram. Because the interfaces between the lumen and the intima and between the media and the adventitia on the sonogram correspond to anatomy, ultrasound is accurate in measuring IMT.

By interpolation, a continuous curve is obtained from the histogram of greyscale density values. The curve is then analysed using a dedicated mathematical algorithm, which defines the exact position of lumen-intima and media-adventitia interfaces. As there are systolic-diastolic differences in the IMT, the M-mode is started synchronously with a trigger derived from the R-peak of the electrocardiograph followed by RF data acquisition; RF data is collected over a period of 4 seconds and stored on the hard disk for off-line analysis.

Off-line analysis of the IMT requires automated digitisation of the B-mode image, after capturing it in a pre-selected phase of the cardiac cycle. To delineate the different boundaries of the intima and media, markers had to be placed manually on the video image. This was time-consuming and introduced some subjectivity, dependent on the reflectivity of the structures, the gain setting and signal processing characteristics of the ultrasound system. The process is improved and speeded up by automated interpolation of the IMT (Figure 3.7). This improved the reproducibility of the procedure and was more user-independent.

Figure 3.7   Typical IMT measurement of the CCA IMT using Wall Tracking System
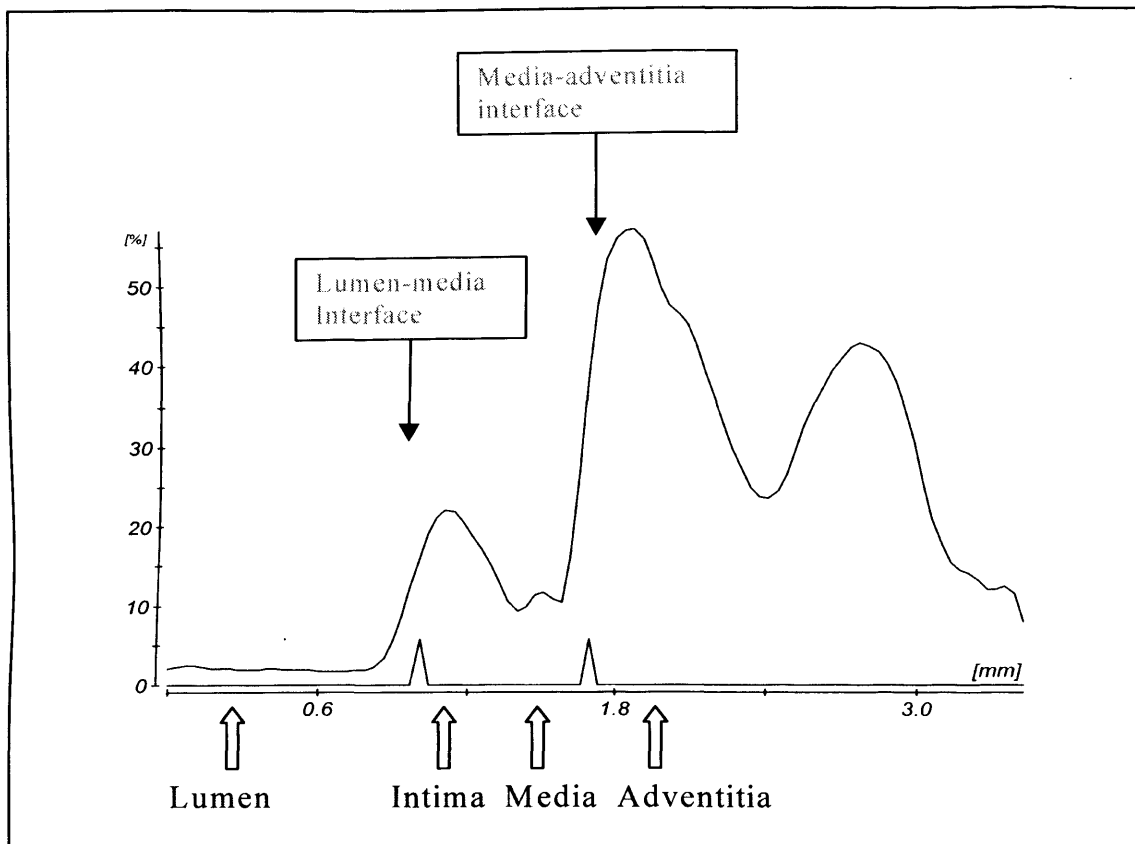
Ultrasound IMT assessment is a non-invasive, reproducible method for the detection of early arterial structural changes associated with various risk factors for atherosclerosis. Arterial wall thickening reflects the influence of multiple risk factors (age, blood pressure, blood lipids, diabetes, and gender) (Linhart et al, 2000) over time and has strong prognostic value for cardiovascular events such as myocardial infarction or stroke (Chambless et al, 1997; Bots et al, 1997; O'Leary et al, 1999). The ease and accuracy of

computer assisted IMT measurement make it a useful and practical marker for cardiovascular dysfunction related to early atherosclerotic disease processes.

3.4.4 Basics of Laser Doppler flowmetry and iontophoresis

Laser Doppler flowmetry

The laser Doppler technique measures blood flow in the very small blood vessels of the skin; tissue thickness of approximately 1 mm and the arteriole diameter of 10 microns (Figure 3.8). Cutaneous iontophoresis of pharmalogically active substances, with simultaneous laser Doppler measurement of dermal blood flux at the site of administration, is a promising non-invasive method for studying the physiology and pathophysiology of the skin microcirculation (Grossmann et al, 1995).

The technique of laser Doppler imaging is based on the frequency shift of low-powered red-laser light caused by moving red blood cells in the arterioles. Due to the minimal penetration of the laser it is used as an index of blood flow specific, uninfluenced by underlying muscle blood flow (Saumet et al, 1988). In this thesis, a 2.0 mW helium-neon laser was used to scan the surface of the skin and the back-scattered light from moving erythrocytes was recorded. The relative measure of volume flow is called laser Doppler flux, and is expressed in arbitrary perfusion units. Perfusion responses were stored on a personal computer and analysed off-line using dedicated software. The advantage of this method is that it provides a non-invasive, instantaneous and continuous measurement of microcirculatory flow offering advantages over the use of transdermal injections. The disadvantage is that it gives information about tissue perfusion only at a focal point.

Furthermore skin blood flow may artefactually be modified by the placement of laser probe on the skin surface.

Figure 3.8 Schematic representation of skin depth penetration with laser Doppler flowmetry (Reproduced with permission from the manufacturer's manual).
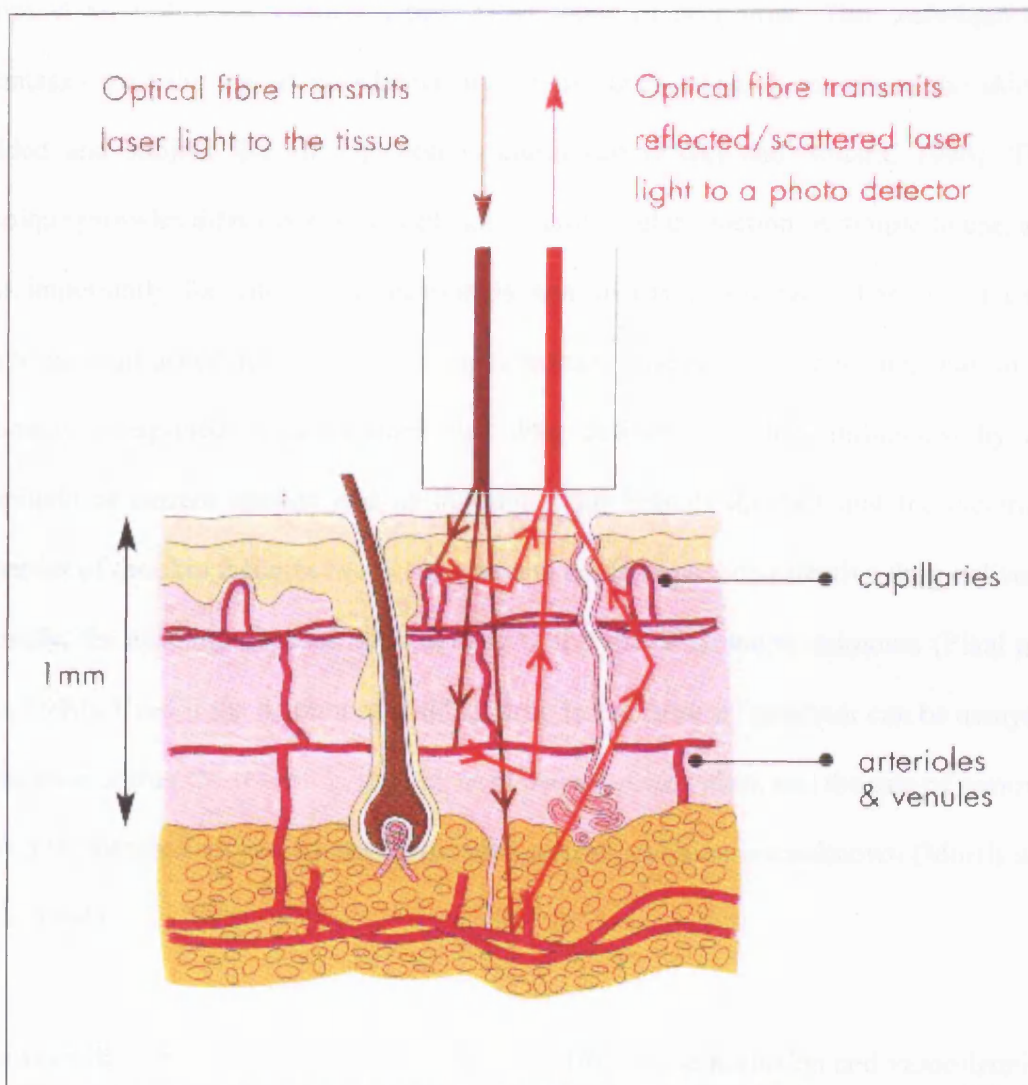
Iontophoresis

Iontophoresis is a non-invasive method of introducing charged substances across the surface of the skin by means of electric charge, allowing a known concentration of drug to be delivered to a local site on the skin (Harris, 1967). The extent of drug delivery is proportional to the magnitude and duration of current applied (Sanderson et al, 1987; Phipps et al, 1989), i.e. current x time is an index of drug dose. This technique has advantages over the use of intradermal injections, since injection trauma to the skin is avoided and subject fear of injection is eliminated (Sloan and Soltani, 1986). This technique provides direct assessment of skin microvascular function, is simple to use, and most importantly for clinical application is non-invasive. However, there are factors which may influence iontophoretic drug responses, and iontophoretic data has to be cautiously interpreted. It is assumed that drug delivery is solely influenced by the magnitude of current applied and its duration. This ignores the fact that the electrical properties of the skin differ between subjects and could impact on effective drug delivery. Secondly, the absolute concentration of drug at the site of action is unknown (Pikal and Shah, 1990). Even if the amount of residual drug in the delivery reservoir can be assayed, degradation within the reservoir, the extent of dermal penetration, and the rate of removal of drug by metabolism and by washout in the microcirculation are unknown (Morris and Shore, 1996).

It has been demonstrated that current alone may affect tissue perfusion and vasodilatation to vehicle has been observed at both anode and cathode, although the increase in erythrocyte flux at cathode has been reported at low current levels than that at the anode

(Morris et al, 1995). The major pathway of iontophoretically applied current across the skin is through areas of low electrical resistance such as sweat ducts, hair follicles and broken skin (Harris, 1967). Sweat glands also contain vasoactive substances such as kinins (Renolds et al, 1991), which may possibly be iontophoresed along with the substances being tested. Transcutaneous nerve stimulation has also been reported to stimulate the skin (Wallengren et al, 1987). The combination of the above effects of iontophoresis could account for the observed response to vehicle alone (Berghoff et al, 2002). However, the use of saline virtually eliminated electrically induced artefact in most subjects (Ferrell et al, 2002). Furthermore, skin blood flow may easily alter by the placement of laser probe on the surface of the skin if any pressure is exerted.

3.4.5 Animal model for PCOS

Considerable species differences exist between human and experimental animals and thus the results from studies in animal models and human disease must be cautiously interpreted. Ideally, an animal model for investigating the vascular changes should have all or as many as possible of the features seen in a woman with PCOS. Such features include distinct ovarian histological appearance together with biochemical features. A number of studies have therefore been attempted to induce a PCOS-like state in rat model, as shown in Table 3.3. As seen in Table 3.3, a fully convincing animal model of PCOS, as a whole, has not been established perfectly.

Table 3.3   Characteristics of rat models for Polycystic Ovary Syndrome

Method	Ovarian Morphology	LH	FSH	Testosterone	Insulin
Exposure of constant light (Singh, 1969; Daane & Parlow, 1971; Campbell & Schwarts, 1980	Polyfollicular	=	?	?	Not done
Neonatal androgenization (Barraclough & Gorski, 1961; Goldzieher & Axelrod, 1963; Lookingland et al, 1982; Jones et al, 1987	Polyfollicular	=	=	=	Not done
HCG treatment to hypothyroid rats (Adams & Sensemann, 1976)	Polycystic	X	=/↓	↑	Not done
Chronic oestrogen exposure (Brawer et al, 1986; McCarthy & Brawer, 1990)	Polycystic/macrocysts	↓	↑	Not done	Not done
DHEA treatment (Knudsen et al, 1975; Mahesh et al, 1987)	Polyfollicular polycystic?	↓	=/↑	X	Not done
Mifepristone (Van den Schoot et al, 1987; Sanchez-Criado et al, 1992; Ruiz et al, 1997)	Polycystic	↑	=/↓	↑	↑
hCG and Insulin treatment (Poretsky et al, 1992)	Polycystic	X	=/↓	↑	↑

(Abbreviations: DHEA – dihydroepiandrosterone, HCG – human chorionic gonadotrophin, LH – luteinising hormone, FSH – follicle stimulating hormone, ↑– increased, ↓– decreased, = no change, X – exogenous administration)

With exposure to constant light and neonatal androgenisation, follicles grow to preovulatory size and become atretic, hence the term polyfollicular. With single injection of oestrogen or insertion of oestradiol implants induces PCO, the LH levels were relatively low. Similarly, daily injections of DHEA produce PCO, but the LH levels were suppressed with increased FSH levels. On the contrary, in the presence of elevated basal levels of LH (hCG treatment and with mifepristone), preovulatory follicles become luteinised, but ovulation does not occur. For the purpose of this thesis administration of mifepristone to cyclic rats has been used to 'create' an animal model because of its similarities with PCOS women (endocrine and ovarian morphology)

Action of mifepristone

It has been reported that mifepristone (also known as RU 486) binds to the progesterone receptor, but does not activate the receptor and therefore acts as a progesterone antagonist (Baulieu, 1991). However, in the rat daily injections of mifepristone induce (Figure 3.9):

1. Ovulatory failure, persistent vaginal cornification, and enlarged ovaries containing follicular cysts (Baulieu, 1991, Sáchez-Criado et al, 1993a).

2. Increased serum concentrations of LH, testosterone, oestrogen and prolactin and decreased levels of FSH (Sáchez-Criado et al, 1992b, Sáchez-Criado et al, 1993b).

3. Increased levels of insulin-like growth factor 1 (Ruiz et al, 1997).

Figure 3.9 The effects and interrelation of mifepristone on serum hormone levels and ovarian follicular development in the rat
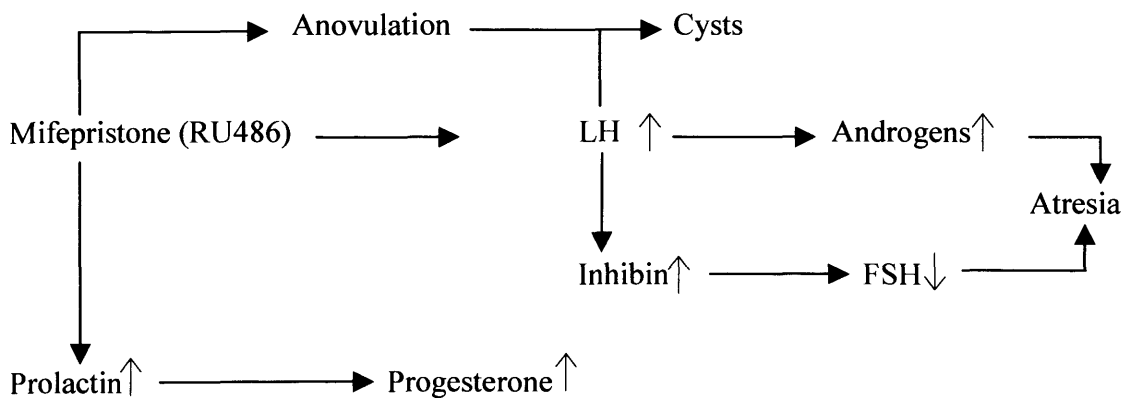
In comparison with the ovaries of control rats, ovaries of rats treated with mifepristone show (1) arrest of follicular growth and increased rate of follicular atresia, (2) increased number of follicular cysts with differing degrees of luteinisation and increased size of corpus luteum, and (3) abundant interstitial glands in the ovary (Sanchez-Criado et al, 1990).

3.5 Endothelium, nitric oxide and vascular disease

Endothelium is a continuous, thin single layer of polygonal flat endothelial cells $\approx$ 0.2 – 0.3 μm thick (except over the bulging nucleus), totaling several hundred grams in an adult, lines the lumen throughout the circulatory system. The enormous area of inner, luminal surface enables endothelium to interact effectively with plasma, white cell and

platelets. Similarly, the enormous area of the outer, abluminal surface enables endothelium to interact effectively with the tissues of the body (Levick, 2000).

The functional importance of the endothelium was suggested by Furchgott and Zawadski (1980), who showed that it is not only the inner lining of the blood vessels, but it also releases humeral factors, which control vessel wall contraction and relaxation, fibrinolysis and platelet activation. Thus the endothelium contributes not only to blood flow but also to vessel patency. Impaired endothelial function is likely therefore to contribute substantially to cardiovascular disorders such as atherosclerosis.

3.5.1 Physiology of the endothelium

Stimulation of intact endothelial cells by neurotransmitters, hormones and shear forces generated by the circulating blood, causes the release of a substance that induces relaxation of the underlying vascular smooth muscle. This substance, the endothelial-derived relaxing factor (EDRF), was found to be highly unstable with a half-life of seconds. In the late 1980s, two different research groups suggested that EDRF was nitric oxide (NO) because the two compounds had similar biological properties (Palmer et al, 1987; Ignarro; 1989).

NO has an intravascular half-life of about 2 ms and an extra-vascular half-life of less than 2 s. It must therefore be generated continuously to be effective. NO is produced from the amino acid L-arginine, which enters the cell via an amino-acid transporter in the cell membrane. NO is cleaved from L-arginine by the enzyme nitric oxide synthase (NOS).

Three isoforms of NOS have been described, endothelial NOS (eNOS), neural NOS (nNOS) and inducible NOS (iNOS); two (eNOS and nNOS) are calcium dependent. The rate of production of NO in blood vessels is determined by the activity of eNOS, which is regulated by humoral factors and shear stress on the vessel wall, via their effects on changes in intracellular calcium (Ca^{2+}) concentration (Channon et al, 2000).

1. Humoral factors: eNOS is bound to the inner cell membrane and activated by calcium-calmodulin, a complex that is formed when cytosolic Ca^{2+} increases and binds to protein calmodulin. Substances such as acetylcholine, bradykinin, thrombin, insulin, testosterone and histamine raise endothelial cytosolic Ca^{2+} concentration and enhance eNOS activity, and ultimately NO production and vasodilation. The rise in cytosolic Ca^{2+} and NO production is by a dual process: (a) humerol factors activate the receptor-operated cation-channels, which allows influx of extracellular Ca^{2+} ions into the cell; (b) in addition humerol factors liberate intracellular Ca^{2+} store. In the latter case, the agonist-receptor complex activates a membrane-bound enzyme called phospholipase C, which catalyses the breakdown of phosphatidyl inositol biphosphate (PIP_2) to form an intracellular 'second messenger', inositol triphosphate (IP_3) and IP_3 activates calcium release channels in the membranes of intracellular Ca^{2+} reservoirs, inducing release of stored Ca^{2+} and an elevation of intracytoplasmic Ca^{2+} concentration.

2. Shear stress: Shear stress, the mechanical force tangential to the endothelial luminal surface, is considered to be the most important stimulus, normally, of eNOS activity increase intracellular Ca^{2+} and thus a continuous production of NO.

3.5.2 How does NO cause vasodilation

NO diffuses from the endothelium into the vascular smooth muscle, where it has two actions, (1) NO combines with the haem group in the enzyme guanylate cyclase, activating the enzyme, which catalyses the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The precise mechanism by which cGMP relaxes vascular smooth muscle is unclear. It is suggested that cGMP activates protein kinase G, which increases calcium entry due to the stimulation of Ca^{2+}-ATPase pump. (2) An alternative proposal suggests that hyperpolarization, increases the probability of the open state for voltage-gated Ca^{2+} channels, leading to an increase in Ca^{2+} concentration, and the sensitivity of the contractile process to Ca^{2+} is reduced because protein kinase A phosphorylates myosin light chain kinase (MLCK), inhibiting its action on the vascular smooth muscle (Levick, 2000; Figure 3.10).

Figure 3.10   Generation and action of nitric oxide (Reproduced with permission from Levick J.R, 2000).
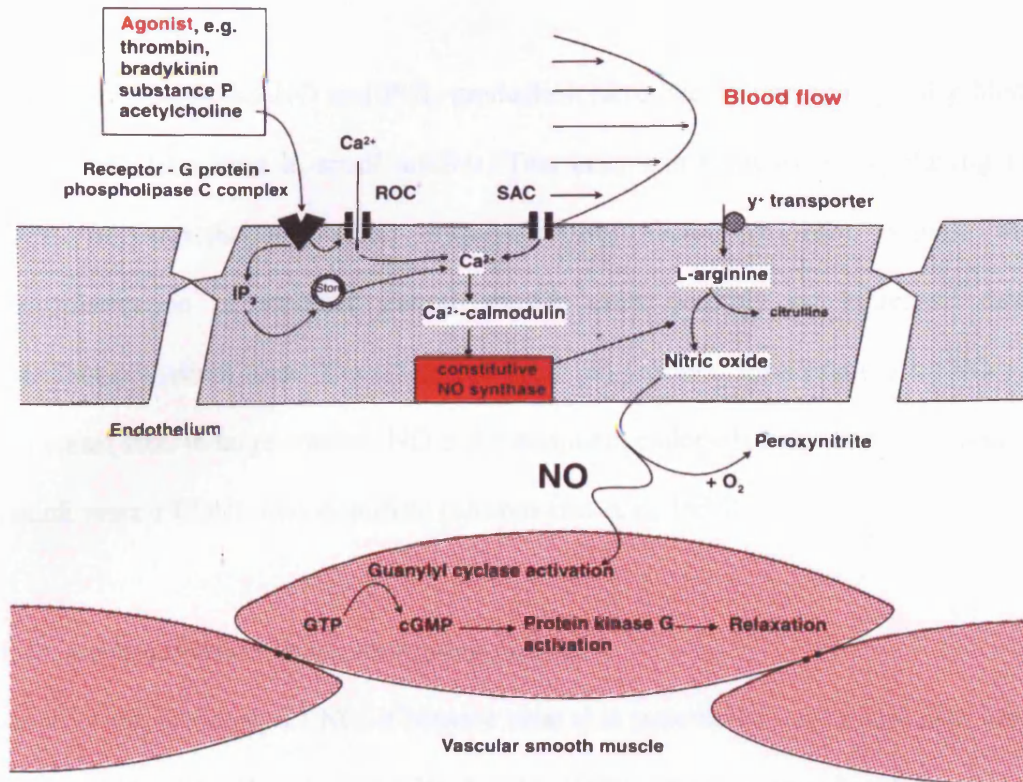
Endothelial cells also release prostacyclin (PGI_2) in response to shear stress, hypoxia and in the presence of substances such as acetylcholine (ACh) and serotonin, which also stimulate the release of NO. Prostacyclin is produced from arachidonic acid by the enzyme cyclo-oxygenase. PGI_2 causes vascular smooth muscle relaxation by activating adenylate cyclate and increasing the production of cyclic 3', 5'-adenosinemonophosphate

(cAMP) from adenotriphosphate and causing a decrease in intracellular Ca^{2+} ion concentration which leads to vascular smooth muscle relaxation. In most blood vessels, the contribution of PGI_2 to vascular tone is limited, and its effect is essentially additive to generally more substantial influence of NO (Levick, 2000).

Even when endothelial NO and PGI_2 production have been pharmacologically blocked, there is still relaxation in small arteries. This occurs in response to a relaxing factor known as endothelium-derived hyperpolarizing factor (EDHF), which causes hyperpolarization of vascular smooth muscle cells possibly by increased calcium dependent-potassium ions. The effect of EDHF on vascular tone relative to NO varies with vessel size. In large arteries, NO is the dominant endothelial-derived vasodilator, but in small vessels EDHF may dominate (Shimokawa et al, 1996).

3.5.3 Endothelium-derived-contracting factors

Soon after the discovery of NO, it became clear that endothelial cells could also mediate vascular contraction (Luscher and Vanhoutte, 1990). These contracting factors include endothelin and prostaglandin.

Endothelin (ET) is a 21 amino acid peptide which is found in three isoforms of endothelin, ET-1, ET-2 and ET-3. ET-2 and ET-3 differ from ET-1 in amino acid residues two and six respectively. Only ET-1 is synthesised by human endothelial cells (Masaki, 2000) and it is released soon after synthesis rather than being stored intracellulary. ET-1 is liberated from the basal surface of the endothelial cell and binds to

114

receptors, ET_A and ET_B on the vascular smooth muscle cells. Although structurally highly similar, ET_A has a higher affinity to ET-1 and ET-2 than ET-3; whilst ET_B binds all three peptides with equal affinity and is also located on the surface of the endothelium. These receptors trigger the opening of receptor-operated Ca^{2+} channels, allowing the influx of calcium ions. ET-1 binding also activates phospholipase C, stimulating IP_3 synthesis, and causes the release of intracellular Ca^{2+} stores. The rise in intracellular Ca^{2+} ion concentrations stimulates smooth muscle contraction resulting in vasoconstriction (Masaki, 2000).

Prostaglandin (PG-H_2) is produced by the endothelium and synthesized from arachidonic acid via the enzyme cyclo-oxygenase. Prostaglandin activates the thromboxane receptors in vascular smooth muscle and counteracts the effects of NO and prostacyclin. In addition, the cyclooxygenase pathway produces superoxide anions (formed by the reduction oxygen molecule–O_2^-), which counteracts the effects of NO and PGI_2 causing vasoconstriction (Rubanyi and Vanhoutte, 1986; Luscher and Vanhoutte, 1990).

3.5.4 Cardiovascular risk factors and endothelial dysfunction

Endothelial dysfunction has been defined as impairment in the ability of vascular endothelium to stimulate vasodilatation, causing changes in endothelial cell morphology, which are associated with functional alterations and intimal thickening (Li and Forstermann, 2000). Endothelial dysfunction precedes structural vascular alterations, indicating a protective role for the functionally intact endothelium. It is therefore possible that a deficiency in local endothelial NO production and availability could be a pathway

that accelerates atherogenesis in humans. There are two general mechanisms by which endothelial NO deficiency might occur in endothelial dysfunction (John and Schmieder, 2000). Firstly, the synthesis of endothelial NO may be diminished due to reduced expression or activity of eNOS (Luscher and Barton, 1997). Secondly, the breakdown of endothelium eNOS-derived NO may be increased by production of superoxide, reducing NO bioactivity and producing peroxynitrite, which readily oxidises lipids and damages the cell membrane (Channon et al, 2000).

Dyslipidaemia

Hypercholesterolaemia and atherosclerosis are associated with impaired endothelium-mediated vasodilatation (Tanner et al, 1991). Clinical studies have shown that patients with dyslipidaemia may have endothelial dysfunction, even in the absence of angiographically detectable disease. In coronary arteries, there is evidence of a direct relationship between serum cholesterol levels and the response to ACh infusions, with greater vasodilatation at lower cholesterol levels (Vita et al, 1990). Small LDL particles are particularly atherogenic since they accumulate in the subendothelial space and impair endothelium-dependent vasodilatation (Dyce et al, 1993), possibly due to increased levels of superoxide radicals (Drexler and Hornig, 1999) and the resultant quenching of endothelium-derived NO signaling.

Endothelial dysfunction is more extensive in advanced stages of atherosclerosis due to impaired bioavailibility of endothelial NO, resulting from reduced synthesis, increased breakdown (Shimokowa et al, 1989) and enhanced production of superoxide (Shimokowa

and Vanhoutte, 1989; Minor et al, 1990). There is some evidence from studies in the aorta of hypercholesterolaemic rabbits that the overall production of NO is not reduced but rather augmented; however, increased production of NO is inactivated by superoxide radicals produced within the endothelium (Minor et al, 1990). Similar observations have also been reported in rabbits with fully developed atherosclerosis (Minor et al, 1990). Thus, conditions of hypercholesterolaemia and atherosclerosis, biologically active NO concentrations are markedly reduced, as suggested by bioassay experiments with coronary arteries from hypercholesterolaemic pigs (Shimikova and Vanhoutte 1989).

Endothelin production is also increased in atherosclerotic vascular disease (Lerman et al, 1991), though the expression of endothelin receptors is down regulated (Winkles et al, 1993). A likely stimulus for the increased endothelin production is LDL, which increases endothelin gene expression and endothelin release from porcine and human aortic endothelial cells *in vitro* (Boulanger et al, 1992); vascular smooth muscle cells that migrate into the intima during the atherosclerotic process also produce endothelin, causing vasoconstriction (Hahn et al, 1990).

Hypertension

Endothelial dysfunction in hypertension may contribute to an increased vascular resistance or to other vascular complications (Luscher and Barton, 1997); high BP is associated with reduced endothelium-dependent relaxation (Luscher and Vanhoutte, 1990). This increase in resistance is due to increased media thickness:lumen diameter, thereby narrowing blood vessels that control vascular resistance (Agabiti et al, 1995). The

mechanism of endothelial dysfunction however differs in various models of hypertension. In the spontaneously hypertensive rat model of genetic hypertension, the activity of the eNOS is increased but is ineffective, probably due to increased inactivation of NO by superoxide (Nava et al, 1995). In contrast, salt-induced hypertension is associated with a marked impairment of eNOS (Hayakawa and Raiji, 1997). Furthermore, studies of the human vasculature in hypertensive patients have demonstrated increased changes in the arterial wall architecture

Diabetes

Diabetes is a risk factor for coronary artery disease, and although patients with diabetes are free of angiographic evidence of atherosclerosis, these patients have abnormal responses to ACh compared with controls (Nitenberg et al, 1996). Other studies have reported endothelial dysfunction in the skin microcirculation in response to endothelium-dependent and–independent vasodilator stimuli in patients with non-insulin-dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) (Williams et al, 1996; Timimi et al, 1998). The underlying mechanism is not clear, but may involve increased synthesis of endothelin and/or impairment of the L-arginine-NO pathway (Yamaguchi et al, 1990), and an increased *in-vitro* expression of NO synthase and the production of superoxide ions (Cosentino et al, 1995).

CHAPTER 4

MATERIALS AND METHODS

4.1 Introduction

A variety of techniques are currently employed to evaluate arterial mechanical properties, however, all have theoretical, technical and practical limitations that impact on their widespread application in the clinical setting. This chapter summaries patient selection/exclusion criteria, physical data, endocrine/biochemical parameters measured and techniques used in this thesis to assess the arterial wall mechanics in women with PCOS.

4.2 Subject selection

Three groups of women aged 18–35 years were recruited from the North Middlesex and the Royal Free Hospitals:

1. Women attending the Reproductive Endocrinology & Gynaecology clinics, with PCOS confirmed by the presence of bilateral polycystic ovaries on TVS (fulfilling the criteria of Fox et al, 1991) and clinical features of menstrual irregularity, infertility, oligomenorrhoea (intermenstrual interval > 35 days) and / or hirsutism (Ferriman and Gallwey (1961) score of > 7).

2. The PCO group was recruited from over 250 asymptomatic (regular menstrual cycles and no hirsutism or acne) women, attending the family planning clinics at the North Middlesex and a neighbouring hospital (St Ann's), between 1998 and 2002. These women attended for localisation of intrauterine contraceptive devices by ultrasound examination and the presence of PCO morphology was also ascertained All those with PCO morphology, defined according to the criteria described by Fox et al, 1999, were asked to participate in the studies; no exclusion criteria were applied other than those stated in Section 4.2.

3. Control group were staff members – radiographers, student midwives and nurses and medical students, with normal ovaries on TVS, no clinical features of hyperandrogenemia (hirsutism or acne), regular menstrual cycles (cycle length of 21–35 days). None of the controls had sought treatment for menstrual disturbance, infertility or hirsutism at any time. No controls who agreed to participate were rejected except on the basis of the exclusion criteria.

One member of staff who agreed to participate in the control group had PCO morphology on TVS with no other clinical or biochemical features of the syndrome. She agreed to be part of the PCO group but had to withdraw due to job relocation.

The same women from the first clinical study were not included in the subsequent studies as many were not traceable due to their relocation. However, the selection criteria described above was followed throughout. Although the controls were not matched with PCOS women for BMI, there is a correlation with respect to BMI and PCOS and control women in all the clinical studies.

Women who smoked, had diagnosed respiratory or cardiovascular disease, or were taking prescribed medication such as oral contraceptives or aspirin, which could influence vascular resistance, were excluded. All measurements were recorded 6 hours post–prandially, between days 4 and 7 in oligomenorrhoeic and control women; there was no special timing for amenorrhoeic women. Ethical committee approval was obtained for the studies and informed consent was obtained in writing from each subject.

Height (m) and weight (kg) measurements were used to calculate the BMI. The minimum waist measurement between the costal margin and the pelvic brim, and the maximum hip measurement at the level of the greater trocanters, were used to calculate the W/H.

The degree of hirsutism was assessed according to the Ferriman and Gallwey score (1961). The Ferriman and Gallwey model quantitates the extent of hair growth in nine anatomic sites (upper lip, chin, chest, higher back, lower back, higher abdomen, lower abdomen, arms, thighs/legs) and graded from scale 0 (no terminal hair) to 4 (maximal growth) for a maximum score of 36. A score of > 7 or more indicates the presence of androgen excess.

Pulse and blood pressure were measured on the left arm in each women, in all clinical experiments, using an automatic Dinamap device (Critikon Inc, Tampa, FL, USA), at 2 minute intervals until the pulse rate varied by less than 5 beats/min and systolic and diastolic blood pressures by less than 5 mmHg over two readings.

Since carotid artery blood flow is influenced by auditory stimulation-noise (Gangar et al, 1991), subjects were exposed to white noise, 50dB above the hearing threshold, via occlusive headphones (Kamplex Diagnostic Audiometer; P.C. Werth Ltd, London UK), worn throughout the procedure.

4.3 Pilot study

A preliminary pilot study was performed for all experiments, prior to main data collection to test the proposed methods and research tools. The results from this pilot study were not included in the main study. The pilot studies served as a means of familiarising with the equipment and analysis package on the ultrasound machines, the audiometer and the continuous pulse and blood pressure machine. Furthermore, the results of these initial studies indicated that the method was appropriate. In addition, the patient information sheet was discussed with the subjects and revised to include a statement of confidentiality and details about the length of time in the hospital.

A justifiable criticism of the study protocol is that it was not possible to assess intra-observer variability especially for the first clinical study as a second trained observer was not available at the time of the study i.e. not trained in vascular studies.

In the assessment of diametrical compliance, stiffness index and IMT, the prime source of measurement error would result from the different abilities of the observers in accurately visualising the correct arterial segment, properly aligning the linear array at right angles to the axis of flow, and minimizing probe movement during vessel wall motion capture. During the initial trials of the wall tracking system, the degree of agreement between observers measuring diametrical compliance, stiffness index and IMT in the same sitting was less than that for consecutive measurements made by a single observer on the same individual (personal communication Tai, N.R. and Cheng, K.S.). It was therefore decided

to concentrate on inter-observer variation as it would be the main contributor to observer error in the technique used.

The protocol for laser Doppler and iontophoresis was agreed between myself together with another researcher and was validated to account for intra-observer variation. There was no difference with respect to erythrocyte flux between left and right arm and therefore one side was assessed.

Due to ethical issues, it was not possible to validate the organ bath technique using thoracic aorta from mifepristone treated animals. Organ bath equipment was validated using human uterine artery and rat aorta obtained from an ongoing research project in the department.

4.4   Ethical implications

This research project involved examination on human subjects, resulting in no immediate direct benefit to women in the study. Although the investigation was relatively non-invasive with no known side effects, approval of the Local Ethics Committee was obtained.

A patient information sheet was provided. Participation was voluntary and informed consent was obtained in writing, after the study had been fully explained. Subjects were informed that they could withdraw at any stage without prejudice.

4.5 Biochemical assays

Biochemical analyses were performed by the Departments of Biochemistry at the North Middlesex and Royal Free Hospitals. Venous blood samples were drawn from an antecubital vein after ultrasound examination (before 10.00 hours in all subjects). Samples were collected in plastic sterile tubes, coded to ensure anonymity, centrifuged and serum stored at $-20^{0}C$ until assay.

North Middlesex Hospital:

Serum levels of LH, FSH, testosterone, oestradiol and prolactin were determined by ACS 180 Chemiluminesce based kits (Ciba-Coring Diagnostics Ltd, Essex, UK) with intra-batch COV of 5.0–8.0% and inter-batch COV of 2.1–7.0%. Serum SHBG was determined using the Chemiluminescent Immulite kit (Diagnostic Products Corporation, LA) with an intra-batch COV of 4.0–6.6% and inter-batch COV of 2.3–5.3%.

Serum concentrations of total cholesterol, HDL and triglyceride were measured using Enzymatic Calorimetric tests (Boehringer Mannheim Immunodiagnostics Systems, UK) with intra-batch COV of 4.0–7.0% and inter-batch COV of 1.1–2.8%. Serum glucose and fasting serum insulin were measured using an automated ELISA ES700 technique (Boehringer Mannheim Immunodiagnostics Systems, UK) with intra-batch COV of 1.1–2.5% and inter-batch COV of 1.3–1.6%.

Royal Free Hospital

Serum levels of LH, FSH, testosterone, oestradiol, prolactin and SHBG were determined by electrochemiluminescence immunoassay (ECLIA) using an E170 analyser (Roche

Diagnostics Corporation, Indianapolis, USA) with intra-batch COV of 3.0–5.0% and inter-batch COV of 1.1– 4.5% .

Serum concentrations of total cholesterol, HDL and triglyceride were measured using an Enzymatic Calorimetric test on a Roche 704 analyser (Roche Diagnostics Corporation, Indianapolis, USA) with intra-batch COV of 2.0–3.0% and inter-batch COV of 1.7–2.4%. Serum insulin was measured using a Microparticle Enzyme Immunoassay (Abbott, Diagnostics, AxSYM System, Japan) with intra-batch COV of 2.0–2.9% and inter-batch COV of 2.9–4.1%; serum glucose was determined using an enzymatic test on a Roche 704 analyser (Roche Diagnostics Corporation, Indianapolis, USA) with intra-batch COV of 1.8 – 2.1% and inter-batch COV of 0.7 – 1.0%.

The free androgen index (FAI) was calculated as (testosterone/SHBG) x 100, which provides an estimate of the active free testosterone in serum. Ratio between fasting plasma insulin (mU/l) and fasting plasma glucose (mmol/l) was used to assess insulin resistance. Serum LDL concentration is calculated according to the formula:

LDL concentration = Total Cholesterol (HDL + Triglyceride / 2.2)

4.6   Scanning protocol

Scans were performed by myself in all the experiments. All subjects rested for 15 min in supine position to allow pulse and blood pressure to stabilise before any measurements were taken. To avoid unnecessary patient movements, a clear and concise explanation of the procedure was given beforehand.

o Vascular resistance and vascular reactivity were assessed using a Toshiba Eccocee SSA 340 ultrasound machine with a 7.5 MHz linear probe and colour Doppler facility. For this experiment the PI, V_{max} and back pressure were recorded and calculated as these parameters are good indicators of a measure of downstream vascular resistance especially in low resistance vascular beds (Taylor, 1990; Gosling et al, 1991; Fish, 1999).

In order to minimise measurement errors care was taken with the placement of the transducer, avoiding excessive pressure against the skin which may result in the deformation of the vessel; more important of the superficial CCA. To further reduce this error, the CCA was scanned using the sternomastoid muscle as a stand-off. As the Doppler shift frequency is proportional to the angle of insonation, an angle of 60° was used in all subjects.

o The viscoelastic properties and IMT were assessed using a Pie 35 machine (Pie Medical Systems, Maastricht, Netherlands) with 7.5 MHz linear probe and colour Doppler facility with signal output to a high resolution wall tracking system (Wall Track, Pie Medical Systems, Maastricht, Netherlands).

As previously described in Section 3.4.2, the diametrical compliance (C) and stiffness index (β) were used to assess the vessel wall properties in the three groups since these two parameters are less pressure dependent. Due to reduced ultrasound resolution of the

anterior vessel wall, the IMT was determined in the far wall and an average of three readings taken within 2 cm from the carotid bulb in all subjects.

o   Iontophoretic drug delivery

After the 15 min acclimatisation period, two perspex iontophoresis chambers (ION1, Moor Instrument Ltd, Axminster, UK) were attached 5.0 cms from the medial condyle with at least 10.0 cm between them on the right forearm (Figure 4.1) by means of double-sided adhesive rings avoiding hair, broken skin, and superficial veins for the reasons given before (Section 3.4.4). 0.25 ml of 1% acetylcholine chloride (ACh) (Sigma Chemicals Ltd, UK) was placed in the anodal chamber and 0.25 ml of 1% sodium nitroprusside (SNP) (Sigma Chemicals Ltd, UK) in the cathodal chamber. A laser Doppler probe was positioned through the centre of each chamber. The vehicle for both drugs was normal saline and mixed prior to administration; sodium nitroprusside was kept in the dark. Skin blood perfusion was measured using a double chamber laser Doppler perfusion monitor (Laser Doppler DRT4 system, Moor Instrument Ltd, Axminster, UK).

Figure 4.1   Laser Doppler Iontophoresis set-up
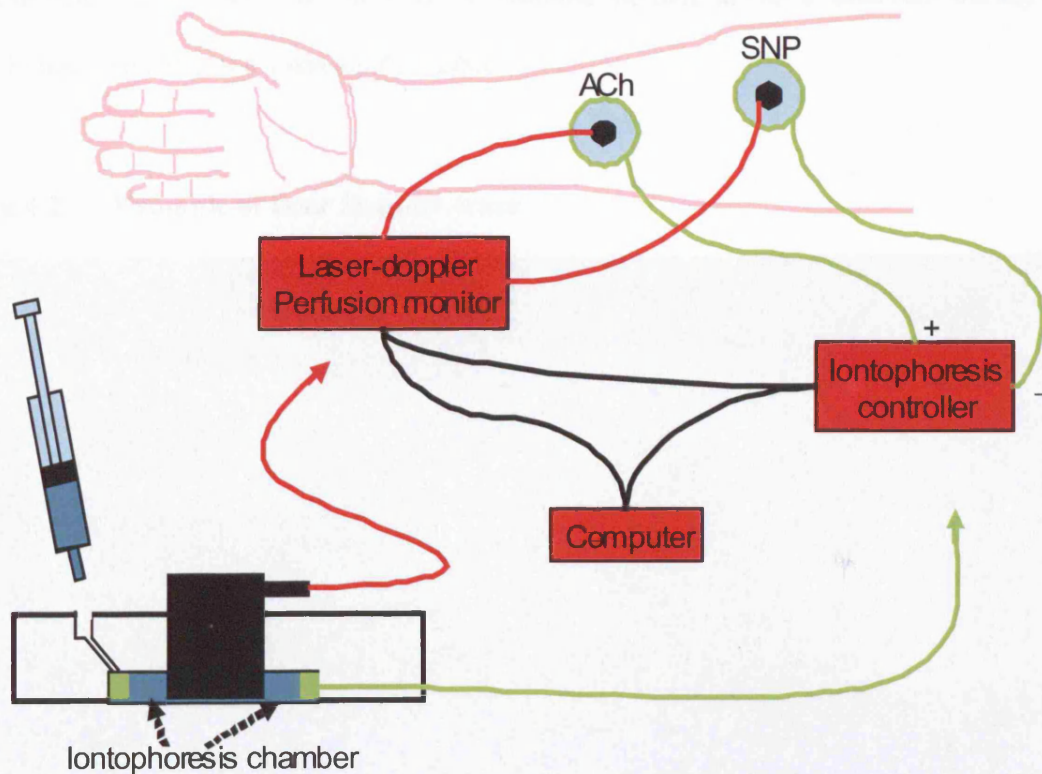
Drug delivery was performed using a battery-powered constant current iontophoresis controller (MIC-1e, Moor Instrument Ltd, Axminster, UK). Low currents were used to limit the iontophoresis dose and to prevent galvanic effects which may cause non-specific vasodilatation (Berghoff et al, 2002) and a cumulative dose-response protocol was used (Morris et al, 1995). Baseline erythrocyte flux was measured for 100 s without current i.e. no drug was iontophoresed. This was followed by drug delivery at 10 µA, 15 µA and 20 µA, sequentially, each for 100 s, followed by 800 s at zero current. Microvascular

perfusion was continually assessed graphically by laser Doppler as shown in Figure 4.2; recording of erythrocyte flux in women with PCOS and controls with iontophoretic application of ACh at predetermined points. In the present study the mean erythrocyte flux estimates are reported at the end of baseline period, at 50 s intervals during iontophoresis, and at 100 s intervals thereafter.

Figure 4.2   Example of laser Doppler trace
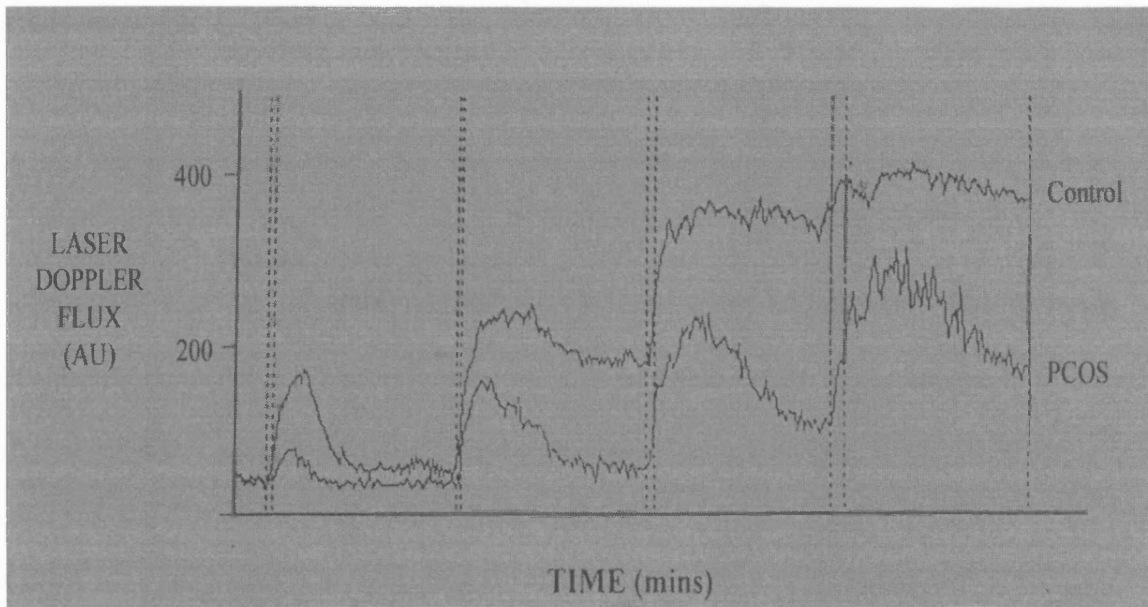
<u>Animal work</u>

All animals were kept in the Comparative Biology Unit and injection protocol was performed by the unit staff. At the end of the injection regimen, general anaesthesia and the *in-vivo* work was performed with the help of Dr W. Yang. *In-vivo* measurements of the thoracic aorta were taken using Pie 35 machine (Pie Medical Systems, Maastricht, Netherlands) with 7.5 MHz linear probe and colour Doppler facility with signal output to a high resolution wall tracking system (Wall Track, Pie Medical Systems, Maastricht, Netherlands).

Ms S. McLellan performed all the immunoassays using dedicated kits. Serum concentrations of LH, FSH and insulin were measured in duplicate in 50 µL samples using an enzyme-linked immunoassay (EIA) supplied by using Biotrak kits and methods (APBiotech, Amersham, UK). Serum testosterone was also assessed in duplicate in 50 µL samples using an ELISA kit (Roche Diagnostics, Welwyn Garden City, Herts, UK).

Organ bath experiments were performed with the help of Ms A. Dooley. The thoracic aorta was placed in a container with oxygenated Krebs buffer solution and tested within 6 hours. The aortic segment was cleaned of fat and adhering connective tissue and cut into 2 mm wide rings. The rings were mounted on hooks in 7 ml organ baths, containing Krebs buffer, maintained at 37° C and oxygenated with 95% O_2, 5% CO_2 (Figure 4.3). The isometric tension was measured with a transducer (Grass Instruments, Quincy, Mass, USA) and digitized using a multi-channel computerised continuous recording system (Linton Instrumentation, Diss, Norfolk, UK) with AcqAcknowledge ACK100W software

(Biopac Systems Inc., Goleta, CA, USA), which allowed simultaneous testing of six rings.

Figure 4.3   Schematic diagram of aortic ring organ bath
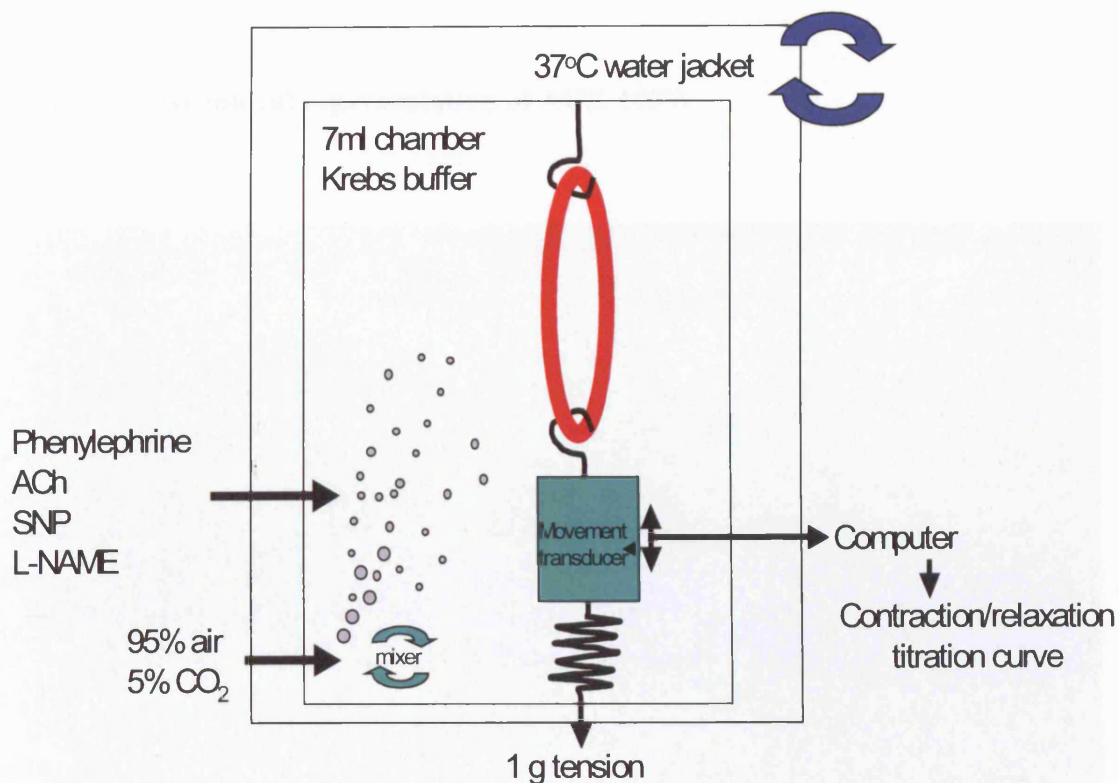
Rings were adjusted to a resting tension of 1 g and equilibrated for 60 min in oxygenated Kreb's buffer. Tissues were then pre-contracted with phenylephrine (PE) 3 μM to give 75% of the maximal contraction. Their dilatory response to cumulative doses of acetylcholine (Ach; 1nM–00μM) was then measured. This PE-ACh treatment cycle was repeated once, then again after addition of 100μM N^G-nitro-L-arginine methyl ester (L-

NAME) to three of the six aortic rings. A final treatment cycle used the cumulative doses of sodium nitroprusside (SNP – a NO donor, 1 nM -100 µM) instead of ACh after PE-mediated contraction and L-NAME addition to the same three samples as before (Liu et al, 2000; Figure 4.4). Aortic tension was expressed graphically, as percentage relaxation, such that the tension induced by 3 µM PE was defined as 0% relaxation, and the tension prior to PE treatment was defined as 100% relaxation.

Figure 4.4   **Graphical representation of ACK 100W**
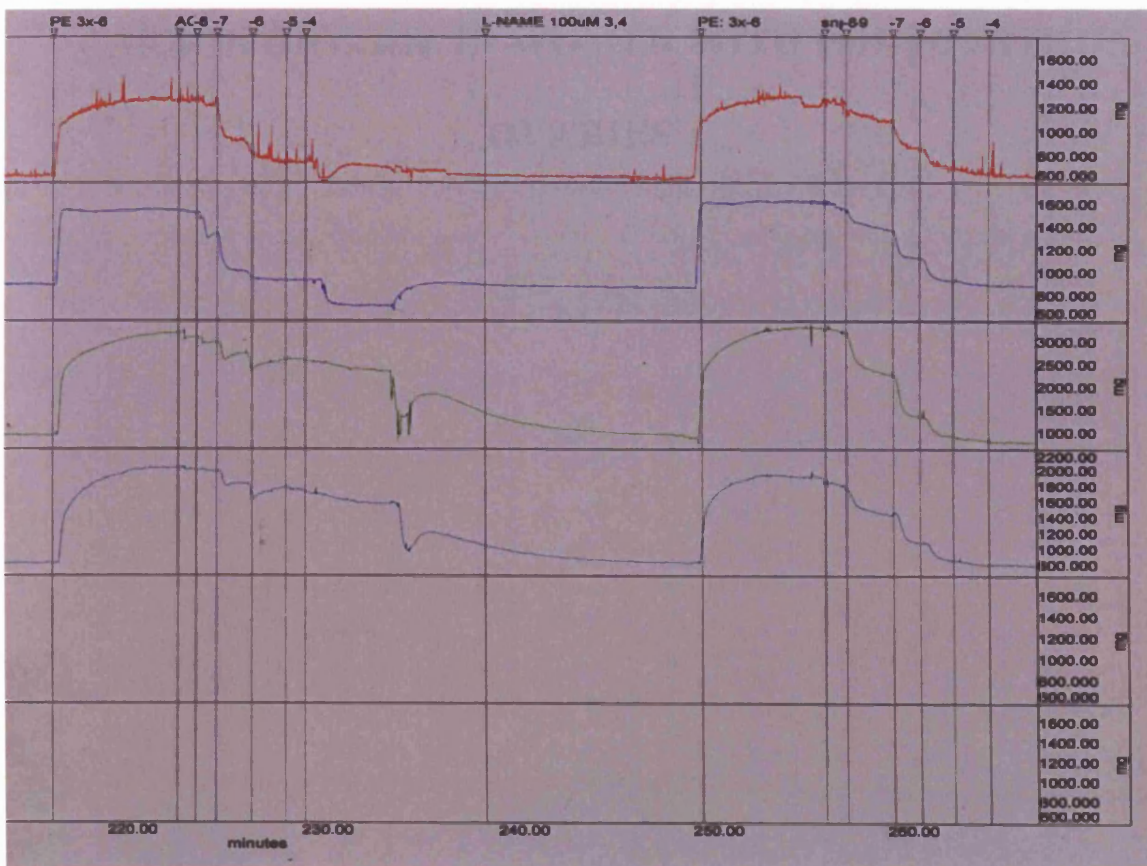
CHAPTER 5

INTERNAL CAROTID ARTERY VASCULAR RESISTANCE AND VASCULAR REACTIVITY TO 5% CARBON DIOXIDE IN WOMEN WITH POLYCYSTIC OVARIES

5.1 Introduction

Haemodynamic changes have been reported in women with PCOS. In a study of 30 oligomenorrhoeic (cycle length $\geq$ 35 days) and 12 amenorrhoeic, hirsute women (no vaginal bleeding for $\geq$ 3 months and Ferriman and Gallwey score $>$ 8), all with PCO morphology on TVS, and 18 control women (normal menstrual cycle), increased uterine artery PI and decreased ovarian stromal RI was observed by Doppler ultrasound in women with PCOS compared with controls (Battaglia et al, 1995). Similarly in 1996, Aleem and Predanic (1996) in a study of 40 women with PCOS (menstrual irregularity, infertility and hyperandrogenism and PCO morphology on TVS) and 50 control women (menstrual cycle 26 – 32 days and normal ovarian morphology on TVS) reported increased uterine artery PI and both decreased PI and RI ovarian parameters in women with PCOS. Since insulin resistance is the central feature in PCOS women, it is of interest that the mean blood flow velocity in the middle cerebral artery measured using transcranial Doppler ultrasound was increased in diabetic subjects (Kawata et al, 1998). However, this study did not mention the type of diabetes and insulin levels, therefore it was very poor. Similar changes have also been reported in cerebral circulation, assessed with radiolabelled microspheres in pancreatomised dogs (Sieber et al, 1993b). As described in Chapter 1, Prelevic (1995) reported decreased flow velocity over the aortic arch assessed by Doppler ultrasound and an increase in resting forearm flow (1996).

The aim of this study was to use Doppler ultrasound to assess vascular resistance at baseline and vascular reactivity following inhalation of 5% carbon dioxide, a known vasodilator (Widder et al, 1986), in the left and right common and internal carotid arteries (CCA and ICA; respectively) in women with PCOS, PCO and healthy controls.

5.2   Methods

The sample size required was calculated using data from a study of the effect of a nitric oxide donor on uterine artery Doppler velocimetry in women with PCOS, where the difference in Doppler parameters in cases and controls was +2.77 ± 0.4 and -0.13 ± 0.06 respectively. Power calculation indicated a sample of 16 subjects in each group was necessary to provide 80% power detecting differences of similar magnitude in the present study and a significance of 5%.

5.2.1   Subjects and study design

A total of 68 women were recruited in this cross sectional study from the North Middlesex Hospital:

- ➢   35 women with PCOS (defined according to the criteria in Section 4.2)

- ➢   15 asymptomatic women with polycystic ovarian morphology on TVS (PCO).

- ➢   18 control women (staff members) with normal ovaries on TVS (controls).

The inclusion and exclusion criteria were followed as described in Section 4.2. Height and weight measurements were used to calculate BMI and the waist and hip measurements to calculate the W/H (Section 4.2). Fasting peripheral blood was obtained from all women for serum endocrine and metabolic parameter measurements (Section 4.5).

5.2.2 *Protocol*

All women were scanned in the supine position with the head hyperextended and turned away from the side being examined. All scans were performed using a Toshiba Eccocee SSA 340 machine with a 7.5 MHz linear probe. The pulse Doppler range gate (2 mm) was placed midway across the CCA and 2.0 cm distal to the carotid bulb for the ICA. The colour pulsed repetition frequency of 20 Hz with a wall filter of 100 Hz was used to achieve an enhanced blood flow image of the vessel. The Doppler exposure time was kept to a minimum.

Once Doppler parameters (PI and V_{max}) were obtained at baseline while the subject breathed room air, the procedure was repeated following inhalation of 5% CO_2 through a tight fitting face mask for 5 minutes at a flow rate of 3 L/min. A mixture of 5% CO_2 and 95% O_2 was chosen as this has been shown to increase the total cerebral blood flow by approximately 75% (Kety and Schmidt, 1948; Sokoloff, 1960 and Breslau *et al*, 1982). 5% CO_2 was inhaled for 2 minutes; a 2 minute interval was chosen as the response to 5% CO_2 is maximal after 20 seconds and remained stable after 2 minutes (Kety and Schmidt, 1948).

The Doppler spectral waveform was simplified by displaying the time dependence of a single parameter, the maximum velocity. At least three consecutive waveforms (representing three cardiac cycles) were displayed, the peak systolic velocity (V_{max} cm/s) and PI were estimated for each vessel on both the left and the right sides. In view of the difficulty in interpreting PI in low impedance vascular beds such as the cerebral

circulation, downstream backpressure was calculated using the model proposed by Gosling et al (1991). According to this model, the back pressure is the sum of arteriolar vasomotor tone and intracranial pressure, and can be estimated using PI, systolic blood pressure (SBP) and diastolic blood pressure (DBP):

$$\text{Back pressure} = DBP + (SBP - DBP) \times (1/3 - [1/PI])$$

Pulse and blood pressure were measured (at baseline and after the inhalation of 5% CO_2) on the left arm in each woman using an automatic Dinamap device (Critikon Inc, Tampa, FL, USA) as described in Section 4.2. Since carotid artery blood flow is influenced by auditory stimulation-noise (Gangar et al, 1991), occlusive headphones were worn throughout the procedure (Section 4.2). Ambient light and temperature (18–20°) were controlled throughout the procedure.

5.2.3 Statistical analysis

Data are mean ± standard deviation (SD). Analysis of variance was used to identify any linear trends in clinical / biochemical parameters and PI, V_{max} and back pressure from controls through PCO to PCOS women where appropriate. The main outcome measures were comparisons of haemodynamic characteristics (PI, V_{max}, back pressure) at baseline and the mean changes in these parameters after inhalation of 5% CO_2, calculated for the PCOS, the PCO and the control groups.

Departure from the normal distribution for PI, V_{max} and back pressure was assessed in

each group with the Kolmogorov Smirnov test. Few data sets were non-normal and a non-parametric test – the Mann-Whitney U test was used to assess the significance of differences with other sets, as was appropriate. Differences between normally distributed data sets were assessed by analysis of variance (ANOVA), with Benferroni's test being used for group-to-group post hoc comparisons.

The following confounding variables were known *a priori* to differ between PCOS and healthy women, and to be associated with haemodynamic disturbances: SBP and DBP, FAI, oestradiol and insulin resistance index. All confounding variables were controlled for within multiple linear regression models. Estimated marginal means (adjusted means) and differences in V_{max}, PI and back pressure between controls and the PCO or the PCOS groups were obtained from general linear models (GLM procedure). This was performed to include in the GLM command the mean value of confounding factors calculated for the control group; 95% confidence interval (CI) and p values for adjusted differences indicated the magnitude and statistical significance of haemodynamic changes, explained only by the presence of polycystic ovaries. Statistical test considered significant for p values $\leq$ 0.05. All analysis was performed using the statistical package SPSS for Windows (version 9.0).

5.3 Results

The V_{max} and PI for each artery on the left and the right sides were highly correlated and the means were therefore combined for analysis (r = 0.81; p < 0.001). The coefficients of variation for ultrasound measurements were between 2% and 8% indicating high reproducibility. Haemodynamic parameters – PI, V_{max} and back pressure were normally

139

distributed and there was minimal difference between the median and mean values; mean values were therefore compared by ANOVA. Median and mean value for each parameter respectively were: PI PCOS – 1.59 vs 1.63, PCO 1.61 vs 1.69, Control 1.83 vs 1.87; V_{max} PCOS - 71.8 vs 71.5 cm/sec, PCO 69.0 vs 68.1 cm/sec, Control 70.2 vs 74.0 cm/sec; back pressure - PCOS 53.1 vs 52.1 mmHg, PCO 56.5 vs 56.1 mmHg; Control 54.7 vs 54.1 mmg/Hg.

Table 5.1 shows the expected upward trend from control through PCO to PCOS women for BMI, waist/hip ratio, BP, FAI, oestradiol, LH, and insulin resistance index, while the PI decreased significantly in the ICA ($p = 0.003$) and just failed to do so in the CCA. There were no significant differences in V_{max} or backpressure in the ICA or in any of the ultrasound parameters in the CCA before adjustment for confounding factors.

On multiple regression analysis (Table 5.2), significant adjusted differences between control and PCO or PCOS women were observed in ICA PI ($p = 0.019$ and $p = 0.016$ respectively), while the differences in V_{max} in the ICA were non-significant ($p = 0.51$ and $p = 0.32$, respectively). The adjusted means of back pressure in the ICA were much lower in both the PCO and PCOS groups than in the controls, especially after removing the confounding effect of blood pressure. Although the differences were of similar magnitude, that between the control and PCO group was statistically significant ($p = 0.038$), whereas that between the control and PCOS women failed to reach significance ($p = 0.070$). No significant adjusted differences were observed in the CCA between control

and PCO or PCOS women, except in V_{max}, which was considerably decreased in PCOS women relative to controls.

Within groups, there was a significant decrease in the ICA V_{max} ($p = 0.024$) and an increase in back pressure ($p = 0.016$) in women with PCOS, after inhalation of 5% CO_2 relative to baseline estimates. Changes in ICA PI in response to 5% CO_2 inhalation were not significant, as were changes in ICA PI, V_{max} and back pressure in normal women and asymptomatic women with PCO in response to this treatment (Figure 5.1). Within groups, a significant change was also observed in the CCA V_{max} in PCOS and PCO women ($p = 0.001$ and $p = 0.029$, respectively) in response to 5% CO_2 treatment, but there was no significant change in PI or back pressure.

Between the control, PCO and PCOS groups, a significant linear trend was found only in the ICA back pressure changes in response to 5% CO_2 , after adjusting for confounding factors ($p = 0.044$). There was no evidence of any linear trend in PI or V_{max} in any parameters in the CCA.

Table 5.1   Physical, endocrine, biochemical and haemodynamic characteristics in controls, PCO and PCOS women

Values are mean ± SD. Analysis of variance was used to test for a significant increase/decrease from control through PCO to PCOS women and p values are given.

Variable	Control (n=18)	PCO (n=15)	PCOS (n=35)	Test for trend* p value
Age, (years)	27.2 ± 2.9	25.8 ± 3.8	26.1± 4.0	0.36
Body Mass Index, (kg/m²)	22.9 ± 3.2	23.3 ± 3.6	25.7 ± 4.9	0.019*
Waist to hip ratio	0.74 ± 0.05	0.77 ± 0.05	0.80 ± 0.06	0.002*
Systolic Blood Pressure, (mmHg)	107 ± 10	112 ± 9	123 ± 10	0.001*
Diastolic Blood Pressure, (mmHg)	63.2 ± 4.9	68.1± 6.8	68.2 ± 5.9	0.007*
Free Androgen Index, (%)	2.6 ± 1.2	3.8 ± 1.5	7.3 ± 4.6	0.001*
Oestradiol (pmol/L)	209 ± 67	181 ± 99	270 ± 122	0.024*
Luteinsing Hormone, (IU/L)	5.6 ± 2.3	5.9 ± 3.5	9.0 ± 6.5	0.022*
Follicle Stimulatin Hormone, (IU/L)	6.0 ± 1.6	5.9 ± 1.6	5.4 ± 1.8	0.20
Prolactin (mU/L)	394 ± 234	352 ± 177	351 ± 379	0.68
Insulin resistance index (mU/mmol)	2.5 ± 0.9	2.6 ± 0.9	3.2 ± 1.3	0.031*
Total Cholesterol (mmol/L)	4.9 ± 1.8	4.4 ± 0.7	5.0 ± 1.0	0.53
Triglycerides (mmol/L)	0.77 ± 0.27	0.83 ± 0.29	1.07 ± 0.74	0.057
High-density lipoprotein, (mmol/L)	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.4	0.17
Low-density lipoprotein, (mmol/L)	3.2 ± 1.8	2.6 ± 0.7	3.3 ± 0.8	0.50
ICA, V_{max} (cm/s)	74.0 ± 17.1	68.1± 8.3	71.8 ± 15.4	0.74
ICA, (PI)	1.87 ± 0.38	1.69± 0.23	1.63 ± 0.21	0.003*
ICA Back pressure, (mmHg)	54.1± 7.3	56.1± 8.3	52.1 ± 8.7	0.29
CCA Vmax, (cm/s)	102.6 ± 12.3	101.3 ± 8.5	101.3 ± 14.6	0.73
CCA, (PI)	2.51± 0.37	2.42± 0.25	2.35 ± 0.27	0.069
CCA Back pressure, (mmHg)	59.9 ± 6.9	64.4 ± 6.7	62.7 ± 7.3	0.25

Table 5.2   Adjusted comparisons of V $_{max}$, PI and back pressure between control and PCO/PCOS women.

Estimated marginal means were calculated using mean values for the control group: BMI = 22.9 kg/m^2, systolic blood pressure = 107 mmHg, diastolic blood pressure 63.2 mmHg, FAI 2.6%, oestradiol = 209 pmol/l, insulin resistance index = 2.5 mU/mmol. Adjusted differences are given with 95% CI. Significant differences indicated by *p < 0.05.

Variable	Group			Adjusted Difference (95% C.I)	
	Control (n=18)	PCO (n=15)	PCOS (n=35)	Control – PCO	Control – PCOS
ICA PI	1.87	1.55	1.58	0.32 (0.06 to 0.58)*	0.29 (0.06 to 0.52)*
ICA Vmax (cm/sec)	74.0	69.7	68.1	4.2 (-8.9 to 17.3)	5.9 (-6.0 to 17.7)
ICA Back pressure (mmHg)	54.1	50.6	50.8	3.5 (0.2 to 6.8)*	3.3 (-0.3 to 6.9)
CCA PI	2.51	2.25	2.39	0.26 (0 to 0.52)	0.12 (-0.12 to 0.36)
CCA V max (cm/sec)	102.6	100.4	92.5	2.3 (-7.6 to 12.2)	10.1 (0.2 to 20.2)*
CCA Back Pressure (mmHg)	59.5	58.5	59.4	1.3 (-0.4 to 3.1)	0.4 (-1.7 to 2.6)

Figure 5.1   Changes in Doppler parameters in the internal carotid artery after inhalation of 5% CO_2 (mean change, 95% confidence interval)
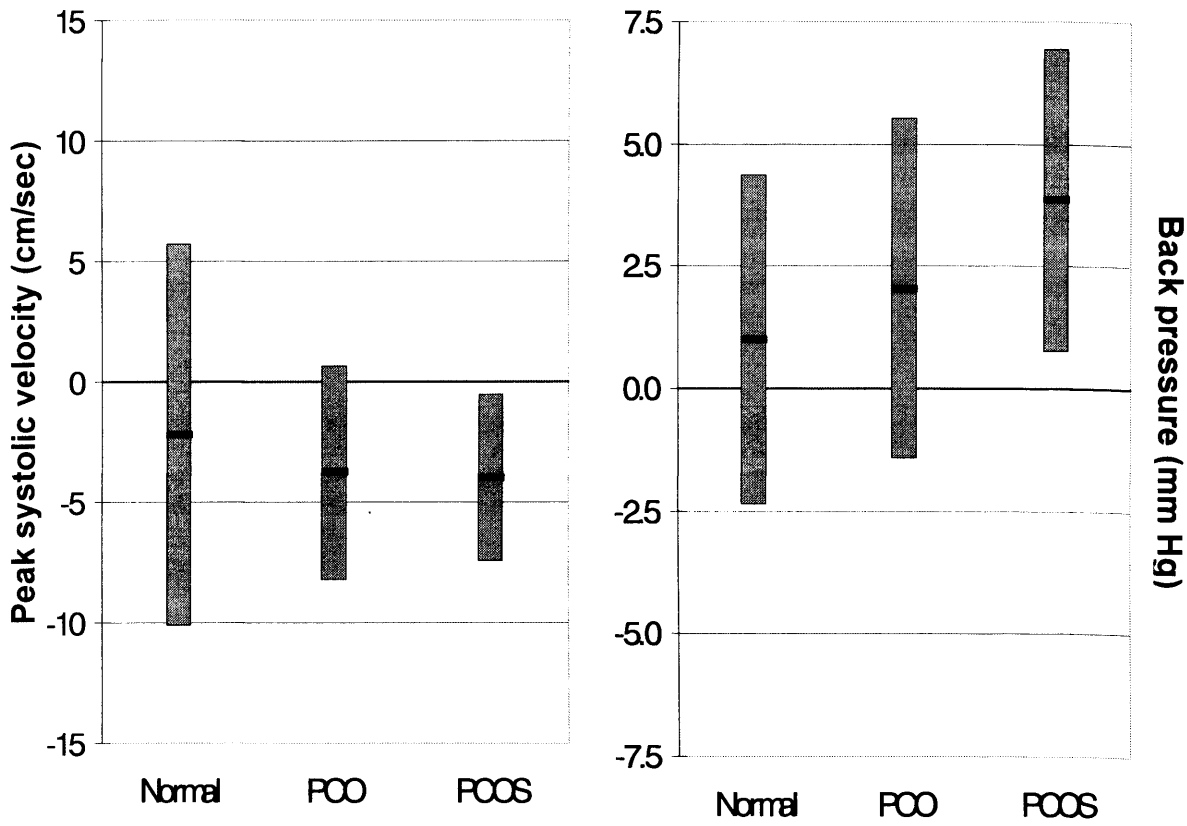
5.4 Discussion

This is the first demonstration of lower PI in the ICA in women with polycystic ovaries and with overt PCOS. Conventionally, a lower PI with similar V_{max} in the internal carotid artery would be considered to be indicative of reduced downstream resistance and increased blood flow.

Gosling and colleagues (1991) have highlighted the difficulties in interpreting PI in low resistance vascular bed such as the ICA. They suggested that small changes in PI do not alter the spectral waveform shape (affected by pressure drop across the vascular bed and not resistance, per se), and proposed a model characterised by two parameters: back pressure (estimated from systemic blood pressure and PI) and 'pure' resistance to flow. According to Gosling et al (1991), the back pressure in the cerebral circulation is the sum of arteriolar vasomotor tone and intracranial pressure, a better indicator of resistance in low impedance vascular beds.

Unadjusted analysis at baseline showed non-significant differences in back pressure between the three groups. However, after adjusting for confounding variables, significantly lower values were seen in women with PCO and a difference of similar magnitude between control and PCOS women, although this was only marginally significant. The finding of lower back pressure in women with polycystic ovaries is consistent with a decrease in vascular tone with lower PI seen in these women. There

were no significant differences in the Doppler parameters and back pressure in the common carotid artery in PCO/PCOS women.

The mechanism responsible for decreased internal carotid artery PI and back pressure in these women is unknown. There is some evidence that hyperglycaemia which is associated with PCOS may influence haemodynamic changes. Griffith and colleagues (1987) reported increased cerebral flow in short duration Type I diabetics with no diabetic complications, and similar findings were reported by Grill et al (1990) in long-duration well-controlled Type I diabetics. PCOS is, however, more closely related to Type II diabetes, in which the regional blood flow may be reduced. Wakisaka et al (1990) reported decreased cerebral blood flow suggestive of increased resistance in Type II diabetic subjects. However, 30% of the subjects studied had retinopathy, neuropathy or nephropathy and since these complications are not seen in PCOS women, this result may not be relevant to this syndrome.

The results of animal studies are also contradictory. Sieber et al (1993a) reported increased cerebral blood flow in chronically hyperglycaemic dogs (after pancreactectomy), but these results were not confirmed when the study was repeated in unanaesthetised dogs or in those anaesthetised with pentobarbital or fentanyl (Sieber et al, 1993b). The authors concluded that the increased cerebral blood flow observed in the first study may have resulted in surgical stimulation during insertion of vascular catheters, and that hyperglycaemia may not be associated with altered cerebral blood flow.

The study also provides evidence of a significant effect of CO_2 inhalation on ICA Doppler variables in women with PCOS but not in women with PCO alone or in controls. Increased back pressure after inhalation of CO_2 is suggestive of an increased vascular tone, an increased intracranial blood pressure, or both. The absence of an effect of 5% CO_2 inhalation in healthy controls cannot be explained by a failure to induce hypercapnia, since the technique was validated by an increase in end-tidal CO_2 concentration, assessed by means of an infrared analyser. Lees and colleagues (1998), in a small study of 10 women with PCOS and nine control women with regular menstrual cycles between 26–29 days and normal ovarian morphology on TVS, reported similar paradoxical response in PCOS women and no difference in control women in the uterine artery resistance assessed with colour Doppler ultrasound following the administration of glyceryl trinitrate.

The increased trend in back pressure, following inhalation of 5% CO_2, from control women through asymptomatic women with PCO to women with PCOS, suggests a progressive alteration in CO_2 reactivity in women with PCO. Very few studies have used ICA Doppler ultrasound to investigate the effect of carbon dioxide. Breslau and colleagues (1982) reported a 96% higher internal carotid artery end-diastolic velocity after inhalation of 6.8% CO_2 in five healthy volunteers. However, this study used male volunteers and the researchers have failed to report the mean change and its significance.

The physiological mechanisms responsible for the vasodilatory response to CO_2 are unclear. Whether CO_2, bicarbonate or hydrogen ions exert effects directly on cerebral vessels, or whether other intermediate processes and/or messenger systems are involved remains unknown. It is also possible that the dilatory effect of CO_2 may be under neurological control involving stimulation of either the aortic or carotid chemoreceptor or both (Madden, 1993).

The results of the present study provide evidence of vascular abnormality but the clinical interpretation of altered vascular reactivity of the cerebral circulation during hypercapnia is still unclear. One possible explanation of these findings may be due to alteration in vascular mechanics in PCOS women, suggestive of reduced resistance in the cerebral vascular beds independent of blood pressure, insulin resistance or other endocrine and metabolic factors. Furthermore, the alteration in cerebrovascular PI could also reflect change in vascular compliance; increased vessel stiffness has been reported in women (but not in men) with diabetes (Ryden-Ahlgren et al, 1996), and this is addressed in the next chapter.

CHAPTER 6

IMPAIRED CAROTID ARTERY ELASTIC

PROPERTIES IN WOMEN WITH POLYCYSTIC OVARIES

6.1   Introduction

As has been discussed previously, PCOS is associated with insulin resistance (Dunaif, 1997), type II diabetes (Wild et al, 1985; Talbott et al, 1995) and gestational diabetes (Urman, 1997). These conditions are associated with decreased vascular compliance and thus increased arterial stiffness (Lehman et al, 1992). This increase has been suggested as an early marker of structural and/or functional changes in the arteries related to the pathogenesis of atherosclerosis. In this thesis (Chapter 5), a paradoxical constrictor response to 5% CO_2, a known vasodilator, was noted in the ICA of women with PCOS (Lakhani et al, 2000b). A similar constrictor response to the vasodilator glyceryl trinitrate reported in the uterine artery of women with PCOS (Lees et al, 1998). These findings suggest endothelial function may be impaired resulting in stiffer vessels in women with PCOS compared to controls. The present study was designed to assess elastic properties in the carotid arteries in women with PCOS, PCO and controls.

6.2   Methods

The sample size required was calculated using data from a study of testosterone suppression in men and controls, where the change in systemic arterial compliance from baseline for controls and cases was +0.06 ± 0.37 and -0.26 ± 0.33 respectively. Power calculation indicated a sample of 18 subjects was necessary to provide 80% power of detecting differences of a similar magnitude in the present study at a significance of 5%.

6.2.1 Subjects and study design

In this cross-sectional study a total of 60 women were recruited from the North Middlesex and Royal Free Hospitals:

> 20 women clinics with PCOS (defined according to the criteria in Section 4.2)

> 20 asymptomatic women with polycystic ovarian morphology on TVS (PCO).

> 20 control women (staff members) with normal ovaries on TVS (controls).

The inclusion and exclusion criteria were followed as described in Section 4.2. Height and weight measurements were used to calculate the BMI, and the waist and hip measurements to calculate the W/H (section 4.2). Fasting peripheral blood was obtained from all women for serum endocrine and metabolic parameter measurement (Section 4.5). In this study the arterial compliance (C) and stiffness index (β) were used to assess the vessel wall properties in the three subject groups (Section 4.6).

6.2.2 Protocol

All women were examined in the supine position, with the head hyperextended and turned away from the side being scanned. Scans were performed by the same operator (KL) using a colour Doppler Pie 350 machine (Pie Medical Systems, Maastricht, Netherlands) with a 7.5 MHz linear probe, the signal output being connected to a high resolution wall tracking system (Wall track, Pie Medical Systems, Maastricht, Netherlands). This system allowed the measurement of vessel wall (CCA and ICA) movement over time by automatically tracking assigned points of the induced radio frequency signal deemed to be representative of the anterior and posterior vessel wall (as described in Section 3.4.2). In brief, the M-mode cursor positioned perpendicularly and

midway to the long axis of the CCA and 2 cm distal to the carotid bulb for the ICA, the change in induced radio frequency signal from the vessel was sampled. The data was transferred to a personal computer for off-line analysis of the anterior and posterior arterial walls.

Pulse and blood pressure were measured on the left arm in all women, using an automatic Dinamap device (Critikon Inc, Tampa, FL, USA), as described in Section 4.2. Since carotid blood flow is influenced by auditory stimulation (Gangar et al, 1991), occlusive headphones were worn throughout the procedure (Section 4.2). Ambient light and temperature (18 – 20°) were controlled throughout the procedure.

6.2.3   Statistical analysis

The CCA and ICA wall movement was obtained over three cardiac cycles, and the average diametrical compliance and stiffness index were calculated according to equations described in Section 3.4.2, and blood pressure estimated measured at the corresponding time and site. The average of three measures of C and β data at a given site was used to calculate to obtain mean values for each site along the vessel in each subject. Intra-observer variability was determined separately for each site, by determining the coefficient of variation of repeated measurements. Coefficients of variations below 10% were considered to indicate good reproducibility.

Normality of data was assessed using the Kolmogorov-Smirnov test. Differences between normally distributed data sets were assessed by ANOVA, with Bonferroni's test being

used for group-to-group post hoc comparisons. Non-normally distributed data were assessed using a non-parametric test – the Mann-Whitney U test to assess the significance with other sets, as appropriate. The following confounding variables were known *a priori* to differ between PCOS and healthy women and are known to be associated with haemodynamic disturbances: age, BMI, SBP and DBP. Inter-group differences in C and β were compared by one-way ANOVA and if significant differences were detected multiple regression analysis were performed, including all known confounders controlled for within regression models. Inter-group comparisons of adjusted means were performed between PCOS versus controls and PCO versus controls. Adjusted means were calculated using general linear models (GLM) procedure. This was achieved by including in the GLM command the mean values of the confounding factors calculated for the control group. Statistical tests considered significant for p values $\leq$ 0.05. All analyses were performed using the statistical package SPSS for Windows (version 9.0).

6.3   Results

Arterial compliance and stiffness index were correlated between the right and the left sides and therefore only the right side was assessed due to easy accessibility of this side. The coefficients of variations for compliance and stiffness index were between 3.5% and 7.0% for the CCA and 7.6% and 10% for the ICA, indicating good reproducibility for the CCA and reasonable reproducibility for the ICA.

Table 6.1 summarises the physical characteristics in controls and in women with PCO and PCOS. There was no significant difference with respect to age between the three groups. There was a significant difference in BMI (p = 0.003) and systolic (p = 0.03) and

diastolic blood pressures (p = 0.04) in women with PCOS compared with healthy controls.

Table 6.1   Physical characteristics for the three groups. Values are mean (SD), HDL high-density lipoprotein, LDL low-density lipoprotein, NS, Not significant

Variable	Control (n=20)	PCO (n=20)	PCOS (n=20)	p value between groups
Age, (years)	27.5 ± 4.00	27.7 ± 4.60	29.2 ± 4.00	NS
Height, (m)	1.68 ± 0.06	1.64 ± 0.08	1.65 ± 0.07	NS
Weight, (kg)	61.0± 11.30	68.1 ±11.60	85.5 ± 25.30	0.008
Body Mass Index, (kg/m^2)	24.2 ± 3.40	22.5 ± 3.80	31.3 ± 8.20	0.003
Systolic blood pressure, (mmHg)	108.2 ± 9.60	103.1 ± 9.7	118.7 ± 18.20	0.03
Diastolic blood pressure, (mmHg)	59.5 ± 8.40	56.3 ± 6.80	68.9 ±14.60	0.04
Fasting Insulin, (mU/L)	10.43 ± 4.60	16.41± 7.12	23.83 ± 10.31	0.02
Total cholesterol, (mmol/L)	4.42 ± 0.52	5.41 ± 2.49	4.59 ± 1.89	NS
High-density lipoprotein, (mmol/L)	1.38 ± 0.20	1.36 ± 0.21	1.33 ± 0.43	NS
Low-density lipoprotein, (mmol/L)	2.72 ± 0.44	3.61 ± 2.51	3.69 ± 1.43	NS
Triglycerides (mmol/L)	0.70 ± 0.17	0.91 ± 0.32	1.27 ± 0.95	NS

There was minimal difference between the median and mean compliance values; mean values were therefore compared ANOVA. The median and mean value for each group respectively were: C_{CCA} - PCOS 14.73 vs 14.86 % mmHg^{-1}x 10^{-2}, PCO 9.8 vs 10.1 % mmHg^{-1}x 10^{-2}, Control 20.53 vs 18.86 % mmHg^{-1}x 10^{-2}; C_{ICA} PCOS 9.9 vs 12.5 % mmHg^{-1}x 10^{-2}, PCO 9.8 vs 10.15 % mmHg^{-1}x 10^{-2}, Control 12.69 vs 13.195 % mmHg^{-1}x 10^{-2}.

While no difference was noted in unadjusted mean compliance and stiffness index between controls, PCO and PCOS groups, on multiple regression analysis and correction for the influence of confounding variable, significant adjusted differences were observed in compliance and stiffness index between controls and PCO or PCOS women (Tables 6.2 and 6.3).

Table 6.2 Adjusted comparison of compliance (C) of the common carotid artery (CCA) and the internal carotid artery between control and PCO/PCOS women.

Estimated marginal means were calculated from the GLM model using mean values of the confounding factors for the control group: Age = 27.5 years; BMI = 24.2 kg/m^2; systolic blood pressure = 108.2 mmHg; diastolic blood pressure = 59.5mmHg and insulin 10.43 m-units/L. Adjusted differences are given with 95% confidence intervals. Significant differences are indicated by:* p < 0.05.

	Adjusted mean			Adjusted Difference (95% C.I)*	
Variable	Control (n=20)	PCO (n=20)	PCOS (n=20)	Control – PCO	Control – PCOS
C_{CCA} (% mmHg^{-1} x 10^{-2})	19.2	14.1	10.7	5.1 (8.1 to 2.6)*	8.5 (10.2 to 4.8)*
C_{ICA} (% mmHg^{-1} x 10^{-2})	16.9	11.0	10.1	5.9 (8.7 to 1.4)*	6.8 (9.2 to 0.4)*

Table 6.3 Adjusted comparison of stiffness index (β) of the common carotid artery (CCA) and the internal carotid artery between control and PCO/PCOS women.

Estimated marginal means were calculated from the GLM model using mean values of the confounding factors for the control group: Age = 27.5 years; BMI = 24.2 kg/m^2; systolic blood pressure = 108.2 mmHg; diastolic blood pressure = 59.5 mmHg and insulin 10.43 m-units/L. Adjusted differences are given with 95% confidence intervals. Significant differences are indicated by:* p < 0.05.

	Adjusted mean			Adjusted Difference (95% C.I)*	
Variable	Control (n=20)	PCO (n=20)	PCOS (n=20)	Control – PCO	Control – PCOS
β_{CCA}	6.7	10.2	12.3	-3.5 (-1.1 to - 4.7)*	-5.6 (-2.8 to –6.2)*
β_{ICA}	8.7	16.2	14.8	-7.5 (-0.9 to –15.2)*	-6.1 (-4.5 to –13.1)*

6.4   Discussion

This is the first demonstration of decreased vascular compliance and increased arterial stiffness of the CCA and ICA in both PCOS and asymptomatic PCO women compared with control women. This is significant in view of the relationship between the functional properties of the arterial wall i.e. atherosclerosis appears to affect blood vessel stiffness by changing the intrinsic properties of arterial wall, and therefore an increase in cardiovascular mortality and morbidity (Lusis, 2000).

Arterial elasticity diminishes with age, hypertension and other risk factors, such as hyperlipidaemia and smoking (Benetos et al, 1993) as well as in patients with frank coronary artery disease (Dart et al, 1991). The results of this study are unlikely to be confounded by these factors, because all the subjects in this study were young (25 – 33 years), non-smoking adults who had no symptoms of coronary artery disease. Similarly, BP, BMI and fasting serum insulin were higher in women with PCOS than in controls, and are all associated with decreased vascular compliance. It is possible therefore that some or all of these confounders could account for the decreased compliance and increases stiffness index in the ICA and CCA in women with PCOS relative to controls. After adjusting for the confounders in the general linear regression model, vascular parameters remained disturbed in the PCOS women. However, in asymptomatic women with PCO, the BP and BMI was non-significant relative to controls, whereas fasting serum insulin levels were just significant. It is possible that after adjusting for confounders in the general linear regression model, insulin could account for the observed disturbed vascular parameters in the PCO women.

156

The decreased vascular compliance in women with PCOS is compatible with earlier onset

of atherosclerosis in these women, arterial elasticity being reduced by fatty streak

formation before other pathological changes occur (Hironaka et al, 1997). Such a

conclusion is supported by other evidence for subclinical atheromatous change in PCOS,

before plaque formation or disturbance in blood flow (O'Leary et al, 1991) – for

example, the observation that intima-media thickness in the carotid artery is increased in

women with PCOS (Talbott et al, 1995; Guzick et al, 1996). This observation is of

significance in view of the correlation between carotid atherosclerosis and the incidence

of coronary artery disease and stroke (Hertzner et al, 1985; Urbinati et al, 1992).

The mechanism responsible for the decreased arterial compliance and increased stiffness

index in women with PCOS and asymptomatic women with PCO is not known, although

similar findings in type II diabetes have been attributed to non-enzymatic glycation of

elastin and collagen in the tunica media (Ilegbusi et al, 1999) and the accumulation of

advanced end products of glycation which inhibit the appropriate muscular tone in the

arterial wall (Irace et al, 1999). It is reasonable to suggest that this mechanism may be

relevant in PCOS, given that the association with hyperglycemia and hyperinsulinaemia

was seen in women with PCOS. The parallel with diabetes may also extend to endothelial

dysfunction, as seen in the brachial artery in women with NIDDM (Goodfellow et al,

1996). The abnormal response to glyceryl trinitrate (Lees et al, 1998) and paradoxical

vasoconstrictor response to 5% carbon dioxide supports the concept of endothelial

dysfunction in women with PCOS.

The unexpected finding of decreased compliance and increased stiffness index in asymptomatic women with PCO may suggest ovarian morphology be the cause, but this seems unlikely. Some other 'causative factor/s' i.e. genetic may be responsible. The hypothesis that obesity leads to hyperinsulinaemia promoting increased ovarian androgen production and the expression of PCOS in asymptomatic women with PCO may be one of the reasons. But this seems unlikely because there was no difference in BMI of PCO women relative to controls. An alternative explanation is that it seems likely that a 'PCO' gene or combination of genes related to insulin secretion and action may be responsible.

The results of this study provide additional evidence of significant vascular dysfunction in women with polycystic ovaries and highlight the need to confirm or refute the present discrepancy between cardiovascular risk and mortality in these women.

CHAPTER 7

INTIMA-MEDIA THICKNESS OF ELASTIC AND MUSCULAR ARTERIES OF YOUNG WOMEN WITH POLYCYSTIC OVARIES

7.1 Introduction

As discussed previously (Chapter 6) the finding of decreased vascular compliance and increased vascular stiffness (Lakhani et al, 2002) in women with PCOS may reflect early atherosclerotic disease. Increased IMT is considered an early morphological marker of atherosclerosis appearing before the formation of plaque and disturbance in blood flow (Cheng et al, 2002). Furthermore, there is evidence of increased IMT in women with PCOS (Guzick et al, 1996; Talbott et al, 2000), though the women investigated were all aged over 40 years.

The present study was designed to test the hypothesis that women with PCOS suffer from precocious arterial disease, by measuring IMT using a reproducible automated technique capable of measuring to within 0.01 mm (Allan et al, 1997; Liang et al, 1998; Willekes et al, 1999; Cheng et al, 2000; Wilson et al, 2000). IMT was assessed in the CCA, the carotid bulb and the common femoral artery (CFA) in three groups of women aged less than 35 years – women with PCOS, asymptomatic women with PCO and healthy controls.

7.2 Methods

The sample size required was calculated using data obtained from a study in which IMT was measured using B-mode ultrasound in women with PCOS and in controls, IMT estimates being 0.68 ± 0.019 and 0.63 ± 0.012, respectively. A power calculation indicated that a minimum sample of 12 subjects in each group was necessary to provide

80% power in detecting differences of a similar magnitude in the present study at a

significance of 5%.

7.2.1 Subjects and study design

Cross-sectional study of 43 women recruited from the North Middlesex and Royal Free

Hospitals:

> 19 women with PCOS (defined according to the criteria in Section 4.2)

> 12 asymptomatic women with polycystic ovarian morphology on TVS (PCO).

> 12 control women (staff members) with normal ovaries on TVS (Controls).

The inclusion and exclusion criteria were followed as described in Section 4.2. Height

and weight measurements were used to calculate the BMI and the waist and hip

measurements to calculate W/H ratio (Section 4.2). Fasting peripheral blood was obtained

from all women for serum endocrine and metabolic parameter measurement (section 4.5).

7.2.2 Protocol

The principles for the assessment of IMT are described in Section 3.4.3. In brief, the

subjects rested for 15 min in supine position to allow pulse and blood pressure to stabilise

before any form of measurements was performed. Pulse and blood pressure were

measured on the left arm in all women using an automatic Dinamap device (Critikon Inc,

Tampa, FL, USA) as described in Section 4.2. Since carotid blood flow is influenced by

noise (Gangar et al, 1991), occlusive headphones were worn throughout the procedure

(Section 4.2). Ambient light and temperature (18 – 20°) were controlled throughout the procedure.

Electrocardiographic (ECG) leads were placed appropriately on the chest wall for ECG R-wave triggered measurements. Real-time B-mode and M-mode images of the arterial wall motion and IMT were recorded using a 7.5 MHz linear probe in the sagittal plane at 90° to the long axis of the CCA or CFA using a specially adapted duplex scanning system (Pie 350, Pie Medical Systems, Maastricht, Netherlands). Data was collected for 4 seconds, i.e. 3–5 heart beats, with the signal output to a high resolution wall tracking system (Wall track, Pie Medical Systems, Maastricht, Netherlands) at a sample frequency of 20 MHz and an M-line update frequency of 400 Hz. This system allowed measurement of the intraluminal diameter and IMT over time, by automatically tracking the assigned points representing the anterior and posterior vessels walls. The automatic system records the IMT defined by two parallel echogenic lines (double line pattern) corresponding to the lumino-intima and media-adventitia interfaces, detected automatically from the RF signal (Section 3.4.3).

For the right CCA, the women were examined in the supine position, with the head hyperextended and tilted to the left at an angle of 45°. The IMT was assessed with the jugular vein in front of the carotid artery, thereby enhancing the ultrasound signal from the underlying CCA. For the CFA, ultrasound examination was performed with an empty bladder as a full bladder affects CFA artery wall properties (Willekes et al, 1998). The IMT was measured midway to the long axis of the CCA and CFA. At the end of the

measurement protocol all recorded files were processed using the wall thickness program. The data were transferred to a personal computer for analysis.

7.2.3 *Statistical analysis*

The intra-observer errors were estimated, separately for each site, by determining the coefficient of variation of repeated measurements. Analysis of variance was used to identify any differences in biochemical and IMT parameters from controls through PCO to PCOS. The following confounding variables were known *a priori* to differ between PCOS and healthy women and are also associated with haemodynamic disturbances: age, BMI, SBP and DBP, cholesterol and insulin. Inter-group differences in parameters were compared by one-way ANOVA and, if significant by, multiple regression analysis. All confounders were controlled for within regression models. Adjusted means were calculated from general linear models (GLM) procedure. This was achieved by including in the GLM command the mean values of the confounding factors calculated for the control group. Inter-group comparisons were performed between PCOS versus controls and PCO versus controls. Statistical tests considered significant for p values ≤ 0.05. All analyses were performed using the statistical package SPSS for Windows (Version 10.05).

7.3 Results

The coefficient of variation for IMT measurements were 7% in the CFA, 8% in the CCA and 23% in the carotid bulb, respectively. Table 7.1 summarises the characteristics of the three groups. There was no significant difference with respect to age, systolic/diastolic blood pressures and the mean vessel wall luminal diameters between the three groups. As expected there were significant differences in BMI (p = 0.02), LH (p = 0.005) and FAI (p = 0.005) between women with PCOS and controls. The heart rate was also increased in women with PCOS compared with controls (p = 0.001).

There were minimal differences between the median and the mean IMT values. Mean values were therefore compared by ANOVA. Median and mean values for each parameter respectively were: IMT_{CCA} - PCOS 0.56 vs 0.54 mm, PCO 0.49 vs 0.51, Control 0.37 vs 0.40 mm; IMT_{BULB} - PCOS 0.54 vs 0.56 mm, PCO 0.55 VS 0.54 mm, Control 0.46 vs 0.44 mm; IMT_{CFA} – PCOS 0.71mm vs 0.74 mm, PCO 0.53 mm vs 0.52 mm, Control 0.41 mm vs 0.42 mm.

The IMT was significantly increased in the CFA and CCA in young women with PCOS compared to controls (Table 7.2). On multiple regression analysis, when controlling for known confounders (age, SBP and DBP, cholesterol and insulin), the differences in IMT still remained significant (Table 7.2).

In asymptomatic women with PCO, the CCA IMT was increased relative to controls but this difference failed to reach statistical significance after adjusting the means for

confounding factors. Although there was a downward trend from PCOS though PCO to control women with respect carotid bulb IMT, this failed to reach the predetermined statistical significance.

Table 7.1   Physical, endocrine, biochemical and mean luminal diameter for controls, PCO and PCOS women.

Variable	Control	PCO	PCOS	p-value between groups
No. of subjects	12	12	19	
Age, (years)	27.7± 4.8	28.2 ± 5.4	29.0 ± 5.0	0.77
Height, (m)	1.67 ± 0.06	1.64 ± 0.06	1.63 ± 0.07	0.25
Weight, (kg)	67.4 ± 9.4	64.2 ± 10.3	74.8 ± 16.1	0.06
Body Mass Index, (kg/m)^2	24.2 ± 3.2	24.0 ± 3.5	28.3 ± 6.5	0.02*
Systolic Blood Pressure, (mm Hg)	110 ± 11	112 ± 15	119 ± 18	0.26
Diastolic Blood Pressure, (mm Hg)	63 ± 80	59 ± 80	65± 13	0.28
Heart rate, (beats/minute)	65 ± 80	63± 80	75± 90	0.001*
Luteinising hormone, (IU/L)	3.21 ± 1.32	5.78 ± 4.33	9.81 ± 6.66	0.005*
Follicle stimulating hormone, (IU/L)	6.53 ± 3.20	6.02 ± 1.68	5.91± 1.98	0.77
Testosterone, (nmol/L)	1.84 ± 0.78	1.96 ± 0.78	2.21 ± 0.79	0.53
Sex-hormone binding globulin, (nmol/L)	79.60 ± 41.9	58.90 ± 29.9	31.90 ± 15.7	0.002*
Free Androgen Index (%)	2.31 ± 1.5	3.33 ± 1.8	6.92 ± 1.6	0.005*
Prolactin, (mU/L)	244.5 ± 90.2	245.0 ± 138.0	223.0 ± 186.0	0.91
Oestradiol, (pmol/L)	123.6 ± 54.3	175.0 ± 89.7	227.0 ± 175.0	0.13
Fasting Glucose, (mmol/L)	5.16 ± 0.62	5.07± 0.48	4.81 ± 0.79	0.37
Fasting Insulin, (mU/L)	8.10 ± 3.95	12.80 ± 18.28	20.44 ± 20.01	0.31
Total cholesterol, (mmol/L)	4.88 ± 0.56	4.70 ± 0.91	4.87 ± 0.79	0.80
High-density lipoprotein, (mmol/L)	1.73 ± 0.41	1.58 ± 0.32	1.49 ± 0.41	0.30
Low-density lipoprotein, (mmol/L)	2.73 ± 0.78	2.79 ± 0.87	2.79 ± 0.92	0.98
Triglycerides, (mmol/L)	1.01 ± 0.52	0.94 ± .49	1.47 ± 1.15	0.19
CFA diameter, (mm)	6.95 ± 1.14	6.97 ± 0.98	7.18 ± 1.60	0.30
CCA diameter, (mm)	6.39 ± 0.53	6.49 ± 0.63	6.45 ± 0.60	0.43

*significant

Table 7.2 Unadjusted and adjusted IMT measurements in the common carotid artery, carotid bulb and common femoral artery

Variable	PCOS	PCO	Control	PCOS-Control (95% CI)	p-value	PCO-Control (95% CI)	p-value
Number of women	19	12	12				
Unadjusted							
IMT_{CCA} (mm)	0.54±0.11	0.51±0.18	0.40±0.09	0.14 (0.04 to 0.23)	0.006*	0.11 (0.006 to 0.22)	0.038*
IMT_{BULB} (mm)	0.56±0.19	0.54±0.18	0.44±0.11	0.12 (-0.008 to 0.24)	0.066	0.10 (-0.04 to 0.24)	0.17
IMT_{CFA} (mm)	0.74 ±0.30	0.52±0.16	0.42 ± 0.07	0.32 (0.16 to 0.49)	0.001*	0.10 (0.09 to 0.28)	0.28
Adjusted							
IMT_{CCA} (mm)	0.53±0.09	0.50±0.15	0.44±0.09	0.09 (047 to 0.98)	0.034*	0.06 (0.81 to 0.70)	0.84
IMT_{BULB} (mm)	0.62±0.25	0.58±0.14	0.47±0.12	0.15 (-0.19 to 0.46)	0.46	0.11 (-0.55 to 0.19)	0.25
IMT_{CFA} (mm)	0.71±0.29	0.53±0.18	0.42±0.04	0.3 (0.02 to 0.53)	0.032*	0.11 (0.09 to 0.42)	0.20

Adjusted for age, systolic and diastolic BP, BMI, cholesterol and insulin; * $p < 0.05$

7.4 Discussion

The results of this study address the critical question in relation to PCOS and the cardiovascular system as to whether the clustering of risk factors in these women translates into disease. An increase in IMT in the CCA and CFA in such women is indicative of atherosclerotic pathology at relatively young age. This is an important issue to address in view of the conflicting nature of the available evidence and the high prevalence of the syndrome. This conflict in evidence may be attributed to the differing criteria used to define PCOS and hence the physical, metabolic and endocrine characteristics of PCOS women varying between different studies. It is noteworthy therefore that the degree of endocrine dysfunction in the subjects in this study was modest, such that the serum concentrations of insulin levels were not significantly increased in PCOS women compared with controls because of high variability. This may reflect to the BMI of these subjects; most of whom were overweight (BMI 25–30 kg/m^2) rather than obese (> 30 kg/m^2). Nevertheless, adjusted differences were still significant.

The results of this study suggests that the risk factors in women with PCOS do translate to disease in both elastic (common carotid) and muscular (common femoral) arteries. The increase in IMT in the PCOS women was however not significant in the carotid bulb. At first sight this is surprising in view of the vascular turbulence at this site, which is thought to be a factor in the development of arterial disease. The lack of statistical significance may however be explained by the increased intra-group variation, which in turn probably resulted from the decreased reproducibility inherently associated with IMT measurement at the carotid bulb (coefficient of variation 23%). The failure to detect any significant

differences in the mean vessel wall luminal diameter in either artery may reflect the mean age of our women and being asymptomatic with normal lipids; luminal narrowing and an increased IMT have been reported in older subjects with peripheral vascular disease (Benetos et al, 1993).

Although evidence demonstrating the progression of atherosclerotic disease from increased IMT through plaque formation to alteration in blood flow is lacking, increased IMT is considered to be an early marker of atherosclerosis (Gariepy et al, 1995; Poredos et al, 1999; Aminbaksh et al, 1999), before any effect on blood flow occurs. Increased IMT is also associated with thinning and fracturing of elastin fibres together with the deposition of collagen, perhaps due to non-enzymatic glycation of elastin and collagen in the tunica (Ilegbusi et al, 1999). These changes lead to thickening of the arterial wall, and result in a reduction in the buffering capacity, decreasing arterial elasticity and compliance (Pupita et al, 1991).

In addition to providing evidence of early atherosclerosis in women with PCOS, the finding of increased carotid and femoral IMT also suggests that they are at increased risk of developing coronary heart, cerebrovascular and peripheral vascular disease. In this study, 7 of the 19 PCOS women (37%) had CCA IMT > 0.56, which in the study by Hodis (1998) is associated with an odds ratio for coronary heart disease of 5.5. According to Bots (1997) the relative risk of cerebrovascular disease is increased by 1.4 for each standard deviation above the mean CCA IMT. It was found that the mean in the PCOS women was 1.6 SD above that for the controls, which would suggest these women have

an odds ratio of 2.2 for cerbrovascular disease (Bots et al, 1997). Similarly from other studies, admittedly in older age groups, the increased CFA IMT in women with PCOS in this study would suggest an association with an increased risk of peripheral vascular disease (Bots et al, 1994; Simons et al, 1999). It has been suggested that any increased cardiovascular morbidity in PCOS women is wholly attributable to their higher BP, BMI, cholesterol and insulin. The findings for younger women in this study, and the study by Talbott and co-workers for older women (2000) show that the increased IMT remains after adjusting for these risk factors. This supports the hypothesis that PCOS is an independent risk factor for arterial disease and refutes the suggestion that the cardiovascular risk related to PCOS is due entirely to BP, cholesterol, insulin and BMI changes.

As previously discussed only one study has investigated mortality in women with PCOS and the results showed no increase in deaths from coronary heart disease. Several reasons have been proposed to explain this. Firstly, the diagnosis of PCOS was based solely on ovarian morphology. However, the main indication for wedge resection was for infertility, not severity of clinical features. Secondly, many patients' records were not followed up or retrieved, raising the possibility of selection bias. Thirdly, standardised mortality rates were derived from UK national database and took no account of regional variations in mortality rates. Finally, the problems of using death certificate data and their accuracy are well known. It is however possible that mortality is not increased in PCOS women, and increased serum oestrogen is protective in these women.

Confirmation of increased cardiovascular mortality in PCOS would provide evidence to support the establishment of a screening programme, and ultimately pharmacological intervention. Many clinicians suggest opportunistic screening for type II diabetes in women presenting with symptoms of PCOS, and this is certainly justified in some PCOS populations in which the prevalence of type II diabetes is as high as 15% (Legro, 2003). A similar argument could be used in favour of screening for dyslipidaemia, but the cost effectiveness of either test has not been evaluated in women with PCOS. As regards intervention, it would seem prudent to recommend lifestyle modification, particularly for obese women with PCOS. There is also some logic in the suggestion that long-term treatment with an insulin sensitiser such as metformin may be appropriate in patients with PCOS. Although the initial results of the use of insulin senstisers in women with PCOS has been promising, there have been no large-scale, randomised, placebo-controlled trial to support its use as frontline agent and its long-term cardiovascular (? beneficial) effects in women with PCOS.

CHAPTER 8

CUTANEOUS MICROVASCULAR FUNCTION IN YOUNG WOMEN WITH POLYCYSTIC OVARY SYNDROME

8.1 Introduction

The evidence of increased IMT in the CFA and CCA (Lakhani et al, 2004; Talbott et al; 2000, Guzick et al, 1996) and decreased compliance in the CCA and ICA (Lakhani et al, 2002) suggest that women with PCOS have accelerated arterial disease. Furthermore, the response of femoral artery blood flow to intra-arterial methacholine chloride (MCh) administration in 12 obese women with PCOS (elevated T, together with hirsutism and amenorrhoea or oligomenorrhoea – less than 6 periods per year) was 50% less than in 13 weight-and age-matched control women (regular menses – 27 to 31 days and no hirsutism or abnormal T levels), suggesting compromised endothelial NO production or release (Paradisi et al, 2001). There is also evidence of impaired cutaneous vascular function in women with previous gestational diabetes (Hannemann et al, 2002) and in patients with type II diabetes mellitus (Morris et al, 1995). The present study was designed to assess endothelial and vascular smooth muscle function in the skin microcirculation, using laser Doppler perfusion with transcutaneous iontophoretic administration of vasoactive compounds, in young women with PCOS and control women with normal ovaries.

8.2 Methods

The sample size required was calculated using data from a study of the response of the skin microcirculation to ACh and SNP in patients with NIDDM, where the increase in skin perfusion with ACh was 0.86 ± 0.09 in the study group and 1.36 ± 0.14 in the control group. Skin perfusion with SNP in the study group was 0.12 ± 0.05 and 0.45 ± 0.11 in the control group. Power calculation indicated minimum sample of 10 subjects in

each group was necessary to provide 80% power detecting differences of a similar magnitude in the present study at a significance of 5%.

8.2.1 Subjects and study design

Cross-sectional study of 24 women recruited from the North Middlesex and Royal Free Hospitals:

> 12 women with PCOS (defined according to the criteria in Section 4.2)

> 12 control women (staff members) with normal ovaries on TVS (Controls).

8.2.2 Protocol

All measurements were performed with the subjects sitting comfortably on an armchair in a quiet room with an ambient temperature of $22 \pm 1°C$. During a 15 min acclimatization period, the volar aspect of the right forearm was gently cleaned with an alcohol wipe and swabbed with deionised water. Blood pressure and pulse were measured on the left arm in all women using an automatic Dinamap device (Critikon Inc, USA) at 2 min intervals as described in Section 4.2.

The drug delivery iontophoresis protocol is described in Section 4.6. In brief, the anodal chamber was filled with 0.25 ml 1% (w/v) ACh and the cathodal chamber with 0.25 ml (w/v) 1% SNP. Baseline erythrocyte flux was measured for 100 s without current, i.e. no drug iontophoresed followed by drug delivery at 10, 15 and 20 μA, sequentially, each for 100 s, followed by 800 s at zero current. Microvascular perfusion was assessed continuously with laser Doppler and the mean erythrocyte flux was measured at baseline,

at 50 s intervals during iontophoresis at 10, 15 and 20 μA and at 100 s intervals thereafter.

8.2.3 *Statistical analysis*:

Intra-observer errors were determined, separately for each increment in current with ACh and SNP, by determining the coefficient of variation of repeated measurements. Normality of data was assessed using the Kolmogorov-Smirnov test. Data sets which were not normally distributed were assessed using the Mann-Whitney U test. Differences between normally distributed data sets were assessed by ANOVA. A Levene test for homogeneity of variance was simultaneously applied, and when test groups failed, the ANOVA result was ignored, and the data sets were $\log_{10}$ transformed, square root and/or reciprocal function, and re-tested for homogeneity of variance, and then by ANOVA. Differences were considered significant if $p \leq 0.05$. Data are expressed as mean ± SD. All analyses were performed using the statistical package SPSS for Windows (version 9.0)

8.3 Results

There was no significant difference between women with PCOS and controls with respect to age, SBP and DBP, and heart rate. These parameters were also comparable before and after iontophoresis (Table 8.1). Serum LH (p = 0.05), testosterone (p = 0.05) and BMI (p = 0.006), were elevated in women with PCOS compared to controls.

Table 8.1 **Physical, endocrine and biochemical parameters between PCOS and controls**

Variable	PCOS	Control	p-value between groups
No. of subjects	12	12	
Age, (years)	30.3 ± 4.4	27.7 ± 4.8	0.18
BMI, (kg/m^2)	31.1 ± 7.1	24.2 ± 3.5	0.006*
W/H ratio	0.77 ± 0.06	0.76 ± 0.04	0.60
Pre systolic blood pressure, (mm Hg)	115 ± 16	110 ± 7	0.30
Pre diastolic blood pressure, (mm Hg)	67 ± 12	63 ± 8	0.28
Pre heart rate, (beats/min)	73 ± 8	66 ± 10	0.09
Post systolic blood pressure, (mm Hg)	117 ± 17	111 ± 12	0.30
Post diastolic blood pressure, (mm Hg)	68 ± 11	67 ± 7	0.90
Post heart rate, (beats/min)	71 ± 10	65 ± 9	0.15
Luteinising hormone, (IU/L)	10.8 ± 6.6	4.8 ± 3.4	0.05*
Follicle stimulating hormone, (IU/L)	6.3 ± 2.30	5.8 ± 2.60	0.65
Testosterone, (nmol/L)	2.35 ± 0.78	1.71 ± 0.78	0.05*
Sex-hormone binding globulin, (nmol/L)	53.70 ± 47.5	57.4 ± 26.4	0.80
Prolactin, (mIU/L)	250.0 ± 113.0	273.0 ± 103.0	0.60
Oestradiol, pmol/L	257.0 ± 152.0	198 ± 126.0	0.60
Fasting Glucose, (mmol/L)	5.05 ± 0.45	4.98 ± 0.50	0.70
Fasting Insulin, (mU/L)	20.3 ± 11.3	11.4 ± 7.8	0.06
Total cholesterol, mmol/L	4.71 ± 0.97	4.50 ± 0.90	0.60
High-density lipoprotein, (mmol/L)	1.42 ± 0.41	1.53 ± 0.41	0.40
Low-density lipoprotein, (mmol/L)	2.99 ± 1.10	2.57 ± 0.85	0.30
Triglycerides, (mmol/L)	1.01 ± 0.39	0.88 ± 0.36	0.99

*significant; pre – before the start of iontophoresis; post – end of iontophoresis protocol.

The COV of repeated flux measurements after iontophoresis of ACh at 10, 15 and 20 were 9.5%, 10.8% and 12.6%, respectively. Corresponding figures for SNP were 5.1%, 9.1% and 16.6%, respectively. Basal forearm skin perfusion was not significantly different in women with PCOS compared with controls (23.58 ± 3.4 vs 24.23 ± 4.2; respectively, erythrocyte flux is in arbitrary units). The peak ACh induced erythrocyte flux occurred at the end of 20 µA iontophoresis period and there was a minimal difference in the median and mean erythrocyte flux at this point. The mean values were

compared by ANOVA. The median and mean values respectively were: Peak - PCOS 98.1 AU vs 125.1 AU, Controls 186.9 AU vs 200.8 AU; Baseline – PCOS 22.9 AU vs 20.8 AU.

The forearm skin blood flow increased in response of ACh (Figure 8.1) and SNP (Figure 8.2) within both groups for all time points compared to baseline, the increase percentage flux in response to ACh iontophoresis was however decreased significantly in women with PCOS relative to controls (p = 0.018; Figure 8.1); there was no difference in response to SNP (p = 0.59; Figure 8.2) between women with PCOS relative to controls.

Figure 8.1   The fold increase (Mean ± SEM) in forearm cutaneous microvascular erythrocyte flux in response to ACh iontophoresis, relative to the baseline flux at 100 s, in PCOS (n = 12) and control (n = 12) women.
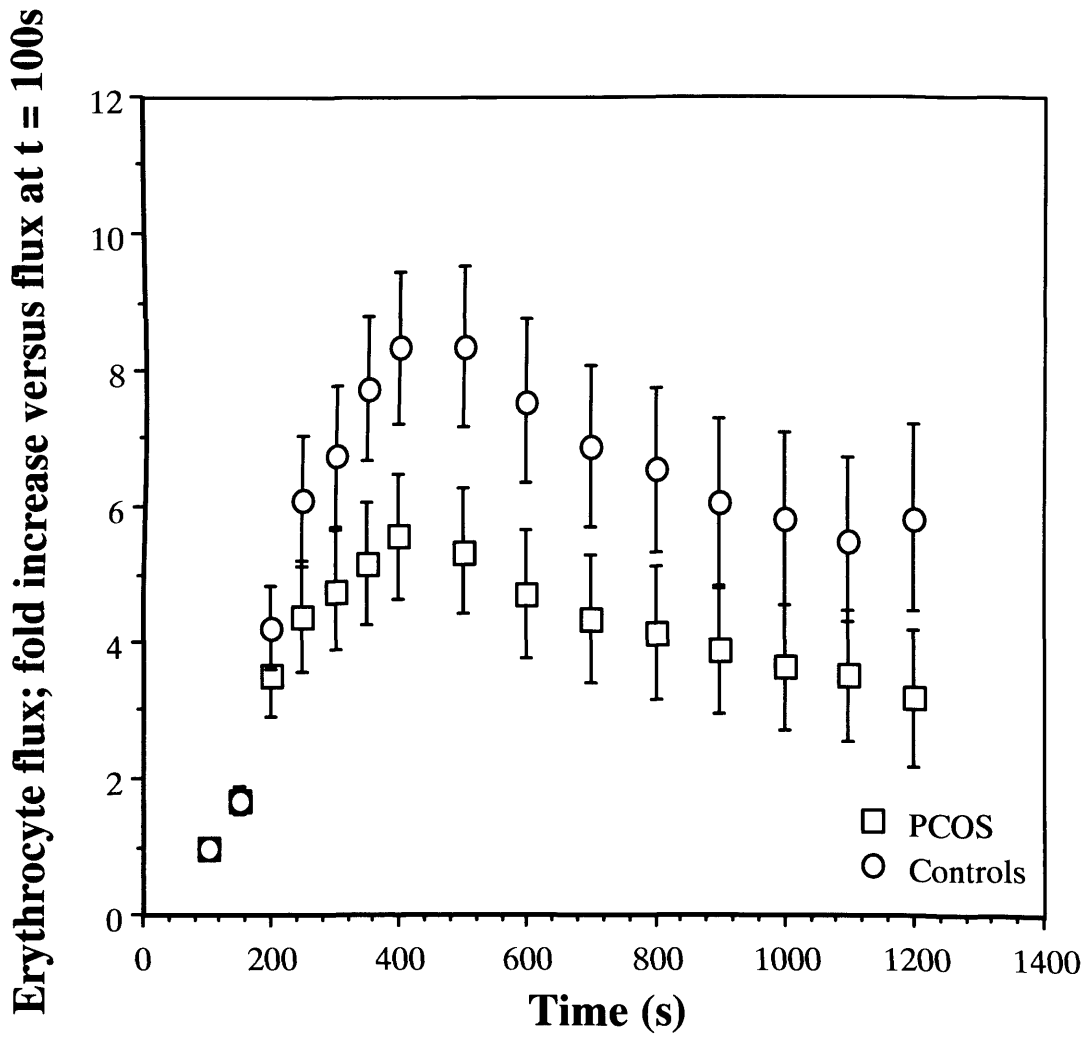
Figure 8.2   The fold increase in forearm cutaneous microvascular erythrocyte
flux in response to SNP iontophoresis, relative to the baseline flux at 100 s, in PCOS
(n = 12) and control (n = 12) women.
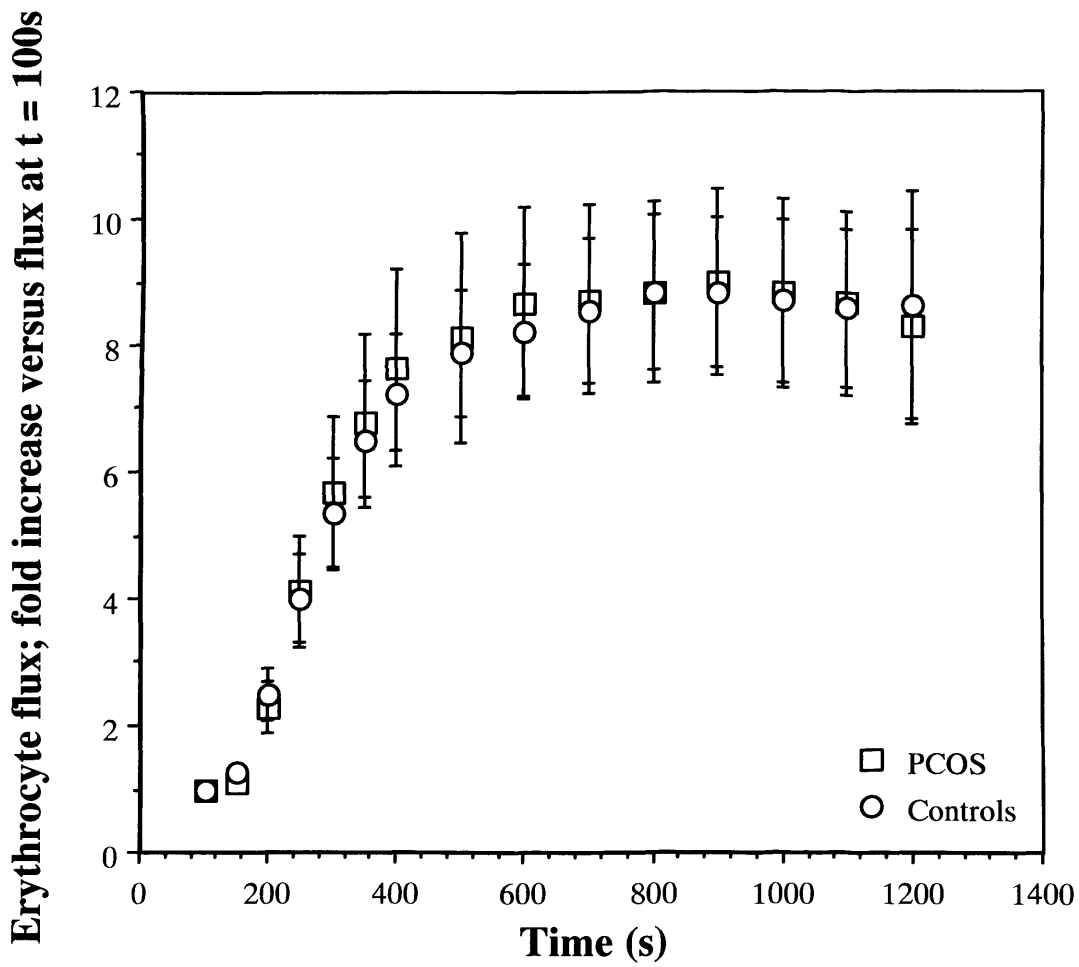
While no difference was noted in unadjusted mean peak erythrocyte flux between controls and PCOS group (p = 0.079), adjusted differences, sequentially adjusted for confounding variables, known *a priori*, to differ between PCOS and control women showed no effect in the mean peak erythrocyte flux in response to ACh between the two groups are shown in Table 8.2.

Table 8.2   **Differences in mean peak erythrocyte flux ratio in PCOS and control women**

Variable	Unadjusted	Adjusted BMI	Adjusted BMI, testosterone	Adjusted BMI, testosterone, Insulin
Number of women	24	24	24	18
Difference	0.17	0.26	0.24	0.21
95% CI	(-0.02 to 0.36)	(0.03 to 0.48)	(0.01 to 0.48)	(-0.10 to 0.52)
P value	0.079	0.026	0.041	0.016

8.4 Discussion

The results of this study demonstrate blunted cutaneous microvascular flux response to the vasodilator ACh in women with PCOS relative to controls, indicating that endothelial dysfunction may occur in the cutaneous microcirculation in young women with PCOS. The response to SNP (a nitric oxide (NO) donor), which acts directly on smooth muscle was comparable in women with PCOS and controls, suggesting normal smooth muscle function.

This altered response could be related to the metabolic or endocrine abnormalities present in women with PCOS. Insulin resistance is an obvious candidate since a similarly impaired microvascular response to ACh has been found in NIDDM (Morris et al, 1995; Pitie et al, 1997; Cabellero et al, 1999), and insulin resistance and glucose intolerance are present in 30 – 60% (Dunaif et al, 1992) and 8 – 40% (Robinson et al, 1993) of PCOS women, respectively. Indeed, mean serum insulin was almost doubled in women with PCOS in the present study, though this result just failed to reach significance (p = 0.06), probably due to limited sample size.

The abnormal response to ACh in diabetic patients is thought to result from decreased NO production or release (Morris et al, 1995; Pitie et al, 1997; Cabellero et al, 1999). It has also been suggested, however, that it may also be due to accumulation of products formed by a nonenzymatic reaction between glucose and collagen in the diabetic microvascular basement membrane (Bucala et al, 1991), which impairs endothelium dependent relaxation through the reduction of NO due to reduce Ca^{2+} influx as described

in Section 3.5.2 (Rodriguez-Manas et al, 1993). There is also evidence from animal studies of altered sensitivity of vascular smooth muscle to NO in diabetes (Okon et al, 2003).

Of these potential mechanisms, the results of the present study would support the possibility that endothelial NO production or release is compromised in women with PCOS, but the other mechanisms are unlikely to occur. Vascular smooth muscle sensitivity to NO is unaltered – there was no difference in the perfusion response to SNP, a direct NO donor. Furthermore, though insulin tended to be elevated in women with PCOS, no differences were seen in serum glucose, perhaps due to the young age of the women, suggesting basement membrane glycosylation would be similar in both groups.

The vasodilatory response to ACh is also impaired in hypercholesterolaemic (Khan et al, 1999), obese (Steinberg et al, 1996) and hypertensive (Panza et al, 1994) patients, independent of insulin resistance. Since BMI is increased in 35 – 60% of PCOS women, and there is evidence of increased cholesterol and blood pressure (Wild et al, 1985; Conway et al, 1992; Talbott et al, 1995), these factors could influence endothelial function in these women with the syndrome. In the present study BMI was elevated in the PCOS group, however, there was no evidence of altered serum cholesterol or BP, perhaps due to relatively young age of the women. In the comparisons of adjusted differences, BMI showed no effect on the cutaneous circulation and the effect of insulin was also not significant, though this may have been due, in part, to the small sample size (n = 18).

The characteristic endocrine feature of PCOS is hyperandrogenaemia. Testosterone is known to influence vasocontractile responses, and impairs endothelium dependent relaxation (Adams et al, 1995; Hutchinson et al, 1997) in hypercholesterolemic rabbits and monkeys. Furthermore, androgen deprivation in adult men enhances endothelium dependent relaxation (Herman et al, 1997). More recent studies suggest, however, that the vascular effects of testosterone may be more complex. For example, acute exposure to even low nanomolar doses of testosterone significantly potentiates endothelin-1 induced vasoconstriction in coronary artery rings (Teoh et al, 2000b). However, in the present study, testosterone showed no effect on the cutaneous circulation in women with PCOS compared with controls since the response differences were still apparent after testosterone had been adjusted for in the analysis.

Questions have been raised regarding the cause of vascular flux increases occurring during iontophoresis. The galvanic effect of current and voltage application causes vasodilation, even in the absence of drug in the iontophoresis chamber (Berghoff et al, 2002). The low current used in this study would have limited such effects (Berghoff et al, 2002). Furthermore, any limited galvanic vasodilatation would have been more apparent in the cathodal (SNP) chamber, where no differences were noted between the two groups (Morris and Shore, 1996). Iontophoresis of ACh can stimulate local C-nociceptive nerve fibres causing indirect nerve axon reflex (Walmsley et al, 1990). It has recently been shown, however, that local anaesthesia has no effect on the total ACh induced vasodilatation and that ACh iontophoresis is a reliable index of skin endothelium-

dependent vasodilation, and is unaffected by local nerve fibre function (Caselli et al, 2003).

In summary, this is the first report of microvascular endothelial dysfunction in women with PCOS. The ACh induced increase in microvascular perfusion due to vasodilation, which is dependent on endothelial NO release, was blunted in women with PCOS. This is perhaps a result of impaired endothelial NO release related to endocrinological or metabolic changes other than BMI, testosterone and insulin in women with the syndrome.

CHAPTER 9

AORTIC FUNCTION IS COMPROMISED IN A RAT

MODEL OF POLYCYSTIC OVARY SYNDROME

9.1 Introduction

The finding of increased vascular stiffness (Lakhani et al, 2002) and IMT in the carotid and femoral arteries in women with PCOS (Lakhani et al, 2003), suggests they exhibit increased atherosclerosis and underlying endothelial dysfunction (Balletshofer et al, 2003). Indeed evidence of endothelial dysfunction in women with PCOS has been observed *in-vivo* in the femoral artery (Paradisi et al, 2003; Kelly et al, 2003) and in the microvasculature (Chapter 8), though not in the brachial artery (Mather et al, 2000).

An animal model would allow *in-vivo* analysis of arterial function followed by *in-vitro* investigation of endothelial function, albeit such data would require cautious interpretation due to species differences. In the rat, daily injections of mifepristone (an antiprogestin, also know as RU 486) for one week or more, induce many features characteristic of PCOS in humans, for example ovulatory failure, persistant vaginal cornification, and enlarged ovaries containing atretic follicles and follicular cysts (Sanchez-Criado et al, 1993a; Ruiz et al, 1996) as well as increased serum concentrations of LH, T, oestradiol and serum insulin-like growth factor 1 levels and decreased prolactin (Sanchez-Criado et al, 1997; Ruiz et al, 1997). The mifepristone-treated rat is a 'fundamentally adequate PCO model' with which to investigate the effect of PCOS-like endocrinological perturbations in the short term (1 – 2 weeks). It is not a suitable model with which to study long-term cumulative changes, likely to occur in older women with PCOS.

This model was used to test the hypothesis that arterial mechanics and endothelial function are modified in PCOS, since it allowed *in-vivo* assessment of the mechanical properties of the aorta followed by precise *in-vitro* assessment of endothelial and vascular smooth muscle function.

9.2 Methods

9.2.1 Study design

The procedures and protocols of the study have been approved by the Home Office under the Animals (Scientific Procedures) Act 1986. It is part of the project licence number 70/5735 and is entitled 'Haemodynamic assessment of PCOS'. In order to do this work, the author had to satisfactorily complete an accredited course laid out by the Home Office to fulfil the Animals (Scientific Procedures) Act 1986 and successfully applied for a personal licence (PIL 70/18075).

Animals

Adult female (12 – 14 weeks) Sprague-Dawley rats (body weight 242 ± 4.5 g) were bred and housed locally at 22°C under a 14 hour on /10 hour off light cycle, with free access to food and water. Vaginal smears were prepared from rats on up to three occasions to assess oestrus i.e. presence of cornified cells only. Only those rats showing at least two consecutive 4 – 5 day oestrus cycles (Figure 9.1) were allocated to the treated group (Group 1) or control group (Group 2).

Group 1 – treated group: Eight rats were subcutaneously injected daily with of mifepristone (11β-17β-hydroxy-17α-estra-4,9-diene-3-one; Sigma Aldrich Chemicals,

Gillingham, Dorset, UK) suspended in olive oil at a dose of 2 mg/0.1 ml olive oil/100 g body weight for 6–10 consecutive days, beginning on the day of oestrus.

Group 2 – control group: Six rats treated daily with subcutaneous injection of 0.1 ml olive oil/100 g body weight daily over 6–10 consecutive days beginning on the day of oestrus.

Figure 9.1   Histology slide demonstrating oestrus: Vaginal smear consisted of cornified cells only – suggesting the female is on 'heat'
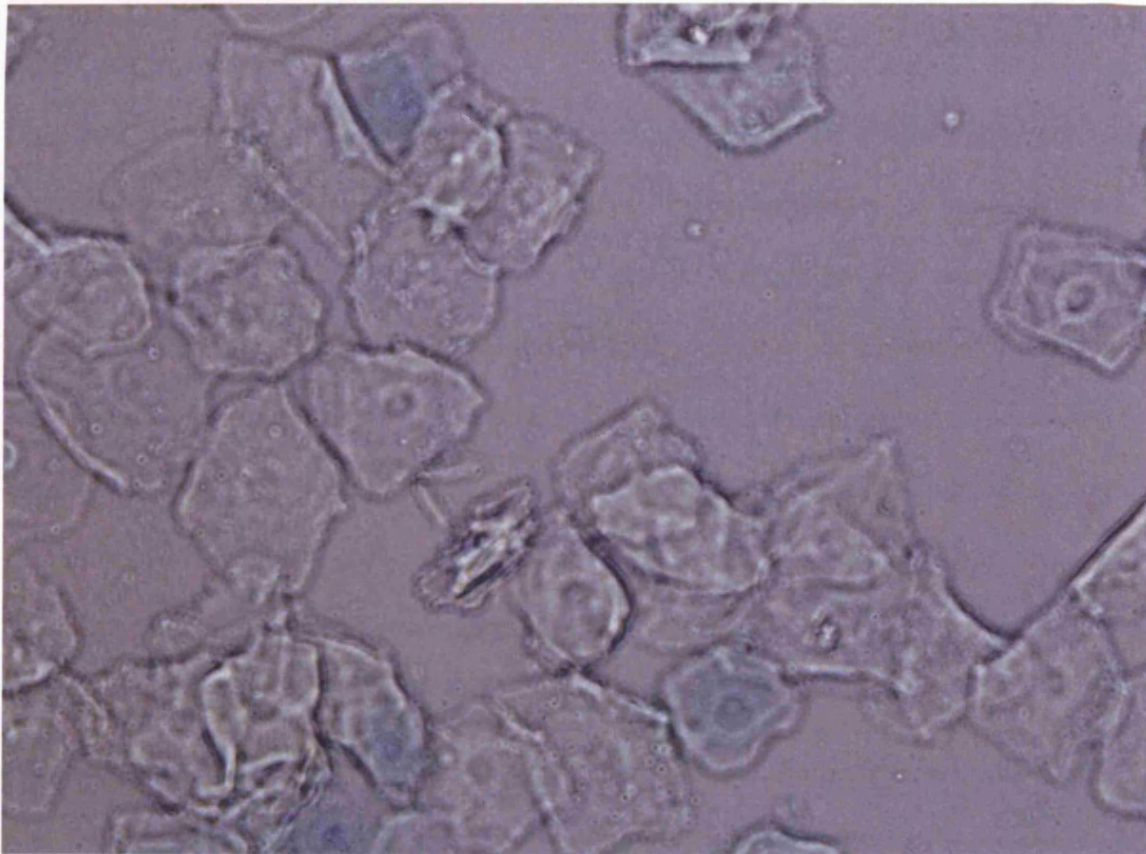
At the end of the injection regimen (6 – 10 days), anaesthesia was induced in each rat with intramuscular injections of Hypnorm (fentanyl citrate and fluanisone; Janssen Animal Health Ltd, Buckinghamshire, UK; 0.5 ml/kg) plus diazepam (Dumex Ltd, Hertfordshire, UK; 2.5 mg/kg). General anaesthesia was maintained using isoflurane (Baxter Health Ltd, Norfolk, UK), nitrous oxide and oxygen through a standard anaesthetic circuit (Yang et al, 2003). Body temperature was monitored with a rectal probe maintained at 37–38°C with an electric heating mat. Arterial oxygen saturation and heart rate were monitored non-invasively with a Biox 3740 pulse oximeter (Ohmeda Inc, Louisville, Colarado, USA), to ensure a consistent level of anaesthesia was maintained.

9.2.2 Measurements

Once stable anaesthesia was established, the abdomen was shaved to allow good contact between the ultrasound probe and the skin, thus facilitating accurate measurements of the aorta. The technique of duplex estimation of arterial compliance has been described previously (Section 3.4.2). In brief, real time B-mode and M-mode images of the arterial wall motion were recorded using a 7.5 MHz linear probe. Measurements were made in the sagittal plane at 90^0 to the long axis of the thoracic aorta with signal output to a high resolution, echo-locked wall-tracking system (Wall Track, Pie Medical Systems, Maastricht, Netherlands) and data analysed off-line.

After ultrasound, a midline laparotomy was performed and the peritoneum was opened. The adipose tissue surrounding the aorta and inferior vena cava was removed with dissection forceps and the aorta was exposed. Aortic SBP and DBP were measured by

intra-aortic probe with Datex Engstron Light Monitor (Datex-Ohmeda Division, Instrumentarium Corp., Helsinki, Finland) and aortic blood flow with a transonic flowmeter system (HT 207; Transonic Medical, New York, USA). Trunk blood was obtained from vena cava and serum prepared by centrifugation and stored at $-20\,^{\circ}\text{C}$ until required. Serum concentrations of LH, FSH, T and insulin were assayed using kits specific for animals. Animals were killed by exsanguination and the thoracic aorta was dissected, cleaned of adherent tissue, washed several times and placed in oxygenated Krebs buffer solution (NaCl 118.6 mM; KCl 2.8 mM; $CaCl_2$ 2.5 mM; $MgSO_4$ 1.2 mM; $NaHCO_3$ 25.1 mM; KH_2PO_4 1.2 mM; glucose 5.5 mM), until *in-vitro* function was assessed. The right ovary was also dissected, fixed in formalin and embedded in paraffin wax. Serial 6 μm sections were cut from wax embedded ovary and assessed to determine the presence of ovarian morphology.

In-vitro endothelial function was assessed as described in Section 4.6. In brief, the dilatory response to graded dose ACh was assessed on PE precontracted aortic rings. The PE-ACh treatment cycle was repeated in the presence of N^G – nitro-L-arginine methyl ester (L-NAME), followed by final treatment with graded doses of SNP. Aortic tension was expressed as percentage relaxation, such that the tension induced by 3 μM PE was defined as 0% relaxation, and the tension prior to PE treatment was defined as 100% relaxation.

9.2.3 Data and statistical analysis

For *in-vivo* data, the intra-observer variability was determined by calculating the coefficient of variation of repeated measurements. Coefficients of variations below 10% were considered to indicate good reproducibility. The average vessel wall movement was calculated from readings over three cardiac cycles, and the diametrical compliance (C) and stiffness index (β) were calculated as previously described (Section 3.4.2) from this data, using the aortic blood pressure and flow estimates taken immediately after laparotomy. Statistical significance was tested by analysis of variance (ANOVA) with Fisher's protected least significant difference test. All data are expressed as mean ± SEM. All analyses were performed using the statistical package SPSS for Windows (version 10.05).

9.3 Results

Treatment of female rats with mifepristone had no significant effect on body weight (244.8 ± 5.2 vs 238.3 ± 8.3 g, treated vs. controls respectively), SBP (74.4 ± 6.6 vs. 62.0 ± 1.6 mmHg) or DBP (59.8 ± 5.80 vs. 54.3 ± 3.3 mmHg). Hormonal disturbances were apparent – treated rats exhibited increased serum concentrations of LH (15.15 ± 1.46 IU/L vs. 9.35 ± 2.16 IU/L; $p < 0.05$) and testosterone (1.81 ± 0.51 nmol/l vs. 0.47 ± 0.10 nmol/l; $p < 0.05$). Serum insulin also tended to be elevated (1.44 ± 0.49 mU/L vs. 0.76 ± 0.22 Mu/L) but this difference failed to attain significance perhaps due to hypervariable insulin concentrations in the treated animals. Mifepristone treatment had no effect on serum FSH levels (143.5 ± 20.6 ng/ml vs. 150.0 ± 30.1 ng/ml). Mifepristone- treated ovaries exhibited an altered morphology relative to controls, as expected, with evidence

of arrested follicular growth, an increase in the abundance of atretic follicles and follicular cysts (Sanchez-Criado et al, 1992; 1993b) (Figure 9.2). Although the histology confirmed ovarian morphology, it was difficult to accurately obtain qualitative data due to the lack of qualified personnel.

Figure 9.2   Histology of ovaries on day 9 in rats treated with mifepristone

A – General view of one ovarian section showing arrested follicular growth

B – Follicular cyst with massive luteinisation
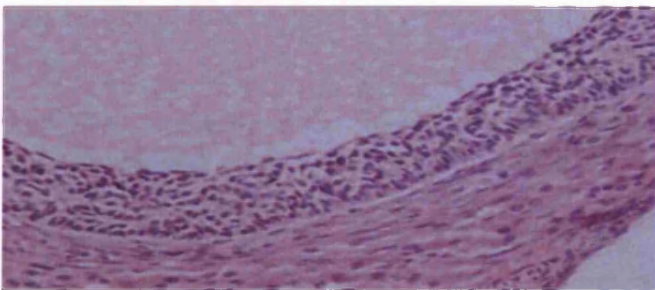
C – Increased atretic follicles
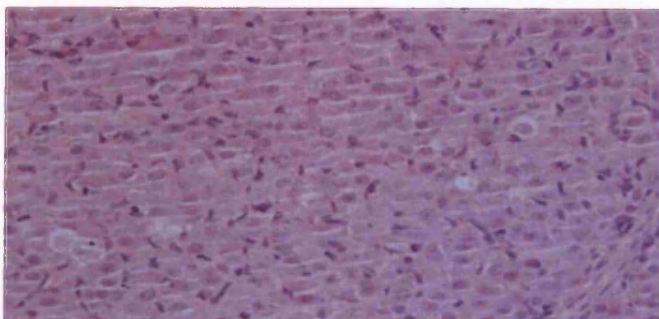
Coefficients of variation for ultrasound estimation of aortic parameters were in nearly all cases < 10%, indicating these measurements were made with an appropriate level of reproducibility. In mifepristone-treated animals, the mean aortic diameter and blood flow were unaffected, relative to the controls, but aortic compliance was reduced by 67%, while stiffness index was increased 2.3-fold (Table 9.1).

There was minimal difference in the median and the mean compliance values between the mifepristone treated rats and controls. The mean values were compared by ANOVA. The median and mean compliance values for each group respectively: Compliance – mifepristone treated rats 109.35 vs 140.88 % mmHg^{-1}x 10^{-2}; Controls 399.0 vs 426.86 % mmHg^{-1}x 10^{-2}. Similarly, the differences in percentage relaxation at each concentration of ACh and SNP between the median and mean values were minimal; therefore the mean values were plotted.

Table 9.1 Aortic diameter, blood flow and mechanical parameters in mifepristone treated rats and control rats (mean ± SEM)

Variable	Control (n = 6)	Mifepristone (n = 8)	p - value
Diameter, (mm)	1.32 ± 0.02	1.44 ± 0.04	NS
Blood flow, (ml/min)	37 ± 5.1	32.0 ± 2.6	NS
Compliance, (%mm Hg^{-1} x 10^{-2})	426.86 ± 17.36	140.88 ± 6.33	0.05
Stiffness index	0.57 ± 0.03	1.30 ± 0.07	0.02

In vitro organ bath assessment of endothelial and smooth muscle function was performed to determine whether the changes in aortic compliance and stiffness might be related to dysfunction of these tissues. ACh induced a concentration-dependent relaxation in PE contracted aortic rings from mifepristone treated and control animals ($p < 0.001$; Figure 9.3), however, relaxation was less in mifepristone-treated animals than in controls ($p = 0.002$), notably at 0.1 µM and 1.0 µM (Figure 9.3).

ACh-stimulated aortic relaxation is endothelium-dependent, and thought to be due to activation of eNOS, producing NO, which diffuses to the underlying smooth muscle causing relaxation (Palmer et al, 1987). Indeed L-NAME, which inhibits 80% of eNOS activity, considerably impaired ACh-induced relaxation in aortic rings from normal and mifepristone-treated animals ($p < 0.001$ for L-NAME effect in both the mifepristone-treated and control groups). This inhibitory effect was, however, less in aortic rings from the mifepristone-treated rats than in controls ($p < 0.001$), notably at ACh concentrations of 1 µM and above (Figure 9.3).

Figure 9.3 ACh-induced relaxation in aortic rings from mifepristone-treated (circles) and control (squares) rats, without (filled) and with (unfilled) L-NAME. *p < 0.05; **p < 0.005, treated *vs* appropriate control, by 1 way ANOVA.

ACh
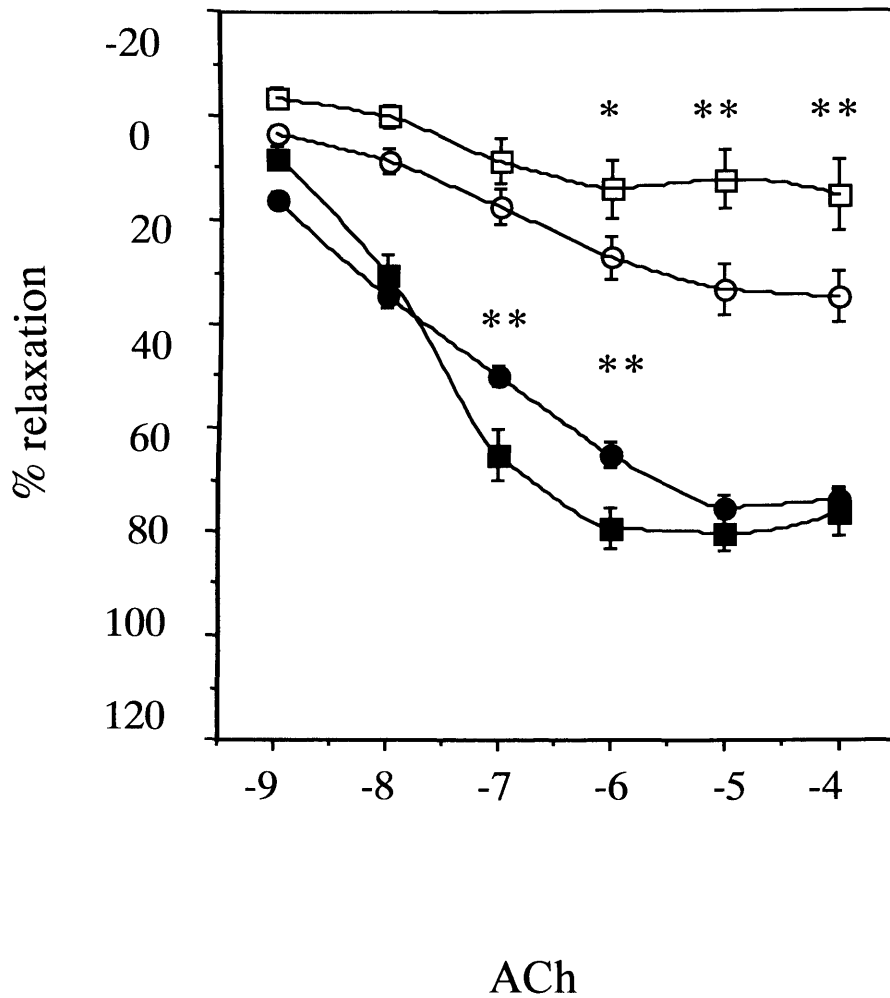
To determine smooth muscle function independent of endothelial NO, the aortic dilation was assessed using graded doses of SNP, a non-endothelium dependent NO donor. As for ACh, SNP-induced concentration-dependent (p < 0.001, SNP effect by 2-way ANOVA) relaxation in aortic rings from mifepristone-treated and control animals (p < 0.001).

Relaxation was however greater in mifepristone-treated animals compared with controls

(p = 0.001, mifepristone treatment effect by 2-way ANOVA), notably at 0.01 µM and 0.1

µM SNP (Figure 9.4)

Figure 9.4   SNP induced relaxation in aortic rings from mifepristone-treated (circles) and control (squares) rats. ***p < 0.001, treated *vs* control, by 1 way ANOVA.
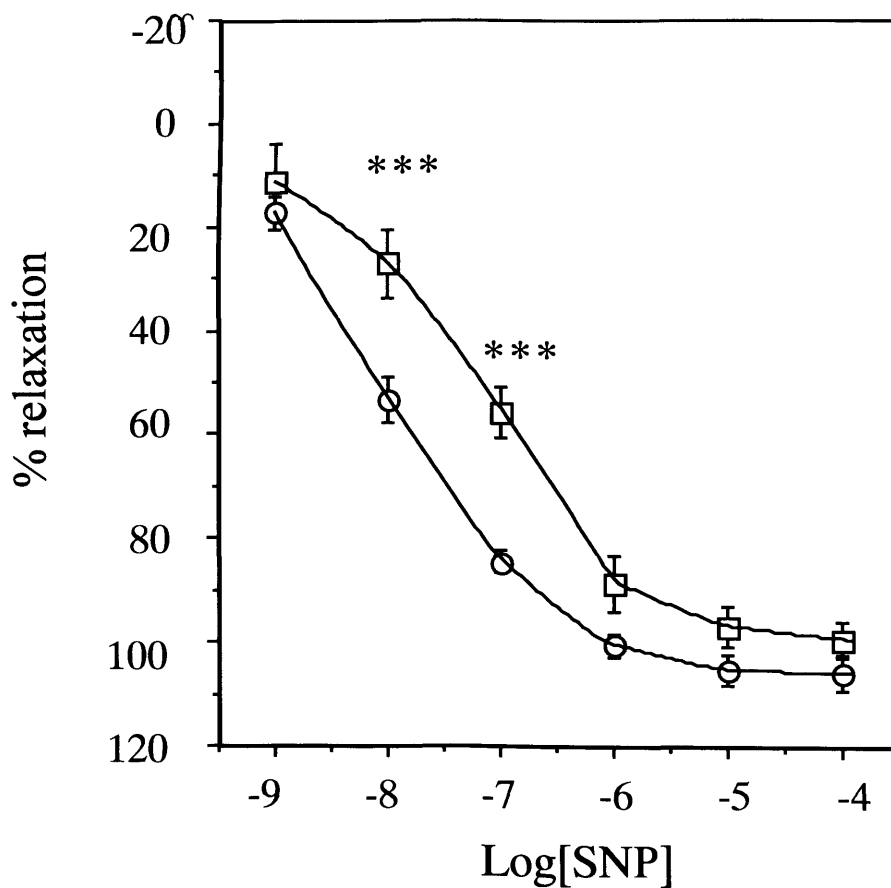
9.4   Discussion

Female rats injected with mifepristone for 7 – 9 days displayed signs similar to those seen before in this model (Sanchez-Criado et al, 1992; 1993a, b; Ruiz et al; 1996) and in women with PCOS (Goldzieher, 1982; Lakhani et al, 2002; Conway et al, 1989; Franks, 1989). Ovarian morphology was disrupted – follicular development was retarded with more small follicles and increased follicular atresia; serum LH and testosterone were also elevated. The lack of an effect on insulin may be related to the young age of the rats (12 – 14 weeks) and the short-term nature of the model. Despite this, the mifepristone-injected rat model displays may other features seen in the human PCOS women. It provides a valid model in which to assess the short-term effects of the associated ovarian and endocrine disturbances on aortic function.

Mifepristone treatment modified rat aortic vascular mechanics – the aortic stiffness index was increased and conversely the compliance was decreased. These results are similar to those seen in women with PCOS, albeit in other vascular beds (Lakhani et al, 2002; Kelly et al, 2003).

In-vitro assessment of aortic endothelial and smooth muscle function revealed significant alterations in aortic rings from mifepristone-treated rats. ACh stimulated endothelium-dependent NO mediated relaxation was reduced, while the endothelium–independent action of SNP on smooth muscle was exaggerated. L-NAME substantially inhibited ACh-induced relaxation in control rings, confirming this process to be NO mediated; residual relaxation may be non-NO mediated or possibly L-NAME inhibition of eNOS was incomplete. Indeed, L-NAME appears to inhibit only 80% of eNOS activity (Li and

197

Fostermann, 2000). The inhibitory effect of L-NAME was less pronounced in aortic rings from mifepristone-treated animals and the putative non-NO mediated, ACh-stimulated, dilatory pathway may be more active in mifepristone-treated animals. These changes in endothelial and smooth muscle function *in vitro* are consistent with the increase in aortic stiffness index and decrease compliance noted *in vivo*.

These results are the first demonstration of *in vivo* and *in vitro* of aortic dysfunction in a rat model of PCOS. The endothelium synthesizes many vasodilators, NO being the primary vasodilator in arteries such as the rat aorta, whereas EDHF is more prominent in small arteries (Shimokova et al, 1996; Ge and He, 2000). Prostacyclin was originally thought to have a lesser vasodilatory role in aorta than NO (Palmer et al, 1987) and its importance may rest in its inhibition of smooth muscle growth and platelet aggregation. Furthermore shear stress promotes endothelial prostacyclin synthesis and activates receptor-operated non-selective cation channels, increasing intracellular Ca^{2+} and K^+ concentrations, stimulating the release of NO and EDHF. The results of this study are consistent with the possibility that aortic endothelial eNOS activity and NO synthesis may be impaired, and/or NO degradation enhanced in mifepristone-treated rats. In partial compensation, the aortic smooth muscle appears to become more sensitive to NO and a non-NO mediated, ACh stimulated vasodilatory pathway may be activated. This may reflect an increase in endothelial EDHF or prostacyclin release in the mifepristone-injected aorta.

The changes in endothelial behaviour and aortic mechanics may result from the endocrine consequences of mifepristone injection. Serum oestradiol is elevated in this model (Sanchez-Criado et al, 1993; Ruiz et al, 1996) but this should enhance, not diminish, vasodilatory responses since oestradiol stimulates NOS expression and endothelial NO synthesis in rat aorta (Goetz et al, 1994; Andersen et al, 1999) and cultured endothelial cells (Lantin-Hermasos et al, 1997). In addition, oestradiol enhances ACh induced rat aortic relaxation *in vitro*, probably via increasing NO release (Huang et al, 1998; Teoh et al, 2000a) and oestrogen replacement also normalises the femoral artery blood flow response to ACh in oophrectomised women (Pinto et al, 1997).

The elevated serum testosterone concentration in mifepristone-treated animals could decrease vasodilatory responses (Herman et al, 1997) in line with the results of the present study. Indeed in PCOS women, increases in leg blood flow induced by methacholine chloride (an ACh mimic) were negatively correlated with testosterone levels (Steinberg et al, 1996), albeit the causal link may be with hyperandrogenism associated with hyperinsulinaemia and insulin resistance (Steinberg et al, 1996). Indeed in type II diabetes, methacholine chloride-induced leg blood flow is negatively correlated with these parameters, perhaps due to the effects on NO degradation and/or smooth muscle sensitivity to NO (Williams et al, 1996). In the present study, however, mifepristone had not much effect on insulin and sensitivity to SNP was elevated; aortic NO degradation might be thought to be disturbed. It is plausible that testosterone influences endothelial function directly (Paradisi et al, 2003) as it stimulates aortic ring dilation *in vitro* by enhancing prostanoid but not NO synthesis, independent of androgen

receptor (Tep-areenan et al; 2003). The non-NO mediated ACh-stimulated vasodilatory pathway in mifepristone-treated rats may be due to the elevated serum testosterone activating an aortic prostanoid vasodilatory pathway.

Serum progesterone is depressed in PCOS women (Goldzieher, 1981) hence the use of mifepristone as a progesterone and glucocorticoid antagonist (Philibert, 1984), to reduce progesterone action in a rat model (Sanchez-Criado et al, 1993a). The effects of mifepristone on the rat aorta may be due to reduced aortic progesterone or glucocorticoid action. Progesterone has a dilatory action on rat aorta (Glusa et al, 1997; Mukerji et al, 2000; Zhang et al, 2002) due to the blockage of smooth muscle Ca^{2+} channels (Glusa et al, 1997) and/or stimulation of endothelial prostacyclin synthesis with consequent NO release (Zhang et al, 2002). These effects do not though involve the mifepristone antagonised nuclear progesterone receptor and are non-genomic (Glusa et al, 1997; Zhang et al, 2002). Whether mifepristone interacts with a progesterone receptor to inhibit vasodilation *in vivo* is unknown. As it is a progesterone analogue it is possible and might explain some of the results in this study. The impact of mifepristone *in vitro* was thought to be minimal, as the aortic rings were repeatedly washed in Krebs buffer before analysis; this is known to prevent acute effects of progesterone and thus mifepristone (Zhang et al, 2002). A long-term effect of mifepristone-progesterone blockade on aortic gene expression cannot be ruled out as the cause of the changes seen in the present study. However, progesterone treatment over 8 days, albeit at supraphysiological levels, had no effect on noradrenaline-contracted aortic rings from ovarectomised rats (Sampaio-Moura and Marcondes, 2001). Glucocorticoids inhibit eNOS expression and prostacyclin

synthesis in rat aorta in a manner blocked by mifepristone (Jeremy and Dandona, 1986; Wallerath et al, 1999). Thus mifepristone could promote vasodilation, as is seen in aortic rings from male rats (Tep-areenan et al, 2003). In the present study vasodilation was inhibited indicating that the anti-glucocorticoid effects of mifepristone had minimal influence.

Endothelial impairment occurs in hyperlipidaemia (Shimokowa et al, 1989) whereas its association with hyperglycaemia is controversial (Poston and Taylor, 1995; Oltman et al, 1997). It is possible that the aortic function in this study is the result of metabolic modifications related to mifepristone injection. This model is, however, poorly characterised at the metabolic level, and it is therefore difficult to address the possible involvement at this time.

Changes in aortic mechanics were observed in mifepristone-treated, anaesthetised rats, albeit under conditions standardised to remove variability due to the depth of anaesthesia and the use of nitrous oxide, which is vasoactive. The changes were therefore considered treatment related, but their impact may be modified by the condition of the model i.e. rats were anaesthetised. Thus aortic changes did not significantly alter aortic blood flow in mifepristone-treated rats. Another methodological caveat is that the mechanical calculations relied on ultrasound measurements recorded pre-laparotomy and blood pressure estimates made post-laparotomy. Blood pressures may have been modified by laparotomy but a consistent level of anaesthesia was achieved in all rats, thus variability in blood pressure related to the stress of laparotomy was thought to be minimal.

In summary, this is the first demonstration that rat aortic mechanical characteristics are disturbed *in vivo* in the mifepristone-treated rat model of PCOS, in a manner comparable with human studies (Lakhani et al, 2002; Lakhani et al, 2003). Aortic ring behaviour is modified *in vitro* suggestive of decreased endothelial NO release or elevated NO degradation. In compensation, smooth muscle sensitivity to NO is elevated and a non-NO mediated ACh stimulated relaxation mechanism appears activated. The latter mechanism may be due to increased prostacyclin or EDHF synthesis or release.

CHAPTER 10

SUMMARY

10.1 Introduction

The studies described in this thesis were undertaken to evaluate the hypothesis that vascular dysfunction is an early consequence of PCOS, and is a prelude to atherosclerosis in later life.

To do this, arterial mechanical vascular properties were assessed in young women with PCOS, PCO alone, and in controls. The initial studies assessed a measure of downstream vascular resistance – the PI and the effect of inhalation of 5% CO_2, a known vasodilator stimulus in the ICA (Chapter 5). Subsequent studies discussed in Chapters 6 and 7 assessed a measure of arterial wall elasticity – compliance and stiffness index in the ICA and the CCA, and a measure of arterial wall integrity – IMT in the CCA and the CFA in these subject groups. Changes in arterial mechanical properties were noted in PCOS women, therefore endothelial function *in vivo,* at least in the cutaneous microvasculature, was investigated in young women with PCOS and in controls, as discussed in Chapter 8. This study used non-invasive laser Doppler ultrasound to measure cutaneous microvascular perfusion responses to transcutaneous iontophoretic administration of endothelial-dependent (ACh) and endothelial-independent vasoactive (SNP) vasoactive compounds. To assess the endocrine and metabolic perturbations seen in PCOS on endothelial function and arterial wall mechanics, and the interaction between the two phenomena, the mifepristone-treated rat model was used, as discussed in Chapter 9. This model allowed ultrasound assessment of aortic mechanical properties *in-vivo*, followed by assessment of endothelial and smooth muscle function *in-vitro*. The purpose of this

final chapter is to summarise the conclusions of all the studies, the chief limitations and consider areas which merit further research.

It should be noted that there are some limitations with respect to the endocrine assessment for other causes of hirsutism in women proposed to have PCOS in this thesis. Hyperandrogenism is key feature of PCOS, the major sources of hyperandrogenism in women are the ovaries and adrenals. Hyperandrogenism arising from the ovary or adrenal does not always result in skin manifestations (acne/hirsutism) and, in general, the expression of skin presentation is viewed to be a peripheral disorder (Lobo, 2002).

Testosterone and dihydrotestosterone are the most biologically active androgens and are derived from the peripheral conversion of androstenedione. In 'normal' women, about 1% of circulating testosterone is unbound, 66% is bound to SHBG and 30% is bound to albumin (Dunn et al, 1981). In hirsute PCOS women where total and free testosterone levels are within normal range, there is evidence of a possible increase in 5-α reductase activity within the hair follicles, which increases the conversion of testosterone to the more potent dihydrotestosterone. Skin biopsies from these women revealed increased 5-α reductase activity, which correlates with an androgen metabolite, 5α-androstane-3α, 17β-diol-17glucuronide (Paulson et al, 1986).

Rare causes of hyperandrogenism include ovarian tumor – Arrhenoblastoma also known as stromal tumour, gonadal stromal tumour, androblastoma, which secretes testosterone. This is a rare tumour and accounts for less than 0.5% of all ovarian tumours. All women

recruited in this thesis had transvaginal scan and none had any evidence of ovarian pathology.

Androgen secreting adrenal tumours is rare; the clinical course of the disease is a good indicator for a detailed evaluation. Endocrine stigmata of Cushing's syndrome are usually clinically recognizable. Hypercortisoleamia for obese women with PCOS can be differentiated by determination of 24-hour urinary free cortisol levels, which will be normal in PCOS women and high in Cushing's syndrome. Sudden onset and rapid progress of clinical hyperandrogenism was not seen in any of the women recruited in the studies described in this thesis and none had features of Cushing's Syndrome – moon face, 'buffalo hump' (collection of fat between shoulders) or purple striations on abdomen, thighs or breasts, furthermore, the blood pressure was also within normal range in all women with PCOS.

The findings of elevated androgen levels also demands exclusion of non-classical adrenal hyperplasia (NCAH), which often mimics PCOS (Lobo et al, 1980). A fasting 17-hydroxyprogesterone level of > 4 ng/ml should arouse suspicion of NCAH. However, a more robust diagnosis can only be confirmed by dynamic test with adrenocorticotrophin stimulation following dexamethasone suppression.

During data collection for this thesis, the European definition for the diagnosis of PCOS was used which required ultrasound documentation of polycystic ovarian morphology mandatory with the presence of clinical manifestations of hyperandrogenism (acne/hirsutism/alopecia) and/or menstrual disturbances

(oligomenorrhoea/amenorrhoea/infertility; Adams et al, 1986; Balen et al, 1995) and not the National Institute of Health (1990) definition which requires the exclusion of secondary causes NCAH and androgen secreting tumours (Zawadzki and Dunaif, 1992). Secondly, the use of 17-hydroxyprogesterone for 'mild/moderate' hirsutism was not performed and available at the time. Furthermore, it is not possible to perform this test at present because all the saved serum samples were lost due to freezer breakdown in the Department of Biochemistry. Therefore, it is possible that some women with PCOS may have NCAH in association with PCO morphology. However, there is evidence that only 4 - 7% of women with PCOS may have NCAH (Benjamin and Deutsch, 1986; Azziz et al, 2004).

One of the limitations in the human studies in this thesis with respect to hyperandrogenism is that the principal biochemical abnormality in women with PCOS is often attributed to enhanced androgen production by both the ovaries and adrenals. Other causes of hyperandrogenism were not investigated in any of the studies in this thesis because none of the women had gross hirsutism or any clinical evidence of Cushing syndrome or androgen secreting tumours.

Another limitation regarding the human studies in this thesis was that the evidence for ovulation was not assessed in the oligomenorrhoeic subjects. Spontaneous ovulation may have occurred in women with PCOS during the time of the examination. This was considered unlikely, although possible. If ovulation did occur in a small number of

women with PCOS, how this may have influenced the vascular mechanical properties is not known.

The cycle length of 21 – 35 days used in the controls in this thesis is the same as that used in previous studies of PCOS (Conway et al, 1989; Clayton et al, 1992; Rodin et al, 1998). It is commonly held that the mean menstrual cycle length is 28 days, with a standard deviation of ca. 2 days (Scommegna & Dmowski, 1977). The range 21 – 35 days defines the limits of the normal set of values in that values outside this range are unlikely (p > 0.05) to belong statistically to the normal set of values. In a more clinical justification for this range, it has been said that 'cycles as short as 21 days and as long as 40 days should be considered within physiologic limits, unless associated with symptoms or findings indicative of pathology' (Scommegna & Dmowski, 1977).

The 21 – 35 day menstrual cycle length range, in defining the control subjects in this study, may however complicate the interpretation of the results. Since cycle length variability is normally due to differences in follicular phase length, subjects with 21 – 35 day cycles may have 7 – 21 day follicular phases. Serum sampling and assessment of vascular parameters at day 4 may, therefore, actually have been at different relative cycle times in different subjects, depending on their menstrual cycle length. This may have influenced the serum levels of hormones and any hormonally regulated vascular parameters measured in this thesis. In mitigation, changes in serum levels of major sex hormones are limited in the first third of the follicular phase, albeit other hormones may be cycle dependent and influence the vasculature. In addition, the potential variability due

to the relative timing of sampling/vascular assessment would be expected to increase the range of values in the normal and PCO groups, thus tending to reduce the significance of any differences between groups, and not to produce spuriously significant results. Furthermore, the effect of such variability, if present, would be to skew the data set, whereas all data passed a statistical test of normality. In retrospect however, it would have been preferable to use a narrower range for 'normal' cycle length in order to minimise this potential influence on the outcome measures.

As previously, the ratio between fasting plasma insulin and fasting plasma glucose was used to assess insulin resistance (Holte, 1996). The hyperinsulinaemic glucose clamp is considered to be the 'gold standard' and should be the preferred method to measure insulin sensitivity. In this test, exogenous insulin is administered as a prime followed by constant infusion to maintain physiological suprabasal insulin levels. Simultaneously, glucose concentration is monitored frequently and clamped at the euglycaemic or isoglycaemic (patients own fasting glycaemia) concentration by glucose infusion at variable rates. When a steady rate is attained (within ~ 2 hrs), the exogenous glucose infusion rate is equal to the rate of whole body glucose disposal. This method is technically complex, requires experience personnel and is invasive (two intravenous lines throughout the study and frequent bedside blood samples) (Yildiz and Gedick, 2004).

Simpler methods, in particular indices calculated using fasting plasma concentrations of glucose and insulin, as well as data obtained using plasma concentrations of insulin and glucose obtained 120 minutes of a standard 75g oral glucose tolerance test have been

reported for quantifying insulin sensitivity (Radikova, 2003). There is a significant correlation ($r = 0.63$; $p< 0.001$) between the simpler technique and euglycaemic hyperinsulinaemic clamp in assessing insulin sensitivity and provides validation of the minimal model which can be adaptable for use in both clinical and research settings (Gutt et al, 2000). The commonly used fasting glucose insulin ratio was therefore used as a method for assessing insulin sensitivity in this thesis.

All the above mentinoned limitations are important and will be addressed in all further future work. Furthermore, where appropriate and possible the more robust hyperinsulinaemic glucose clamp method will be the preferred method to measure insulin sensitivity, with ethical approval.

10.2 Vascular resistance and vascular reactivity to 5% carbon dioxide in women with polycystic ovaries

o This study is the first to demonstrate decreased PI in the ICA in PCOS women, suggesting decreased downstream vascular resistance.

o This study is the first to demonstrate low back pressure in the ICA in asymptomatic women with PCO alone, and a difference of similar magnitude between PCOS and controls.

o Within groups, there was a significant decrease in PI and a paradoxical increase in V_{max} in PCOS women following inhalation of 5% CO_2.

Increased ICA PI is a risk factor for cerebral infarction in patients with type II diabetes (Nakatou et al, 2004). However, in PCOS women PI in this vessel was reduced compared

to controls. This could result either from increased luminal diameter of the ICA or decreased downstream vascular resistance and therefore increased blood flow. Although it was not possible to measure luminal diameter in this study, since the required software was not available on the ultrasound machine within the host Department at the time the work was performed, results from a later pilot study indicate there were no differences in luminal diameter between women with PCOS, women with PCO alone and controls. Thus it is likely that the decreased PI is related to a reduction in downstream cerebral vascular resistance in women with PCOS.

The finding of decreased PI in the present study differs from that reported in patients with type II diabetes (Lee et al, 2000). Lee and colleagues (2000) reported increased PI in the ICA and middle cerebral arteries in patients with diabetic complications in relation to those without complications, as well as in controls. This is not an unusual finding because, compared with health controls, patients with diabetes have more extensive atherosclerosis of the extracranial and intracranial vessels (Grunett, 1963). The differences noted in this thesis may suggest that PCOS women have insulin resistance but no diabetic complications and it would be of interest to assess vascular changes in these women who may develop type II diabetes in later life. Vascular responses in women with PCOS may be modulated by oestrogen profile. Oestrogen is known to enhance blood flow and the increase in oestradiol concentration from controls through PCO to PCOS may suggest its influence on ICA resistance i.e. low PI. However, after controlling its effect within multiple regression models, the difference was still significant.

Non-invasive evaluation of middle cerebral artery blood flow velocity, with transcranial Doppler ultrasound, and CO_2-induced vascular stimulation has been used in the assessment of cerebral vascular reactivity in patients with ICA occlusions (Ringelstein et al, 1988; Markus and Cullinane, 2001). In the present study, vascular reactivity was only assessed in the extracranial vasculature, possibly suggesting changes distal to the point of measurement. There is no published data to suggest that CO_2 could act as a vasoconstrictor. The paradoxical response to 5% CO_2 in the ICA in the present study in comparison to Ringelstein et al (1988) and Markus and Cullinane (2001) may reflect the concentration of CO_2 used (5% vs. 8%), and age of patients (mean age 26.1 vs. 51years) and the technique used in assessing vascular reactivity (spectral colour Doppler ultrasound vs. transcranial ultrasound with computer assisted programme). Secondly, this response after inhalation of CO_2, suggests some form of neurohumoral dysregulation may be the cause of altered reactivity in women with PCOS. However, as previously described, Lees (1998) reported similar unexpected finding in the uterine artery of PCOS women with glyceryl trinitrate as a vasodilator. Whilst rapidly developing atherosclerosis with loss of elasticity and ability to dilate may relate to the loss of vascular reactivity in older subjects, this is unlikely to be the cause in younger subjects. Further studies are on-going using Near-Infrared Spectrophotometry to measure cerebral blood flow and vascular reactivity with 5% CO_2 in women with PCOS and controls.

As previously described, insulin resistance is a central feature of PCOS and there is increasing evidence of type II diabetes in these women (Dunaif, 1997). The importance of assessing haemodynamic parameters results from a cross-sectional study which showed

PI being an independent parameter for estimating previous risk of cerebral infarction in patients with type II diabetes (Nakatou et al, 2004).

10.3 Viscoelastic properties and intima-media thickness in women with polycystic ovaries

o This is the first demonstration of decreased vascular compliance in the CCA and ICA and conversely an increased stiffness index in these vascular beds in PCOS women.

o Similar findings were also observed in asymptomatic women with PCO.

o The IMT was also increased in the elastic CCA and muscular CFA in *young* PCOS women

Alterations of the mechanical properties of arteries – compliance in relation to risk factors i.e. hypertension, diabetes, dyslipidaemia, smoking and age (McVeigh, et al, 1991) – are major contributors to cardiovascular morbidity and mortality (Lusis, 2000).

With respect to the evaluation of the vascular elastic properties, the main limitation is in the assessment of blood pressure. Ideally blood pressure should be measured from the same segment of artery under investigation. Intra-arterial blood pressure recording would be difficult to justify in this research project due to its invasive nature. Non-invasive assessment of brachial artery blood pressure introduces a small error to the estimation, but has been used as the reference point in much published research (Benetos et al, 1993; Sonesson et al, 1993; Salomaa et al, 1994). Brachial artery blood pressure was measured in all women using an automated device at the start of the procedure with a cuff

appropriate for the circumference of the arm. Measurements were taken at 2 min intervals until the systolic and diastolic blood pressures varied by less than 5 mmHg and the pulse rate by 5 beats/min, over two readings and an average of three readings were taken. This same technique was used for all the patients and the method is therefore not responsible for the observed differences between the groups.

The limitation with duplex scanning is the inaccuracy and inability to recognize the IMT in the near wall (Wong et al, 1993; Linhart et al, 2000). Therefore, the IMT is determined in the far arterial wall, which in general is significantly thicker when compared with histological measurement (Wong et al, 1993; Gamble et al, 1993; Persson et al, 1994). Another limitation against the use of duplex determination of the IMT is that there is no consensus of where the measurement should be taken. The Edinburgh Artery Study (EAS) (Allan et al, 1997) defined the CCA IMT as the maximum value measured from both left and right common carotid arteries, 2.0 cm below the bifurcation. The Rotterdam study (Bots et al, 1994) used the average value from both left and right CCA, each with three recordings obtained from the distal 1.0 cm from the carotid bulb. The Atherosclerosis Risk In Community (ARIC) study (Burke et al, 1995) used the average of the readings taken from the CCA, the carotid bifurcation and the ICA of both left and right sides.

In this thesis, the carotid IMT was measured according to a modified version of the EAS study i.e. an average of three readings taken within 2.0 cm of the bifurcation from the carotid artery. The use of average reading rather than the maximum reading of IMT has

the advantage of better reproducibility (Frost et al, 1998) and allows the evaluation of observer errors. The coefficient of variations for IMT measurements in this thesis was 8% for CCA and 23% for the carotid bulb, respectively. The lack of statistical significance at the carotid bulb may be explained by the increased intra-group variation possibly due to the anatomical shape of the segment resulting in the inability to produce an adequate image. The IMT was not measured in the internal carotid artery due to its localisation i.e. the CCA bifurcating behind the mandible, rendering the identification and positioning difficult.

There is evidence suggesting that reduced vessel wall compliance (Winer et al, 2001) and increased IMT of the CCA (Jerrard-Dunne et al, 2002) are independent risk factors for myocardial infarction in young subjects. The mechanism of impaired vascular compliance may be genetic in origin and mediated through alterations in the vessel wall matrix composition independent of age, gender, smoking, mean arterial pressure, BMI, family history of hypertension and activity scores (Brull et al, 2001). Brull et al (2001) reported an association between collagen type 1-alpha1 gene (COL1A1) polymorphism and arterial compliance. Furthermore, there is evidence of a possible linkage between genetic alteration of angiotensin converting enzyme gene (ACE-1D) and functional large artery properties (CCA and CFA) in a white population (Balkestein et al, 2001).

10.4 Skin microcirculation in women with polycystic ovaries

o This is the first demonstration of reduced cutaneous response to ACh (endothelial-dependent) but no difference in response with SNP (endothelial-independent) in women with PCOS.

There is some evidence that skin vessel reactivity to SNP and, to a lesser extent, ACh decreases with age and is gender dependent (Algotsson et al, 1995). Although skin reactivity declined with age in both men and women, women exhibited greater perfusion after iontophoresis than men. The influence of age in the present study is unlikely since all subjects in the present study were young adults. Although there was no difference in serum cholesterol levels and BP (before and after iontophoresis) in women with PCOS in relation with controls, there are *in-vitro* studies to suggest evidence of impaired endothelial function in small arteries i.e. skin (as a result of hypertension and dyslipidaemia), which do not develop atherosclerotic plaques (Heagerty, 1999).

Steinberg et al (1996) and Paradisi et al, (2001) reported abnormal vascular reactivity with infrafemoral infusions of MCh in PCOS women, suggesting impaired endothelial function, but did not assess vascular smooth muscle function. The findings of impaired perfusion response confirm endothelial dysfunction rather than vascular smooth muscle function of small vessels in these women with the syndrome. Although iontophoresis only assesses the cutaneous microcirculation, it is effectively a robust surrogate marker of vascular function in other vascular beds. Reduced response has been reported in women with previous gestational diabetes mellitus (Hannemann et al, 2002) and there is a link between pre-eclampsia and maternal coronary heart disease (Ramsay et al, 2002; 2003) as well as in heart transplant recipients (Andreassen et al, 1998).

216

A limitation of this study is that it only showed that endothelium dysfunction is mediated by NO but did not demonstrate other systems localised in the endothelial and smooth muscle cells which may regulate microvascular tone. It did not assess the extent to which other substances such as prostaglandins and EDHF contribute and act as intermediates in relation of the L-arginine/nitric oxide pathway. The goal of this work was to assess functional change in skin microcirculation using simultaneous introduction of endothelium-dependent ACh and endothelium-independent stimulus SNP in women with PCOS and to establish and complement the invasive technique used before for the assessment of endothelial function. Whether abnormal endothelial function observed in women with PCOS is a more generalised defect rather than an abnormality at the endothelial muscarinic receptor level remains to be determined.

The prognostic value of endothelial function independently predicting acute coronary events has been reported in patients with and without coronary artery disease by measuring change in vascular resistance with ACh and SNP. This provides both functional and prognostic information that complements angiographic and risk factor assessment i.e. subjects with increased response to ACh had improved survival as did those without coronary atherosclerosis in the total population (Halcox et al, 2002). Once again the mechanism responsible for these findings is unclear, but genetic factors could be contributory. As described before, NO plays an important role in atherogenic events (Channon et al, 2000). Polymorphism in the eNOS activity influences the functional action of the enzyme and affects the susceptibility of atherogenesis. A recent study has

demonstrated the 4a allele and eNOS combined genotypes (4a4a + 4a4b/ -786CC + TC) are independent predisposing factors for carotid atherosclerosis (Fatini et al, 2004).

10.5   Aortic function in a rat model of polycystic ovary syndrome

- o The principal morphological and endocrine features seen in PCOS women (Goldzieher, 1962; Conway et al, 1989; Franks, 1989) were replicated in this model.

- o This is the first *in-vivo* demonstration of thoracic aortic vascular changes, in parallel to those seen in humans, of decreased vascular compliance and an increased vascular stiffness index.

- o For the first time, an *in-vitro* study has confirmed endothelial dysfunction in a young animal model (15–18 year old female).

Although the findings of abnormal vascular reactivity in the cutaneous microcirculation (Chapter 8) and that by Paradisi et al (2001), in the femoral artery suggests endothelial dysfunction in women with PCOS relative to controls, PCOS-related vascular modifications may result from the influence of endocrine and metabolic disturbances on endothelial function, and in the longer term, increase the risk of atherosclerosis. Because it is not feasible to assess the mechanism(s) responsible for our findings in human subjects, an animal model of PCOS was used to confirm the endocrine, metabolic, ovarian morphology, endothelial and mechanical properties seen in women with PCOS. Further studies are now planned to use this animal model for assessing the mechanism(s) responsible for our findings.

Unfortunately the PCOS-like symptoms do not last beyond three weeks in the mifepristone-injected rat model, and it is therefore difficult to determine whether longer period treatments would lead to symptoms of metabolic disorder seen in older PCOS sufferers. Although the reproducibility of repeated measurements of the vascular compliance and stiffness index were made with reasonable levels of reproducibility and accuracy, the effect of anaesthesia on the central nervous system and vascular system may be different depending on the degree of disease. But the effect of disease seems unlikely in this short-term model.

A limitation of the *in-vitro* study is that local concentration of ACh is unknown, and the assumption that the levels at which the differences were significant between mifepristone treated rats and controls are significantly less than those required to produce vascular effects *in-vivo* studies, the accuracy of the model in relation with human situation is not validated. Secondly, the effects of vasoactive substances such as prostacyclin, EDHF on vascular smooth muscle, which are derived and released from the endothelium, were not assessed.

As discussed previously, there is no ideal animal model of PCOS. Most of the published models involve the administration of sex steroids or, in this case, an antagonist. Although using this model we demonstrated many of the endocrine, metabolic and morphological features of the syndrome, it is possible that some of the vascular abnormalities found resulted from the direct effects of mifepristone or progesterone antagonism. Although progesterone has a dilatory effect on rat aorta (Glusa et al, 1997; Mukerji et al, 2000;

Zhang et al, 2002), there is no evidence to suggest that it influences vascular compliance. The evidence for this lack of effect derived from studies of post-menopausal women (Honisett et al, 2003) and one study of a third-generation combined oral contraceptive pill (Willekes et al, 1999). There are no published studies relating to the effects of mifepristone on arterial compliance. There is therefore no reason to attribute the decreased arterial compliance seen in the mifepristone-induced PCOS rat model to a direct effect of this drug or progesterone antagonism.

10.6 Final summary

The unique finding in this thesis is that asymptomatic women with PCO had similar, but smaller, significantly different changes in Doppler parameter V_{max} and vascular compliance, seen in women with PCOS in relation to controls; bearing in mind these women had normal hormonal and metabolic features. The explanation for this observation is that either phenotypic or genotypic factor(s) may be common to both PCO and PCOS women. The typical phenotypic appearance common to both asymptomatic and symptomatic women with PCO is ovarian morphology. However, this characteristic feature seems unlikely to be the cause of observed vascular changes seen in women with PCO. Therefore, one can hypothesise that common genotypic risk factor(s), either endocrine, metabolic or both, may underlie both PCO and PCOS women. Further studies are necessary to confirm or refute this explanation.

The results demonstrate that PCO, *per se*, accelerates the earlier presentation of atherogenesis i.e. the initiation of the 'disease', not only in young women with PCOS but

also in asymptomatic women with PCO. It is now important not to consider PCO, present in around 20% of the female population, as a simple, uncomplicated condition with low morbidity / mortality. Life style modification by weight management through the promotion of exercise and eating habits should be an essential educational approach in women with PCO.

There is little doubt that women with PCOS cluster risk factors for cardiovascular disease and there is more evidence from experiments in this thesis of both increased macro- and micro-vascular disease in young women with PCOS and in a comparable animal model. Furthermore the independent associations of PI, vascular compliance, IMT and endothelial dysfunction with cardiovascular disease and stroke as well as the genetic predisposition suggests that doctors in all specialities should not only be aware of the association of PCOS with diabetes, dyslipidaemia and hypertension but also of possible long-term cardiovascular/cerebrovascular consequences in these women.

Insulin sensitising agents such as metformin are now widely used to treat infertile women with PCOS (Ehrmann et al, 1997; Paradisi et al, 1999) and there is some evidence of its beneficial effect on blood pressure, plasma cholesterol, triglycerides (Wulffele et al, 2004) and haemostasis and vascular function (Grant, 2003). However, the use of metformin in a large study by the Diabetes Prevention Program Research Group (2002) reported that life style management was more effective than metformin in the reduction of type II diabetes in persons at high risk. Furthermore, in a recent 2-year prospective study reported that metformin attenuates progression of CCA-IMT in patients with type II

diabetes and did not alter BMI, BP and serum lipids relative to controls (Matsumoto et al, 2004). Obviously metformin shows potential for short-term gain, and we should not overlook the long-term objectives of improving gynaecological and general health over many years, including into the menopausal years.

10.7 Suggested topics for future research arising from this work

<u>Human studies</u>

o Insulin resistance is a common feature of PCOS and insulin-sensitising drugs are currently being used regularly. Although some doctors are advocating long-term treatment (with metformin) for all women with PCOS (Nestler, 2002), this practice has been critised (Harborne et al, 2003) on the basis that an association between increased cardiovascular mortality and PCOS, let alone the benefits of any intervention to prevent this increase, are also unproven. The effects of such drugs on vascular mechanics may provide information on the beneficial effects, if any, on cardiovascular mortality.

o Insulin resistance, increased incidence of hypertension and dyslipidaemia are all associated with abnormal blood rheology (Rillaerts et al, 1989) resulting in increased blood viscosity (Lowe et al, 1982) and leading to increased shear stress on the vessel wall (Shaaban and Duernickx, 2000). A non-invasive, *in-vivo* technique for the assessment of vessel wall shear stress in atherosclerotic-prone

haemodynamic profile of women with PCOS would provide and support the vascular changes seen in this thesis.

<u>Laboratory research</u>

o Further studies are planned to characterise the endocrinological and metabolic changes in the mifepristone rat model and possibly in a larger species (Home Office Ethics Approval granted) and to clarify the possible mechanism(s) involved.

o Vasodilator action of EDHF on thoracic aortic rings from mifepristone-treated rats and control animals. Repeat organ bath experiments with aortic rings pre-contracted with PE and relaxed with cumulative increments of ACh in the presence of L-NAME and indomethacin which blocks the production of NO and prostacyclin respectively.

o Measure nitric oxide concentration in either serum or plasma as nitrate/nitrite using the established and accurate chemiluminescent technique

o The only active component of the aortic wall is the smooth muscle. There is evidence of increased expression of platelet-derived growth factor (PDGF) especially β-receptors and fibronectin (the important factor in the phenotypic change of smooth muscle cell from contractile to synthetic with the expression of PDGF β-receptor) from smooth muscle cells and arterial thickness is increased after balloon catheter injury before the onset of NIDDM in Otsuka Long-Evans Tokushima fatty rats (Tamura et al, 2000). The increased vascular stiffness and

223

decreased response to ACh in PCOS model could be attributed to increased expression of these vasoactive agents in the smooth muscle wall, leading to migration from the media into the intima and their proliferation in the intima, typically observed after percutaneous transluminal coronary angioplasty. Assessment of PGDF β-receptors and fibronectin from smooth muscle cells of mifepristone treated rats may provide the mechanism(s) responsible for macro vascular disease.

Mortality studies

o Since the criteria for defining PCOS have been agreed, large, multi-centre, collaborative studies with universally accepted definition of PCOS are now necessary to establish if any increased risk of cardiovascular mortality is apparent in these women.

REFERENCES

Adams, W.C., & Sensemann, D.M. Aromatization of androstenedione by induced polycystic ovaries in the rat. J.Steroid Biochem. 1976; 7: 309–310.

Adams, J., Franks, S., Polson, D.W., Mason, H.D., Abdulwahid, N., Tucker, M., Morris, D.V., Price, J., & Jacobs, H.S. Multifollicular ovaries: clinical and endocrine features and response to pulsatile gonadotrophin releasing hormone. Lancet 1985; 2: 1375–1379.

Adams, J., Polson, D.W., & Franks, S. Prevalence of polycystic ovaries in women with anovulation and idiopathic hirsutism. Br.Med.J.Clin.Res. 1986; 293: 355–359.

Adams, M.R., Williams., J.K., & Kaplan, J.R. Effects of androgens on coronary artery atherosclerosis and atherosclerosis-related impairment of vascular responsiveness. Arterioscler.Thromb.Vasc.Biol. 1995; 15: 562–570.

Agabiti, R.E., Rizzoni, D., & Castellano, M. Media: lumen ratio in human resistance arteries is related to forearm minimal vascular resistance. J Hypertens 1995;13: 349–355.

Aleem, F.A. & Predanic, M. Transvaginal color Doppler determination of the ovarian and uterine blood flow characteristics in polycystic ovary disease. Fertil.Steril. 1996; 65: 510–516.

Algotsson, A., Nordberg, A., & Winbald, B. Influence of age and gender on skin vessel reactivity to endothelium-dependent and endothelium-independent vasodilators tested with iontophoresis and a laser Doppler perfusion imager. J. Gerontology 1995; 50: M121–M127.

Allan, P.L. Doppler ultrasound in medicine; part 1. Hospital Update 1992; 82–86. Ref Type: Abstract.

Allan, P.L., Mowbray, P.I., Lee, A.J., & Fowkes, F.G. Relationship between carotid intima-media thickness and symptomatic and asymptomatic peripheral vascular disease. The Edinburgh Artery Study. Stroke 1997; 28: 348–353.

Amer, S.A.K.S., Li, T.C., Bygrave, C., Sprigg, A., Saravelos, H., & Cooke, I.D. An evaluation of the inter-observer and intra-observer variability of the ultrasound diagnosis of polycystic ovaries. Hum. Reprod. 2002; 17: 1616–1622.

Aminbaksh, A. & Mancini, G.B. Carotid intima-media thickness measurements? What defines an abnormality? A systemic review. Clin. Invest. Med. 1999; 22: 149–157.

Andersen, H.L., Weis, J.U., Fjalland, B., & Korsgaard, N. Effect of acute and long-term treatment with 17-beta estradiol on the vasomotor responses in rat aorta. Br. J. Pharmacol. 1999; 126: 159–168.

Andreassen, A.K., Kvernebo, K, Jorgensen, B, & et al. Exercise capacity in heart transplant recipients: relation to impaired endothelium-dependent vasodilatation of the peripheral microcirculation. Am.Heart J. 1998; 136: 320–328.

Anonymous. British Cardiac Society, British Hyperlipidaemia Association and British Hypertension Society, endorsed by the British Diabetic Association. Joint British recommendations on prevention of coronary heart disease in Clinical Practice. Heart (Suppl) 1998; 2: S1–S29. Ref Type: Abstract

Apridonidze, T., Essah, H., Iuono, M.J & Nestler, J.E Prevalence and characteristics of the Metabolic Syndrome in women with polycystic ovary syndrome. J. Clin.Endocrinol.Metab. 2004; 28: 245–252.

Archard, G. & Thiers, H. Hirsutes and its link with glycolytic insufficiency (diabetes of the bearded women). Bull. Acad. Natl. Med. 1921; 86: 51–85.

Ardaens, Y., Robert, Y., Lemaitre, L., Fossati, P., & Dewailly, D. Polycystic ovarian disease: contribution of vaginal endosonography and reassessment of ultrasonic diagnosis. Fertil.Steril. 1991; 55: 1062–1068.

Arnett, D.K., Chambless, L.E., Kim, L.H., Evans, G.W., & Riley, W.A. Variability in ultrasonic measurements of arterial stiffness in the Atherosclerosis Risk in Communities study. Ultrasound Med.Biol. 1999; 25: 175–180.

Atiomo, W.U., Pearson, S., Shaw, S., Prentice, A., & Dubbins, P. Ultrasound criteria in the diagnosis of polycystic ovary syndrome (PCOS). Ultrasound Med.Biol. 2000; 26: 977–980.

Azziz, R., Sanchez, L.A., Knochenhauer, E.S., Moran, C., Lazenby, J & et. Al. Androgen excess in women: experience with over 1000 consecutive patients. 2004; 89: 453–462.

Balbarini, A., Buttitta, F., Limbruno, U., Petronio, A.S., Baglini, R., Strata, G., Mariotti, R., Ciccone, M., & Mariani, M. Usefulness of carotid intima-media thickness measurement and peripheral B-mode ultrasound scan in the clinical screening of patients with coronary artery disease. Angiology 2000; 51: 269–279.

Balen, A.H., Conway, G.S., Kaltsas, G., Techatrasak, K., Manning, P.J., West, C., & Jacobs, H.S. Polycystic ovary syndrome: the spectrum of the disorder in 1741 patients. Hum.Reprod. 1995; 10: 2107–2111.

Balen, A. The pathogenesis of polycystic ovary syndrome: the enigma unravels. Lancet 1999; 354: 966–967.

Balen, A & Michelmore, K.F. What is polycystic ovary syndrome? Are national views important? (Debate). Hum. Reprod. 2002; 17: 2219–2227.

Balen, A., Laven, J., Tan, S.L., & Dewailly, D. Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum.Reprod. 2003; 9: 505–514.

Balkestein, E.J., Staessen, J.A., Wang, J.G., Van Der Heijden-Spek, J.J., Van Bortel, L.M., Barlassina, C., Bianchi, G., Brad, E., Herrmann, S.M., & Struijker-Boudier,H.A. Carotid and femoral artery stiffness in relation to three candidate genes in a white population. Hypertension 2001; 38: 1190–1197.

Balletshofer, B.M., Rittig, K., Stock, J., Lehn-Stefan, A., Overkamp, D., Dietz, K., & Haring, H. Insulin resistant young subjects at risk of accelerated atherosclerosis exhibit a marked reduction in peripheral endothelial function early in life but not differences in intima-media thickness. Atherosclerosis 2003;171: 303–309.

Barraclough, C.A. & Gorski, R.A. Evidence that the hypothalamus is responsible for androgen induced sterility in the female rat. Endocrinology 1989; 68: 68–79.

Battaglia, C., Artini, P.G., D'Ambrogio, G., Genazzani, A.D., & Genazzani, A.R. The role of color Doppler imaging in the diagnosis of polycystic ovary syndrome. Am.J.Obstet.Gynecol. 1995; 172: 108–113.

Baulieu, E.E. The anti-steroid RU486. Its cellular and molecular mode of action. TEM 1991; 2: 233–239.

Beach, K.W., Isaac, C.A., Phillips, D.J., & Strandness, D.E.J. An ultrasonic measurement of superficial femoral artery wall thickness. Ultrasound Med.Biol. 1989; 15: 723–728.

Benetos, A., Laurent, S., Hoeks, A.P., Boutouyrie, P., & Safar, M. Arterial alterations with aging and high blood pressure - a non invasive study of carotid and femoral arteries. Arterioscler.Thromb. 1993; 13: 90–97.

Benetos, A., Rudnichi, A., Safar, M., & Guize, L. Pulse pressure and cardiovascular mortality in normotensive and hypertensive subjects. Hypertension 1998; 560–564.

Benjamin, F., Deutsch, S., Saperstein, H & Seltser, V.L. Prevalence of and markers for the attenuated form of congenital adrenal hyperplasia and hyperprolactinemia masquerading as polycystic ovarian disease. Fertil. Steril. 1986; 46: 215–221.

Berghoff, M., Kathpal, M., Kilo, S., Hilz, M., & Freeman, R. Vascular and neural mechanisms of ACh-mediated vasodilation in the forearm cutaneous microcirculation. J.Appl.Physiol. 2002; 92: 780–788.

Bili, H., Laven, J., Imani, B., Eijkemans, M.J., & Fauser, B.C. Age related differences in features associated with PCOS in normogonadotrophic oligo-amenorrheic infertile women of reproductive years. Eur.J.Endocrinol. 2001: 145: 749–755.

227

Birdsall, M.A., Farquhar, C.M., & White, H.D. Association between polycystic ovaries and extent of coronary artery disease in women having cardiac catheterization. Ann.Intern.Med. 1997; 126: 32–35.

Bots, M.L., Hofman, A., & Grobbee, D.E. Common carotid intima media thickness and lower extremity arterial atherosclerosis. Arterioscler.Thromb. 1994; 14: 1885–1891.

Bots, M.L., Hoes, A.W., Koudstaal, P.J., Hofman, A., & Grobbee, D.E. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. Circulation 1997; 96: 1432–1437.

Boulanger, C.M., Tanner, F.C., Bea, M.L., Hahn, A.W., Werner, A., & Lüscher, T.F. Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and porcine endothelium. Circ.Res. 1992; 70: 1191–1197.

Bramwell, J.C. & Hill, A.V. Velocity of transmission of the pulse wave and elastic arteries. Lancet 1922; 1: 891–892.

Brands, P.J., Hoeks, A.P., Willigers, J., Willekes, C., & Reneman, R.S. An integrated system for the non-invasive assessment of vessel wall and hemodynamic properties of large arteries by means of ultrasound. Eur.J.Ultrasound 1999; 9: 257–266.

Brawer, J.R., Munoz, M., & Farookhi, R. Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. Biol.Reprod. 1986; 35: 647–655.

Bray, G.A. Obesity: basic aspects and clinical implications. Mes.Clin.North Am. 1981; 73: 261–264.

Breslau, P.J., Knox, R., Fell, G., Greene, F.M., Thiele, B.L., & Strandness Jr, M.D. The effect of carbon dioxide on flow patterns in normal extracranial arteries. J. Surg. Res. 1982; 32: 97–103.

Brull, D.J., Murray, L.J., Boreham, C.A., Ralston, S.H., Montgomery, H.E., Gallagher, A.M., McGuigan F.E, Davidson, B.R., Davey Smith, G., Savage, M., Humphries, S.E., & Toung, I.S. Effect of a COL1A1 Sp1 binding site polymorphism on arterial pulse wave velocity: an index of compliance. Hypertension 2001; 38: 444–448.

Bucala, R., Tracey, K.J., & Cerami, A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J. Clin. Invest. 1991; 87: 432–438.

Burghen, G.A., Givens, J.R., & Kitabehi, A.E. Correlation of hyperandrogenism with hyperinsulinaemia in polycystic ovarian disease. J. Clin. Endocrinol. Metab. 1980; 50: 113–116.

Burke, G.L., Evans, G.W., Riley, W.A., Sharrett, A.R., Howard, G., Barnes, R.W., Rosamond, W., Crow, R.S., Rautaharju, P.M & Heiss, G. Arterial wall thickness is associated with prevalent cardiovascular disease in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study. Stroke 1995; 26: 386–391.

Burton, A.C. Relation of structure to function of the tissues of blood vessels. Physiology Review 1954; 34: 619–642.

Caballero, A.E., Arora, S., Saouaf, R., Lim, S.C., Smakowski, P., Park, J.Y., King, G.L., LoGerfo, F.W., Horton, E.S & Veves, A. Microvascular and macrovascular reactivity is reduced in subjects at risk for type II diabetes. Diabetes 1999; 48: 1856–1862.

Campbell, C.S. & Schwartz, N.B. Impact of constant light on the estrous cycle of the rat. Endocrinology 1980; 106: 1230–1238.

Cannon, R.O. Role of nitric oxide in cardiovascular disease: focus on the endothelium. Clin.Chem. 1998; 44: 1809–1819.

CAPRIE Steering Committee. A randomised, blinded trial of clopidogres versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996; 1329–1339.

Carmina, E., Wong, L., Chang, L., Paulson, R.J., Sauer, M.V., Stanczyk, F.Z., & Lobo, R.A. Endocrine abnormalities in ovulatory women with polycystic ovaries on ultrasound. Hum.Reprod. 1997; 12: 905–909.

Caselli, A., Rich, J., Hanane, T., Uccioli, L., & Veves, A. Role of C-nociceptive fibres in the nerve axon reflex-related vasodilation in diabetes. Neurology 2003; 60: 297–300.

Chae, C.U., Pfeffer, M.A., Glynn, R.J., Mitchell, G.F., Taylor, J.O., & Hennekens, C.H. Increased pulse pressure and risk of heart failure in the elderly. JAMA 1999; 634–639.

Chambless, L.E., Heiss, G., Folsom, A.R., Rosamond, W., Szklo, M., Sharrett, A.R., & Clegg, L. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the atherosclerosis risk in communities (ARIC) study 1987- 993. Am.J.Epidemiol. 1997; 146: 483–494.

Channon, K.M., Qian, H., & George, S.E. Nitric oxide synthase in atherosclerosis and vascular injury. Arterioscler.Thromb.Vasc.Biol. 2000; 20: 1873–1881.

Cheng, K.S., Tiwari, A., Baker, C.R., Morris, R., Hamilton, G., & Seifalian, A.M. Impaired carotid and femoral viscoelastic properties and elevated intima-media thickness in peripheral vascular disease. Atherosclerosis 2000; 164: 113–120.

Cheng, K.S., Mikhailidis, D.P., Hamilton, G., & Seifalian, A.M. A review of carotid and femoral intima-media thickness as an indicator of the presence of vascular disease and cardiovascular risk factors. Cardiovasc.Res. 2002; 54: 528–538.

Cibula, D., Cifkova, R., Fanta, M., Poledne, R., Zivny, J., & Skibova, J. Increased risk of non-insulin dependent diabetes mellitus, arterial hypertension and coronary artery disease in perimenopausal women with a history of the polycystic ovary syndrome. Hum.Reprod. 2000; 15: 785–789.

Cibula, D., Hill, M., & Starka, L. The best correlation of the new index of hyperandrogenism with the grade of increased hair. Eur.J.Endocrinol. 2000; 143: 405–408.

Clayton, R.N., Ogden, V., Hodgkinson, J., Worswick, L., Rodin, D.A., Dyer, S., & Meade, T.W. How common are polycystic ovaries in normal women and what is their significance for the fertility of the population? Clin.Endocrinol.(Oxf.) 1992; 37: 127–134.

Cohn, J.N. Vascular wall function as a risk marker for cardiovascular disease. J.Hypertens. 1999; 17: S41–S44.

Conway, G.S., Honour, J.W., & Jacobs, H.S. Heterogeneity of the polycystic ovary syndrome: clinical, endocrine and ultrasound features in 556 patients. Clin.Endocrinol. (Oxf.) 1989; 30: 459–470.

Conway, G.S., Jacobs, H.S., Holly, J.M., & Wass, J.A. Effects of luteinising hormone, insulin, insulin-like growth factor I and insulin-like growth factor small protein I in polycystic ovary syndrome. Clin Endocrinol 1990; 33: 593–603.

Conway, G.S., Agrawal, R., Betteridge, D.J., & Jacobs, H.S. Risk factors for coronary artery disease in lean and obese women with the polycystic ovary syndrome. Clin.Endocrinol. (Oxf.) 1992; 37: 119–125.

Cosentino, F., Hishikawa, K., Katusic, Z.S., & Lüscher, T.F. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997; 96: 25–28.

Daane, T.A. & Parlow, A.F. Serum FSH and LH in constant light induced persistent estrous: short-term and long-term studies. Endocrinology 1971; 964–968.

Dahlgren, E., Janson, P.O., Johansson, S., Lapidus, L., & Oden, A. Polycystic ovary syndrome and risk for myocardial infarction. Evaluated from a risk factor model based on a prospective population study of women. Acta Obstet.Gynecol.Scand. 1992a; 71: 599–604.

Dahlgren, E., Johansson, S., Lindstedt, G., Knutsson, F., Oden, A., Janson, P.O., Mattson, L.A., Crona, N., & Lundberg, P.A. Women with polycystic ovary

syndrome wedge resected in 1956 to 1965: a long-term follow-up focusing on natural history and circulating hormones. Fertil.Steril. 1992b; 57: 505–513.

Dandona, P., James, I.M., Newbury, P.A., Woollard, M.L., & Beckett, A.G. Cerebral blood flow in diabetes mellitus: evidence of abnormal cerebrovascular reactivity. Br.Med.J. 1978; 2: 325–326.

Dart, A.M., Lancombe, F., Yeoh, J.K., Cameron, J.D., Jennings, G.L., Laufer, E and Esmore, D.S. Aortic distensibility in patients with isolated hypercholesterolaemia, coronary artery disease, or cardiac transplant. Lancet 1991; 3: 270–273.

Dewailly, D. Definition and significance of polycystic ovaries. Baillieres Clin.Endocrinol.Metab 1997; 11: 349–368.

de Simone, G., Roman, M.J., Koren, M.J., Mensah, G.A., Ganau, A., & Devereux, R.B. Stroke volume/pulse pressure ratio and cardiovascular risk in arterial hypertension. Hypertension 1999; 800–805.

Diabetes Prevention Program Research Group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N.Engl.J.Med. 2002; 346: 393–403

Drexler, H. & Hornig, B. Endothelial dysfunction in human disease. J. Mol.Cell.Cardiol. 1999; 31: 51–60.

Dunaif, A., Graf, M., Mandeli, J., Laumas, V., & Dobrjansky, A. Characterization of groups of hyperandrogenic women with acanthosis nigricans, impaired glucose tolerance, and/or hyperinsulinemia. J.Clin.Endocrinol.Metab. 1987; 65: 499–507.

Dunaif, A., Graf, M., Mandeli, J., Laumas, V., & Dobrjansky, A. Characterization of groups of hyperandrogenic women with acanthosis nigricans, impaired glucose tolerance, and/or hyperinsulinemia. J.Clin.Endocrinol.Metab. 1987; 65: 499–507.

Dunaif, A., Segal, K.R., Shelley, D.R., Green, G., Dobrjansky, A., & Licholai, T. Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. Diabetes 1992; 41: 1257–1266.

Dunaif, A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr.Rev. 1997; 18: 774–800.

Dunn, J.F., Nisula, B.C & Rodbard, D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid binding in human plasma. J. Clin. Endocrinol. Metab. 1981; 53: 58–68.

Dyce, M.C., Anderson, T.J., Yeung, A.C., et al. Indices of LDL particle size closely relate to endothelial function. Circulation 1993; 88: 466.

Edwards, E.M. & Evans, K.T. Pelvic pneumography in the Stein and Leventhal syndrome. Br.J.Radiol. 1961; 36: 46.

Ehrmann, D.A., Barnes, R.B., Rosenfield, R.L., Cavaghan, M.K., & Imperial, J. Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. Diabetes Care 1999; 22: 141–146.

Elting, M.W., Korsen, T.J., & Schoemaker, J. Obesity, rather than menstrual cycle pattern or follicle cohort size, determines hyperinsulinaemia, dyslipidaemia and hypertension in ageing women with polycystic ovary syndrome. Clin.Endocrinol. (Oxf.) 2001; 55: 767–776.

Emoto, M., Nishizawa,Y., Kawagishi,T., Maekawa, K., Hiura, Y., Kanda, H., Izumotani, K., Shoji, T., Ishimura, E., Inaba, M., Okuno, Y & Morii, H. Stiffness indexes b common carotid and femoral arteries are associated with insulin resistance in NIDDM. Diabetes Care 1998; 21: 1178–1182.

Evans, D.J., Hoffman, D.G., Kalkhoff, R.K., & Kissebah, A.H. Relationship of androgenic activity in body fat topography, fat cell morphology and metabolic aberrations in premenopausal women. J. Clin. Endocrinol. Metab. 1983; 57: 304–310.

Evans, D.J., Barth, J.H., & Burke, C.W. Body fat topography in women with androgen excess. Int.J.Obes. 1988; 12: 157–162.

Fatini, C., Sofi, F., Gensini, F., Sticchi, E., Lari, B., Pratesi, G., Pulli, R., Dorigo, W., Pratesi, C., Gensini, G.F., & Abbate,R. Influence of eNOS gene polymorphism on carotid atherosclerosis. Eur. J. Vasc. Endovasc. Surg. 2004; 27: 540–545.

Fauser, B.C., Pache, T.D., Lamberts, S.W., Hop,W.C., de Jong, F.H., & Dahl, K.D. Serum bioactive and immunoactive LH and FSH levels in women with cycle abnormalities, with or without PCOD. J. Clin. Endocrinol. Metab. 1991; 73: 811–817.

Ferrara, L.A., Mancini, M., Celentano, A., Galderisi, M., Iannuzzi, R., Marotta, T., & Gaeta, I. Early changes of the arterial carotid wall in uncomplicated primary hypertensive patients. Study by ultrasound high-resolution B-mode imaging. Arterioscler.Thromb. 1994; 14: 1290–1296.

Ferrell, W.R, Ramsay, J.E, Brooks, N, Lockhard, J.C, Dickson, S, McNeece, G.M., Greer, I.A, & Sattar, N. Elimination of electrically induced iontophoretic artefacts: implications for non-invasive assessment of peripheral microvascular function. J.Vasc.Res. 2002; 39: 447–455.

Ferriman, D.M. & Gallwey, J.D. Clinical assessment of body hair growth in women. J. Clin. Endocrinol. Metab. 1961; 21: 144–147.

Fish, P.J. Flow Imaging. In: Clinical Diagnostic Ultrasound. G.M.Baxter, P.L.P. Allan, & P. Morley, editors. Blackwell Science Ltd UK. 1999: 53–64.

Fleming, J.E.E., Spencer, I.H., & Nicholson, M. Clinical diagnostic ultrasound. In: Medical ultrasound-germination and growth. G. Baxter, P.L. Allan, & P. Morley, editors. Blackwell Science Ltd, Oxford. 1999: 1–17

Forsberg, F. Physical principles of Doppler ultrasound and colour flow imaging. In: An Atlas of Ultrasound Colour Flow Imaging. B.G. Goldberg, D.A. Merton, & C.R. Deane, editors. Martin Dunits, UK. 1997: 1–13

Fox, R., Corrigan, E., Thomas, P.A & Hull, M. G. The diagnosis of polycystic ovaries in women with oligo-amenorrhoea: predictive power of endocrine test. Clin. Endocrinol. 1991; 34: 127–131.

Fox, R. Transvaginal ultrasound appearances of the ovary in normal women and hirsute women with oligomenorrhoea. Aust.N.Z.J.Obstet.Gynaecol. 1999; 39: 63–68.

Franklin, S.S., Khan, S.A., Wong, N.D., Larson, M.G., & Levy, D. Is pulse pressure useful in predicting risk of coronary heart disease? The Framingham Heart Study. Circulation 1999; 100: 354–360.

Franks, S. Polycystic ovary syndrome: a changing perspective. Clin.Endocrinol. (Oxf.) 1989; 31: 87–120.

Frishman, W.H. Increased vascular compliance/decreased cardiovascular risk: what the studies tell us. Heart Dis. 2000; 2: 384–388.

Frost D, Friedl A, Beischer W. Determination of intima-media thickness of the carotid artery: influences of methods, proband and examination variables. Ultraschall Med 1998; 19:168–173.

Fulghesu, A.M., Ciampelli, M., Belosi, C., Apa, R., Pavone, V., & Lanzone, R. A new ultrasound criterion for the diagnosis of polycystic ovary syndrome: the ovarian stroma: total area ratio. Fertil.Steril. 2001; 76: 326–331.

Furchgott, R.F. & Zawadzki, J.V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-376.

Gamble, G., Beaumont, B., Smith, H., Zorn, J., Sanders, G., Merrilees, M., MacMahon, S., & Sharpe, N. B-mode ultrasound images of the carotid artery wall: correlation of ultrasound with histological measurements. Atherosclerosis 1993; 102: 163–173.

Gangar, K.F., Vyas, S., Whitehead, M., Crook, D., Meire, H., & Campbell, C.S. Pulsatility index in the internal carotid artery is influenced by transdermal oestradiol and time since menopause. Lancet 1991; 338: 839–842.

Gariepy, J., Simon, A., Massonneau, M., Linhart, A., & Levenson, J. Wall thickening of carotid and femoral arteries in male subjects with isolated hypercholesterolemia. PCVMETRA Group. Prevention Cardio-Vasculaire en Medecine du Travail. Atherosclerosis 1995; 113: 141–151.

Ge, Z.D. & He, G.W. Comparison of University of Wisconsin and St.Thomas' Hospital solution on EDHF-mediated function in coronary micro-arteries. Transplantation 2000; 70: 22–31.

Geroulakos, G., O'Gorman, D.J., Kalodiki, E., Sheridan, D.J., & Nicolaides, A.N. The carotid intima media thickness as a marker of the presence of severe symptomatic coronary artery disease. Eur. Heart. J. 1994; 15: 781–785.

Gill, R.W. Measurement of blood flow by ultrasound: accuracy and sources of errors. Ultrasound Med.Biol. 1985; 11: 625–641.

Glasser, S.P., Arnett, D.K., McVeigh, G.E., Finkelstein, S.M., Bank, A.J., Morgan, D.J., & Cohn, J.N. Vascular compliance and cardiovascular disease: a risk factor or a marker? Am. J. Hypertension. 1997; 10: 1175–1189.

Glusa, E., Gräser, T., Wagner, S., & Oettel, M. Mechanisms of relaxation of rat aorta in response to progesterone and synthetic progestins. Maturitas 1997; 28: 181–191.

Goetz, R.M., Morano, I., Calovini, T., Studer, R., & Holtz, J. Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. Biochem.Biophys.Res.Commun. 1994; 205: 905–910.

Goldzieher, J.W. & Green, J.A. The polycystic ovary: Clinical and histological features. J Clin Endocrinol Metab 1962; 22: 325–338.

Goldzieher, J.W. & Axelrod, L.R. Clinical and biochemical features of polycystic ovarian disease. Fertil.Steril. 1963; 14: 631–653.

Goldzieher, J.W. Polycystic ovarian disease. Fertil.Steril. 1981; 35: 371-394.

Goodfellow, J., Ramsey, M.W., Luddington, L.A., Jones, C.J., Coates, P.A., Dunstan, F., Lewis, M.J., Owens, D.R., & Henderson, A.H. Endothelium and inelastic arteries: an early marker of vascular dysfunction in non-insulin dependent diabetes. BMJ 1996; 312: 744–745.

Gosling, R.G., Lo, P.T.S., & Taylor, M.G. Interpretation of pulsatility index in feeder arteries to low-impedance vascular beds. Ultrasound Obstet.Gynecol. 1991; 1: 175–179.

Grant, P.J. Beneficial effects of metformin on haemostasis and vascular function in man. Diabetes Metab. 2003; 29: 6S44–6S52.

Griffith, D.N., Saimbi, S., Lewis, C., Tolfree, S., & Betteridge, D.J. Abnormal cerebrovascular carbon dioxide reactivity in people with diabetes. Diabet.Med. 1987; 4: 217–220.

Grill, V., Gutniak, M., Bjorkman, O., Lindqvist, M., Stone-Elander, S., Seitz, R.J., Blomqvist, G., Reichard, P., & Widen, L. Cerebral blood flow and substrate utilization in insulin-treated diabetic subjects. Am.J.Physiol 1990; 258: E813–E820.

Grossmann, M., Jamieson, M.J., Kellogg, D.L.J., Kosiba, W.A., Pergola, P.E., Crandall, C.G., & Shepherd, A.M.M. The effect of iontophoresis on the cutaneous vasculature: evidence for current induced hyperemia. Microvasc. Res. 1995; 50: 444–452.

Grunett, M. L. Cerebrovascular disease: diabetes and cerebral atherosclerosis. Neurology 1963; 13: 486–491. Ref Type: Abstract

Gutt, M., Davis, C.L., Spitzer, S.B., Llabre, M. M., Kumar, M., Czarnecki, E.M & et.al. Validation of the insulin sensitivity index (IS(0,120): comparison with other measures. Diabetes Res. Clin. Prac. 2000; 85: 177–184.

Guzick, D.S., Talbott, E.O., Sutton-Tyrrell, K., Herzog, H.C., Kuller, L.H., & Wolfson, S.K., Jr. Carotid atherosclerosis in women with polycystic ovary syndrome: initial results from a case-control study. Am.J.Obstet.Gynecol. 1996; 174: 1224–1229.

Hahn, A.W., Resink, T.J., Scott-Burden, T., Powell, J., Dohi, Y., & Buhler, F.R. Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: A novel autocrine function. Cell.Regul. 1990; 1: 649–659.

Halcox, J.P., Schenke, W.H., Zalos, G., Mincemoyer, R., Prasad, A., Waclawiw, M.A., Nour, K.R., & Quyyumi, A.A. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 6: 640–642.

Hannemann, M.M., Liddell, W.G., Shore, A.C., Clark, P.M., & Tooke, J.E. Vascular function in women with previous gestational diabetes mellitus. J.Vasc.Res. 2002; 39: 311–319.

Hansen, F., Bergqvist, D., Mangell, P., Rydén, Å, Sonesson, B., & Länne, T. Non-invasive measurement of pulsatile vessel diameter change and elastic properties in human arteries: a methodological study. Clin. Physiol. 1993; 13: 631–643.

Harborne, L., Fleming, R., Lyall, H., Norman, J., & Sattar, N. Descriptive review of the evidence for the use of metformin in polycystic ovary syndrome. Lancet 2003; 361: 1894–1901.

Harrington, D.J. & Balen, A.H. Polycystic ovary syndrome: aetiology and management. Br.J.Hosp.Med. 1996; 56: 17–20.

Harris, R. Iontophoresis: In Physical Medicine Library. E. Light, editor. Connecticut. 1967: 156

Hayakawa, H. & Raiji, L. The link among nitric oxide synthase activity, endothelial function, and aortic and ventricular hypertrophy in hypertension. Hypertension 1997; 29: 235–241.

Heagerty, A.M. In vitro studies of the human microcirculation in cardiovascular disorders. In: Endothelium, Nitric Oxide, and Atherosclerosis. J.A. Panza & R.O. Cannon, editors. Futura Publishing Company, Inc, NY. 1999: 213–223

Herman, S.M., Robinson, T.J., McCredie, R.J., Adams, M.R., & Boyer, M.J. Androgen deprivation is associated with enhanced endothelium-dependent dilation in adult men. Arterioscler.Thromb.Vasc.Biol. 1997; 17: 2004–2009.

Hertzner, N.R, Young, J.R, Baren, E.G, Groar, R.A, O'Hara, P.J, Ruschhaupt, W.F., de Wolfe, V.G and Maljovec, L.C. Coronary angiography in 506 patients with extracranial cerebrovascular disease. Arch.Intern.Med. 1985; 145: 849–852. Ref Type: Abstract

Hickler, R.B. Aortic and large artery stiffness: current methodology and clinical correlations. Clin. Cardiol. 1990; 13: 317–322.

Hirai, T., Sasayama, S., Kawasaki, T., & Yagi, S. Stiffness of systemic arteries in patients with myocardial infarction. A noninvasive method to predict severity of coronary atherosclerosis. Circulation 1989; 80: 78–86.

Hironaka, K., Yano, M., Kohno, M., Tanigawa, T., Obayashi, M., Konishi, M., Umemoto, S., & Matsuzaki, M. In vivo aortic wall characteristics at the early stage of atherosclerosis in rabbits. Am.J.Physiol. 1997; 273: H1142–H1147.

Hodis, H.N., Mack, W.J., Liu, C., & Stanley, P.A. The role of carotid arterial intima-media thickness in predicting clinical coronary events. Ann. Intern. Med. 1998; 128: 262–269.

Hoeks, A.P., Smeets, F.A.M., & Reneman, R.S. Assessment of the distensibility of superficial arteries. Ultrasound Med.Biol. 1990;16: 121–128.

Hoeks, A.P., Willekes, C., Boutouyrie, P., Brands, P.J., Willigers, J.M., & Reneman, R.S. Automated detection of local artery wall thickness based on M-line signal processing. Ultrasound Med.Biol. 1997; 23, 1017–1023.

Holte, J. Disturbances in insulin secretion and sensitivity in women with polycystic ovary syndrome. In Polycystic ovary syndrome. K.G.M.M. Alberti, G. M. Besse, H. G. Burger, R.D. Cohen, M.B. Ranke, S. Reichlin & J.D. Wilson editors. Bailliers Clinical Endocrinology and Metabolism, Bailliere Tindall, London. 1996:221–247.

Holte, J.G.G., Berne, C., Bergh, T., & Lithell, H. Elevated ambulatory day-time blood pressure in women with polycystic ovary syndrome: a sign of pre-hypertensive state? Hum. Reprod. 1996; 11: 23–28.

Holte, J., Genarelli, G., Wide, L., Lithell, H & Berne, C. High prevalence of polycystic ovaries and associated clinical, endocrine and metabolic features in women with previous gestational diabetes mellitus. J. Clin. Endocrinol. Metab. 1998; 83: 1143–1150.

Homburg, R. What is polycystic ovary syndrome? A proposal for a consensus on the definition and diagnosis of polycystic ovary syndrome. Hum. Reprod. 2002; 17: 2495–2499.

Honisett, S.Y., Pang, B., Stojanovska, L., Sudhir, K., & Komesaroff, P.A. Progesterone does not influence vascular function in post menopausal women. J. Hypertens. 2003; 21: 1145–1149.

Hopkinson, Z.E., Sattar, N., Fleming, R., & Greer, I.A. Polycystic ovarian syndrome: the metabolic syndrome comes to gynaecology. BMJ 1998; 317: 329–332.

Hoskins, P.R. Spectral Doppler systems. In: Clinical Diagnostic Ultrasound G.M. Baxter, P.L.P. Allan, & P. Morley, editors. Blackwell Science Ltd, UK. 1999: 39–52.

Huang, A., Sun, D., Koller, A., & Kaley, G. Gender difference in flow-induced dilation and regulation of shear stress: role of estrogen and nitric oxide. Am.J.Physiol. 1998; 275: R1571–R1577.

Hughesdon, P.E. Morphology and morphogenesis of the Stein-Levanthal ovary and the so-called hyperthecosis. Obstet.Gynecol.Surv. 1982; 37: 59–77.

Hutchinson, S.J., Sudhir, K., Chou, T.M., Sievers, R.E., Zhu, B.Q., Sun, Y.P.,Deedwania, P.C., Glantz, S.A., Parmley, W.W & Chatterjee, K. Testosterone worsens endothelial dysfunction associated with hypercholesterolemia and environmental tobacco smoke exposure in male rabbit aorta. J. Am. Coll. Cardiol. 1997; 29: 800–807.

Ignarro, L.J. Biological actions and properties of endothelium-derived nitric oxide formed and released from arteries and veins. Circ.Res. 1989; 65: 1-21.

llegbusi, O.J., Hu, Z., Nesto, R., Waxman, S., Cyganski, D., Kilian, J., Stone, P.H., & Feldman, C.L. Determination of blood flow and endothelial shear stress in human coronary artery in vivo. J.Invasive.Cardiol. 1999: 11: 667–674.

Irace, C., Carallo, C., Crescenzo, A., Motti, C., De Franceschi, M.S., Mattioli, P.L., & Gnasso, A. NIDDM is associated with lower wall shear stress of the common carotid artery. Diabetes 1999; 48: 193–197.

Jacobs, H.S. Polycystic ovaries and polycystic ovary syndrome. Gynecol.Endocrinol. 1987; 1: 113–131.

Jacobs, H.S. FSH and ovarian regulation. In: Polycystic ovary syndrome and Cardiovascular disease. B.C.Fauser, editor. 1997: 247-252.

Jeremy, J.Y. & Dandona, P. Inhibition by hydrocortisone of prostacyclin synthesis by rat aorta and its reversal with RU486. Endocrinology 1986; 119: 661–665.

Jerrard-Dunne, P., Markus, H.S., Steckel, D.A., Buehler, A., Von Kegler, S., & Sitzer, M. Early carotid atherosclerosis and family history of vascular disease. Specific effects on arterial sites have implications for genetic studies. Arterioscler.Thromb.Vasc.Biol. 2002; 23: 302–316

John, S & Schmieder, R.E Impaired endothelial function in arterial hypertension and hypercholesterolemia: potential mechanisms and differences. J. Hyperten. 2000; 18; 363–365

Jonard, S., Robert, Y., Cortet-Rudelli, C., Decanter, C., & Dewailly, D. Ultrasound examination of polycystic ovaries: is it a worth counting the follicles? Hum. Reprod. 2003; 18: 598–603.

Jones, H.M., Vernon, M.W., & Rush, M.E. Systematic studies invalidate the neonatally androgenised rat as a model for polycystic ovary disease. Biol.Reprod. 1987; 36: 1253–1265.

Kahn, C.R., Flier, J.S., Bar, R.S., Archal, J.A., & Gorden, P. The syndrome of insulin resistance and acanthosis nigricans. N. Eng. J. Med. 1976; 294: 739–745.

Kannel, W.B. Blood pressure as a cardiovascular risk factor: prevention and treatment. JAMA 1996; 275: 1571–1576.

Kawasaki, T., Sasayama, S., Yagi, S., Asakawa, T., & Hirai, T. Non-invasive assessment of the age related changes in stiffness of major branches of the human arteries. Cardiovasc.Res. 1987; 21: 678–687.

Kawata, R., Nakakimura, K., Matsumoto, M., Kawai, K., Kunihiro, M., & Sakabe, T. Cerebrovascular CO_2 reactivity during anesthesia in patients with diabetes mellitus and peripheral vascular disease. Anesthesiology 1998; 89: 887–893.

Kelly, C.J, Spiers, A., Gould, G.W., Petrie, J.R., Lyall, H. & Connell, J.M Altered vascular function in young women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 2003; 87: 742–746.

Kety, S & Schmidt, D.F. The effects of altered arterial tension of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. J. Clin. Invest. 1948; 27: 484. Ref Type: Abstract

Khan, F., Litchfield, S.J., Stonebridge, P.A., & Belch, J.J Lipid-lowering and skin vascular responses in patients with hypercholesterolaemia and peripheral arterial obstructive disease. Vasc. Med. 1999; 33: 805–812.

Knudsen, J.R., Costoff, A., & Mahesh, V.B. Dehydroepiandrosterone induced polycystic ovaries and acyclicity in the rat. Fertil.Steril 1975; 26: 807–817.

Korhonen, S., Hippelainen M., Niskanen L., Vanhala L., Saarikoski S. Relationship of the metabolic syndrome and obesity to polycystic ovary syndrome: a controlled, population-based study. Am.J.Obstet.Gynecol. 2001; 184: 289–296.

Kuller, L.H., Sutton-Tyrrell, K., & Matthews, K.A. Blood pressure levels and measurement of subclinical vascular disease. J.Hypertens. 1999; 17: S15–S19.

Kyei-Mensah, A., Maconochie, N., Zaidi, J., Pittrof, R., Campbell, S., & Tan, S.L. Transvaginal three dimensional ultrasound: accuracy of ovarian follicular volume measurements. Fertil.Steril. 1996a; 65: 371–376.

Kyei-Mensah, A., Maconochie, N., Zaidi, J., Pittrof, R., Campbell, S., & Tan, S.L. Transvaginal three dimensional ultrasound: reproducibility of ovarian and endometrial volume measurements. Fertil.Steril. 1996b; 66: 718–722.

Kyei-Mensah, A., Tan, S.L., Zaidi, J., & Jacob, H.S. Relationship of ovarian stromal volume to serum androgen concentrations in patients with polycystic ovary syndrome. Hum.Reprod. 1998; 13: 1437–1441.

Lakhani, K., Purcell, W. M., Fernando, R., & Hardiman, P. Ovarian volume and polycystic ovaries. Eur. J. Ultrasound, 1998; S21–S22. Ref Type: Abstract.

Lakhani, K., Constantinovici, N., Purcell, W.M., Fernando, R., & Hardiman, P. Internal carotid artery haemodynamics in women with polycystic ovaries. Clin.Sci.(Lond.) 2000a; 98: 661–665.

Lakhani, K., Constantinovici, N., Purcell, W.M., Fernando, R., & Hardiman, P. Internal carotid-artery response to 5% carbon dioxide in women with polycystic ovaries. Lancet 2000b; 356: 1166–1167.

Lakhani, K., Seifalian, A.M., & Hardiman, P. Impaired carotid viscoelastic properties in women with polycystic ovaries. Circulation 2002; 106: 81–85.

Lakhani, K., Hardiman, P & Seifalian, A.M Intima-media thickness in elastic and muscular arteries in young women with polycystic ovaries. Atherosclerosis 2004; 175: 353–358.

Langlois, Y.E., Green, F.M., Roederer, G.O., Jager, K.A., Philips, D.J., Beach, K.W., & Strandness, D.E. Computer based pattern recognition of carotid artery Doppler signals for disease classification: prospective validation. Ultrasound Med.Biol. 1984; 10: 581–595.

Lantin-Hermosos, R.L., Rosenfeld, C.R., Yuhanna, I.S., German, Z., Chen, Z., & Shaul, P.W. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. Am.J.Physiol. 1997; 273: L119–L126.

Lee, K.Y., Sohn, Y.H., Baik, J.S., Kim, G.W., & Kim J. Arterial pulsatility as an index of cerebral microangiopathy in diabetes. Stroke 2000; 31: 1111–1119.

Lees, C., Jurkovic, D., Zaidi, J., & Campbell, S. Unexpected effect of a nitric oxide donor on uterine artery Doppler velocimetry in oligomenorrheic women with polycystic ovaries. Ultrasound Obstet.Gynecol. 1998; 11: 129–132.

Leeson, C.P., Whincup, P.H., Cook, D.G., Mullen, M.J., Donald, A.E., Seymour, C.A., & Deanfield, J.E. Cholesterol and arterial distensibility in the first decade of life: a population-based study. Circulation 2000; 101: 1533–1538.

Legro, R.S., Driscoll, D., Strauss, J.F., Fox, J., & Dunaif, A. Evidence for genetic basis for hyperandrogenemia in polycystic ovary syndrome. Proc. Natl. Acad. Sci. USA 1998; 95: 14956–14960.

Legro, R.S., Blanche, P., Krauss R.M., & Lobo, R.A. Alterations in low-density lipoprotein and high-density lipoprotein subclasses among Hispanic women with polycystic ovary syndrome: influence of insulin and genetic factors. Fertil.Steril 1999; 72: 990–995.

Legro, R.S Polycystic Ovary Syndrome and Cardiovascular Disease: A Premature Association? Endocr.Rev. 2003; 24: 302–312.

Lehmann, E.D., Gosling, R.G., & Sönksen, P.H. Arterial wall compliance in diabetes. Diabetic Medicine 1992; 9: 114–119.

Lehmann, E.D., Hopkins, K.D., & Gosling, R.G. Multiple definitions of 'compliance'. Clin.Sci.(Lond) 1996; 90: 433–434.

Lerman, A., Edwards, B.S., Hallett, J.W., Heublein, D.M., Sandberg, S.M., & Burnett, J.C.J. Circulating and tissue endothelium immunoreactivity in advanced atherosclerosis. N. Eng. J. Med. 1991; 325: 997–1001.

Levick, J.R. An Introduction to Cardiovascular Physiology. Arnold Publishers, London. Oxford University Press Inc. New York. 2000.

Li, H & Forstermann, U. Nitric oxide in the pathogenesis of vascular disease. J Pathol. 2000; 190: 244–254

Liang, Y.L., Cameron, J.D., Teede, H., Kotsopoulos, D., & McGrath, B.P. Reproducibility of arterial compliance and carotid wall thickness measurements in normal subjects. Clin. Exp.Pharmacol.Physiol. 1998; 25: 618–620.

Linhart, A., Gariepy, J., Massonneau, M., & Dauzat, M. Carotid intima media thickness: the ultimate surrogate end-point of cardiovascular involvement in atherosclerosis. Appl. Radiol. 2000; 29: 25–39.

Liu, X.D., Skold, C.M., Umino, T., Spurzem, J.R., Romberger, D.J & Rennard, S.I Sodium nitroprusside augments human lung fibroblast collagen gel contraction independently of NO-cGMP pathway. Am. J. Physiol. Lung Cell Mol. Physiol. 2000; 278: L1032–1038.

Lloyd-Jones, D.M. & Kannel, W.B. Coronary risk factors: an overview. In: Cardiovascular Medicine. J.T. Willerson & J.N. Cohn, editors. Churchill Livingstone, Philadelphia. 2000: 2193–2215

Lobo, R.A & Goebelsmann. U.W.E. Adult manifestation of congenital adrenal hyperplasia due to incomplete 21-hydroxylase deficiency mimicking polycystic ovarian disease. Am. J. Obstet.Gynecol. 1980; 138: 720–726.

Lobo, R.A. Treatment of hyperadrogenism. In Polycystic ovary syndrome. R.J Cheng, J.J. Heindel & A. Dunaif editors. Marcel Dekker, Inc. New York. 2002; 327–347.

Lookingland, K.J., Wise, P.M., & Barraclough, C.A. Failure of the hypothalamic noradrenergic system to function in adult androgen-sterilized rats. Biol.Reprod. 1982; 27: 268–281.

Lowe, G.D.O. Increased blood viscosity and fibrinolytic inhibitor in type II hyperlipoproteinaemia. Lancet 1982; i: 472–475.

Lüscher, T. F and Vanhoutte, P. M. The Endothelium: Modulator of Cardiovascular Function. Boca.Raton, Fla, CRC Press. 1990. Ref Type: Report

Lüscher, T.F. & Barton M. Biology of the Endothelium. Clin Cardiol 1997; 20: II-3-II-10.

Lusis, A.J. Atherosclerosis. Nature 2000; 407: 233–241.

Madden, J.A. The effect of carbon dioxide on cerebral arteries. Pharmac. Ther. 1993; 59: 229–250.

Mahesh, V.B., Mills, T.M., Bagnell, C.A., & Conway, B.A. Animal models for study of polycystic ovaries and ovarian atresia. In: Regulation of Ovarian and Testicular Function. V.B. Mahesh, D.S. Dhindssa, E. Anderson, & S.P. Kalra, editors. Plenum Press, New York. 1987: 237–257.

Markus, H and Cullinane, M. Severely impaired cerebrovascular reactivity predicts stroke and TIA risk in patients with carotid artery stenosis and occlusion. Brain 2001; 124: 457-467. Ref Type: Abstract

Masaki.T. The endothelin family: an overview. J. Cardiovasc. Pharmacol. 2000; 35: 3-5

Mather, K.J., Verma, S., Corenblum, B., & Anderson, T.J. Normal endothelial function despite insulin resistance in healthy women with the polycystic ovary syndrome. J.Clin.Endocrinol.Metab. 2000; 85: 1851–1856.

Matsumoto,M., Sera,Y., Abe,Y., Tominaga,T., Yeki,Y., & Miyake,S. Metformin attenuates progression of carotid arterial wall thickness in patients with type 2 diabetes. Diabetes. Res. Clin. Pract. 2004; 64: 225–228.

McCarthy, G.F. & Brawer, J.R. Induction of Stein-Leventhal-like polycystic ovaries (PCO) in the rat: a new model for cystic ovarian disease. Anat. Res. 1990; 228: 137–144.

McKeigue, P. Cardiovascular disease and diabetes in women with polycystic ovary syndrome. In: Baillieres Clinical Endocrinology and Metabolism. H.S. Jacob, editor. W.B Saunders Co. Ltd, London. 1996: 311–318.

McVeigh, G.E., Burns, D.E., & Finkelstein, S.M. Reduced vascular compliance as a marker for essential hypertension. Am. J. Hypertension 1991; 4: 245–251.

McVeigh, G.E., Brennan, G.M., Johnston, G.D., McDermott, B.J., McGrath, L.T., Henry, W., Andrews, J.W & Hayes, J.R Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin dependent) diabetes mellitus. Diabetologia 1992; 35: 771–776.

McVeigh, G.E., Hamilton, P.H., & Morgan, D.R Evaluation of mechanical arterial properties: clinical, experimental and therapeutic aspects. Clin.Sci.(Lond.) 2002; 102: 51–67.

Michelmore, K.F., Balen, A.H., Dunger, D.B., & Vessey, M.P. Polycystic ovaries and associated clinical and biochemical features in young women. Clin.Endocrinol.(Oxf.) 1999; 51: 779–786.

Minor, R.J., Myers, P.R., Guerra, R.J., Batas, J.N., & Harrison, D.G. Diet induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. J. Clin. Invest. 1990; 86: 2109–2116.

Morris, S.J., Shore, A.C., & Tooke, J.E. Response of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. Diabetologia 1995; 38: 1337–1344.

Morris, S.J. & Shore, A.C. Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanism. J. Physiol. 1996; 496: 531–542.

Mukerji, M.S., Leathard, H.L., & Huddart, H. The effects of progesterone on spontaneous and agonist-evoked contractions of the rat aorta and portal vein. J. Pharm. Pharmacol. 2000; 52: 843–849.

Nakatou T, Nakata K, Nakamura A, Itoshima T. Carotid haemodynamic parameters as risk factors for cerebral infarction in Type 2 diabetes. Diabet. Med. 2004; 21:223–229.

Nava, E., Noll, G., & Lüscher, T.F. Increased cardiac activity of nitric oxide synthase in spontaneous hypertension. Circulation 1995; 91: 179–181.

Nestler, J.E. Should patients with polycystic ovarian syndrome be treated with metformin: an enthusiastic endorsement. Hum. Reprod. 2002; 17: 1950–1953.

Nicolini, U., Ferrazzi, E., Bellotti, M., et al. The contribution of sonographic evaluation of ovarian size in patients with polycystic ovarian disease. J Ultrasound Med 1985; 4: 347–351.

Nitenberg, H., Valensi, P., Sacho, R., Dali, M., Aptecar, E & Attali, J.R Impairment of coronary vascular reserve and ACh-induced coronary vasodilation in diabetic patients with angiographically normal coronary arteries and normal left ventricular function. Diabetes 1996; 43: 1017–1025.

Norman, R.J., Hague, W.M, Masters, S.C., & Wang, X.J. Subjects with polycystic ovaries without hyperandrogenaemia exhibit similar disturbances in insulin and lipid profiles as those with polycystic ovary syndrome. Hum.Reprod. 1995; 10: 2261.

Okon, E.B., Szado, T., Lajer, I., McManus, B.M., & Van Breeman, C. Augmented contractile response of vascular smooth muscle in a diabetic mouse model. J.Vasc.Res. 2003; 40: 520–530.

O'Leary, D.H., Polak, J.F., Wolfson, S.K., Bond, M.G., Bommer, W., Sheth, S., Psaty, B.M., Sharrett, A.R., & Manolio, T.A. Use of sonography to evaluate carotid atherosclerosis in the elderly: the Cardiovascular Health Study. Stroke 1991; 22: 1155–1163.

O'Leary, D.H., Polak, J.F., & Kronmal, R.A. Carotid artery intima and media thickness as a risk factor for myocardial infarction and strike in older adults. Cardiovascular Health Study Collaborative Research Group. N. Eng. J. Med. 1999; 340: 14–22.

Oltman, C.L., Gutterman, D.D., Scott, E.C., Bocker, J.M., & Dellsperger, K.C. Effects of glycosylated hemoglobin on vascular responses in vitro. Cardiovasc.Res. 1997; 34: 179–184.

O'Rourke, M. Vascular impedance in studies of arterial and cardiac function. Physiol. Rev. 1982; 62: 570–623.

O'Rourke, M.F. & Mancia, G. Arterial Stiffness. J. Hypertens. 1999; 17: 1–4.

Orsini, L.F., Venturoli, S., Lourusso, R., Pluchinotto, V., Paradisi, R & Bovicelli, L. Ultrasound findings in polycystic ovarian disease. J. Ultrasound Med. 1985; 4: 341–351.

Pache, T.D., Hop, W.C., Wladimiroff, J.W., Schipper, J., & Fauser, B.C. Transvaginal sonography and abnormal ovarian appearance in menstrual cycle disturbances. Ultrasound Med.Biol. 1991; 17: 589–593.

Pache, T.D., Wladimiroff, J.W., Hop, W.C., & Fauser, B.C. How to discriminate between normal and polycystic ovaries: transvaginal US study. Radiology 1992; 183: 421–423.

Pache, T.D., de Jong, F.H., Hop, W.C., & Fauser, B.C. Association between ovarian changes assessed by transvaginal sonography and clinical and endocrine signs of the polycystic ovary syndrome. Fertil.Steril. 1993; 59: 544–549.

Palmer, R.M., Ferrige, A.G., & Moncada, S. Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature 1987; 327: 524–526.

Panza, J.A., Casin, P.R., Kilcoyne, C.M., & Quyyumi, A.A. Impaired endothelium-dependent vasodilation in patients with essential hypertension: evidence that the abnormality is not at the muscaranic receptor level. J.Am.Coll.Cardiol. 1994; 23: 1610–1616.

Paradisi, G., Steinberg, H.O., Hempfling, A., Cronin, J., Hook, G., Shepard, M.K., & Baron, A.D. Polycystic ovary syndrome is associated with endothelial dysfunction. Circulation 2001; 103: 1410–1415.

Paradisi, G., Steinberg, H., Shepard, M., Hook, G., & Baron, A. Troglitazone therapy improves endothelial function near normal levels in women with polycystic ovary syndrome. Clin. Endocrinol. Metab. 2003; 88: 576–580.

Parisi, L., Tramonti, M., Casciano, S., Zurli, A., & Gazzarini, O. The role of ultrasound in the study of polycystic ovarian disease. J.Clin.Ultrasound 1982; 10: 167–172.

Pasquali, R., Casimirri, F., Cantobelli, S., Labate, A.M., Venturoli, S., Paradisi, R., & Zannarini, L. Insulin and androgen relationships with abdominal body fat

distribution in women with and without hyperandrogenism. Horm.Res. 1993; 39: 179–187.

Paulson, R.J., Serafini, J.A., Catalino, J.A & et al. Measurement of 3 alpha, 17-beta-androstenediol glucuronide in serum and urine and the correlation with skin 5 alpha-reductase activity. Fertil. Steril. 1986; 46: 222–226.

Persson, J., Formgren, J., Israelsson, B., & Berglund, G. Ultrasound-determined intima-media thickness and atherosclerosis. Direct and indirect validation. Arterioscler.Thromb. 1994; 14: 261–264.

Petersen, L.H., Jensen, R.E., & Parnell, J. Mechanical properties of arteries in vivo. Circ.Res. 1960; 8: 622–639.

Peterson, S., Mockford C., Rayner M. British Heart Foundation Health Promotion Research Group. Coronary Heart Disease Statistics, University of Oxford. 1999. Ref Type: Abstract

Philibert, D. An original multifaceted antihormone in vivo. In: Andrenal Steroid Antagonism. M.K. Agarwal, editor. Walter de Gruyter and Co, Berlin. 1984: 77–101

Philibert, D., Moguilewsky, M., Mary, I., Lecaque, D., Tournemine, C., Decchi, J., et.al The antiprogestin steroid RU486 and human fertility control. In: Pharmacological profile of RU486 in animals. E.E. Baulieu & S. Segal, editors. Plenum Press, New York. 1985: 49–68.

Phipps, J., Padmanabhan, R.V., & Lattin, G.A. Iontophoretic delivery of model inorganic drug ions. J.Pharmacol.Sci. 1989; 78: 365–369.

Pierpoint, T., McKeigue, P.M., Isaacs, A.J., Wild, S.H., & Jacobs, H.S. Mortality of women with polycystic ovary syndrome at long-term follow-up. J.Clin.Epidemiol. 1998; 51: 581–586.

Pignoli, P., Tremoli, E., Poli, A., Oreste, P., & Paoletti, R. Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. Circulation 1986; 74: 1399–1406.

Pikal, M.J. & Shah, S. Transport mechanisms in iontophoresis. II. Electroosmotic flow and transference number measurements for hairless mouse skin. Pharmacol.Res. 1990; 7: 213–221.

Pinto, S., Virdis, A., Ghiadoni, L., Bernini, G., Lombardo, M., Petraglia, F., Genazzani, A.R., Taddei, S & Salvetti, A. Endogenous estrogen and acetylcholine-induced vasodilation in normotensive women. Hypertension 1997; 29: 268–273.

Pitie, D., Watkins, P.J., & Edmonds, M.E. NO-dependent smooth muscle vasodilatation is reduced in NIDDM patients with peripheral sensory neuropathy. Diabet.Med. 1997; 14: 284–290.

Pocock, G & Richards, C. The basis of medicine. In Human physiology. G. Pocock, C. Richards & M de Burgh Daly editors. Oxford University Press, London. 1999.

Polson, D.W., Adams, J., Wadsworth, J., & Franks, S. Polycystic ovaries-a common finding in normal women. Lancet 1988; 1: 870–872.

Poredos, P., Orehek, M., & Tratnik, E. Smoking is associated with dose-related increase of intima-media and endothelial dysfunction. Angiology 1999; 50: 208.

Poretsky, L., Clemons, J., & Bogovich, K. Hyperinsulinemia and human chorionic gonadotrophin synergistically promote the growth of ovarian follicular cysts in rats. Metabolism 1992; 41: 903–910.

Poston, L. & Taylor, P.D. Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. Clin.Sci.(Lond.) 1995; 245–255.

Pourcelot, L. Applications cliniques de l'examen Doppler transcutane Vélocimétrie Ultrasonore Doppler. Seminaire INSERM , 1974; 213-240.Ref Type: Abstract

Prelevic, G.M., Beljic, T., Balint-Peric, L., & Ginsburg, J. Cardiac flow velocity in women with the polycystic ovary syndrome. Clin.Endocrinol. (Oxf.) 1995; 43: 677–681.

Prelevic, G.M., Wood, J., Okolo, S., and Ginsberg, J. Peripheral blood flow in young women with the polycystic ovary syndrome. J. Endocrinol. 1996; 151, 13.1–1 Ref Type: Abstract

Pupita, F., Ansuini, R., & Frausini, G. Peripheral vascular damage in hypertension. Cardiologia 1991; 36: 343–354.

Quyyumi, A.A. Endothelial function in health and disease: new insights into the genesis of cardiovascular disease. Am.J.Med. 1998; 105: 32S–39S.

Radikova, Z. Assessment of insulin sensitivity/resistance in epidemiological studies. Endocr. Regul. 2003; 37: 189–194.

Ramsay, J.E., Ferrell, W.R., Greer, I.A., & Sattar, N. Factors critical to iontophoretic assessment of vascular reactivity: implications for clinical studies of endothelial dysfunction. J. Cardiovasc. Pharmacol. 2002; 39: 9–17.

Ramsay, J.E., Stewart, F., Greer, I. A., & Sattar, N. Microvascular dysfunction: a link between pre-eclampsia and maternal coronary heart disease. Br.J.Obstet.Gynaecol. 2003; 110: 1029–1031.

Reaven, G.M. Banting Lecture. Role of insulin resistance in human disease. Diabetes. 1988; 37: 1597–1607.

Reaven, G.M. Pathophysiology of insulin resistance in human disease. Physiol Rev. 1995; 75: 473–486.

Rebuffe-Scrive, M., Cullberg, G., Lundberg, P.A., Lindstedt, G., & Bjorntorp, P. Anthropometric variables and metabolism in polycystic ovarian disease. Horm.Metab. Res. 1989; 21: 391–397.

Reneman, R.S,. & Hoeks, A.P. Non-invasive vascular ultrasound: an asset in vascular medicine. Cardiovasc.Res. 2000; 45: 27–35.

Renolds, N. J., Figueroa, C. D., Burton, J. L., Muller-Esterl, W., and Bhoola, K. D. Tissue kallikrein and kininogen in human sweat glands and psoriatic skin. Br. J.Dermatol. 124, 236-241. 1991. Ref Type: Abstract

Rillaerts, E., van Gaal, L., Xiang, D.Z., Vansant, G., & De, L.I. Blood viscosity in human obesity: relation to glucose tolerance and insulin status. Int.J.Obes. 1989; 13: 739–745.

Ringelstein, E.B., Sievers, C., Ecker, S., Schneider, P.A., & Otis, S.M. Noninvasive assessment of CO_2 - induced cerebral vasomotor response in normal individuals and patients with internal carotid artery occlusions. Stroke 1988; 19: 963–969.

Rittgers, S.E., Thornhill, B.M., & Barnes, R.W. Quantitative analysis of carotid artery Doppler spectral waveforms: diagnostic values of parameters. Ultrasound Med.Biol. 1983; 9: 255–264.

Roach, M.R. & Burton, A.C. The reason for the shape of the distensibility curves of arteries. Can.J.Biochem.Physiol. 1957; 35: 681–690.

Robeer, G.G., Brandsma, J.W, van den Heuvel, S.P., Smit, B., Oostendorp, R.A., & Wittens, C. Exercise therapy for intermittent claudication: a review of the quality of randomised clinical trials and evaluation of prediction factors. Eur.J.Vasc.Endovasc.Surg. 1998; 15: 36–43.

Robert, Y., Dubrulle, F., Gaillandre, L., Ardaens,Y., Thomas-Desrousseaux, P., Lemaitre, L., & Dewailly, D. Ultrasound assessment of ovarian stroma hypertrophy in hyperandrogenism and ovulation disorders: visual analysis versus computerized quantification. Fertil.Steril. 1995; 64: 307–312.

Robinson, S., Kiddy, D., Gelding, S.V., Willis, D., Niththyananthan, R., Bush, A., Johnston, D.G., & Franks, S. The relationship of insulin insensitivity to menstrual pattern in women with hyperandrogenism and polycystic ovaries. Clin.Endocrinol.(Oxf.) 1993; 39: 351–355.

Rodin, D.A., Bano, G., Bland. J.M, Taylor, K, & Nussey, S. Polycystic ovaries and associated metabolic abnormalities in Indian subcontinent Asian women. Clin. Endocrinol. 1998; 49: 91–99.

Rodriguez-Manas, L., Arribas, S., Giron, C., Villamor, J., Sanchez-Ferrer, C.F., & Marin, J. Interference of glycosylated human hemoglobin with endothelium-dependent responses. Circulation 1993; 88: 2111–2116.

Rosenberg, I. The influence of cigarette smoking on the risk of myocardial infarction in women. Cardiovasc. Risk Factors 1991; 1: 141–148.

Ross, R. The pathogenesis of atherosclerosis: a prospective for the 1990's. Nature 1993; 362: 801–809.

Rubanyi, G.M. & Vanhoutte, P.M. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am.J.Physiol. 1986; 250: H822–H827.

Ruiz,A., Aguilar,R., Tébar, M., Gaytán, F., & Sánchez-Criado, J.E. RU486 treated rats show endocrine and morphological responses to therapies analogous to responses in women with polycystic ovary syndrome treated with similar therapies. Biol.Reprod. 1996; 55: 1284–1291.

Ruiz, A., Tébar, M., Pérez-Romero, A., Rol de Lama, M.A., & Sánchez-Criado, J.E. Serum levels of GH, IGF-1,LH and ovarian steroids in cyclic and RU 486 treated rats. J.Endocrinol.Invest. 1997; 20: 611–615.

Ryden-Ahlgren A., Lanne,T., Wollmer, P., Sonesson, B., Hansen, F., & Sundkvist,G. Increased arterial stiffness in women, but not in men with IDDM. Diabetologia 1995; 38: 1082–1089.

Salomaa, V., Ward, R., Kark, J.D., Nardo, C., & Folsom, A.R. Non-insulin dependent diabetes mellitus and fasting glucose and insulin concentrations are associated with arterial stiffness indexes. Circulation 1994; 19: 1432–1443.

Salonen, R., Haapanen, A., & Salonen, J.T. Measurement of intima-media thickness of common carotid arteries with high resolution B-mode ultrasonography: inter- and intra-observer variability. Ultrasound Med.Biol. 1991; 17: 225–230.

Sampaio-Moura, M.J. & Marcondes, F.K. Influence of estradiol and progesterone on the sensitivity of rat thoracic aorta on noradrenaline. Life Sci. 2001; 68: 881–888.

Sample, W.F., Lippe, B.M., & Gyepes, M.T. Grey-scale ultrasonography of the normal female pelvis. Radiology 1977; 125: 477–483.

Sampson, M., Kong, C., Patel, A., Unwin, R., & Jacobs, H.S. Ambulatory blood pressure profiles and plasminogen activator inhibitor (PAI-1) activity in lean

women with and without the polycystic ovary syndrome. Clin.Endocrinol.(Oxf.) 1996; 45: 623–629.

Sanderson, J.E., Caldwell, R.W., Hsiao, J., Dixon, R., & Tuttle, R.R. Noninvasive delivery of a novel ionotropic catecholamine: Iontophoretic versus intravenous infusion in dogs. J.Pharmacol.Sci. 1987; 76: 215–218.

Sánchez-Criado, J.E., Bellido, C., Galiot, F., López, F.J., & Gaytán, F. A possible dual mechanism of the anovulatory action of antiprogesterone RU 486 in the rat. Biol. Reprod. 1990; 42: 877–886.

Sánchez-Criado, J.E., Bellido, C., López, F.J., & Galiot, F. Antiprogesterone RU 486 induces dissociation of LH and FSH secretion in the cyclic rat: effect of anti-inhibin serum. J. Endocrinol. 1992a; 134: 43–49.

Sánchez-Criado, J.E., Uilenbroek, J.Th.J, & de Jong, F.H. Antiprogesterone RU486 increases serum immunoreactive inhibin levels and LH:FSH and testosterone: oestradiol ratios in cyclic rats. J.Endocrinol. 1992b; 51–57.

Sánchez-Criado, J.E., Galiot, F., Bellido, C., Gonzalez, D., & Tébar, M. Hypothalamus-pituitary-ovarian-axis in cyclic rats lacking progesterone actions. Biol.Reprod. 1993a; 48: 916–925.

Sánchez-Criado, J.E., Tébar, M., Sánchez, A., & Gaytán, F. Evidence that androgens are involved in atresia and anovulation induced by antiprogesterone RU486 in rats. J.Reprod.Fertil. 1993b; 99: 173–179.

Satumura, S. A study on examining heart with ultrasonics. Japanese Circulation J. 1956; 20: 227. Ref Type: Abstract

Saumet, J.L., Kellogg, D.L.J., Taylor, W.F., & Johnson, J.M. Cutaneous laser-Doppler flowmetry: Influence of underlying muscle blood flow. J.Appl.Physiol. 1988; 65: 478–481.

Scommegna, A & Dmowski, W.P. Mesntruation. In Scientific foundations of obstetrics and gynaecology. E.E. Philipp, J. Barnes & M. Newton editors. William Heinemann Medical Books Ltd. London, 1977: 127–136.

Shaaban AM., & Duerinckx AJ. Wall shear stress and early atherosclerosis: a review. Am. J. Roentgenol. 2000; 174(6):1657–1665.

Shimokowa, H., Flahavan, N.A., & Vanhoutte, P.M. Natural course of the impairment of endothelium-dependent relaxations after balloon endothelium removal in porcine coronary arteries: possible dysfunction of a pertussis toxin-sensitive G-protein. Circ.Res. 1989; 65: 740–753.

Shimokowa, H. & Vanhoutte, P.M. Hypercholesterolemia causes generalized impairment of endothelium-dependent relaxation to aggregating platelets in porcine arteries. J. Am. Coll. Cardiol. 1989; 13: 1402–1408.

Shimokowa, H., Yasutake, K., Fujii, M.K., Owada, R., Nakaike, Y., & Takayanagi, T. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium dependent relaxations in rat mesenteric circulation. J. Cardiovasc. Pharmacol. 1996; 28: 703–711.

Sieber, F.E., Brown, P.R., Wu, Y., Koehler, R.C., & Traystman, R.J. Cerebral blood flow and metabolism in dogs with chronic diabetes. Anesthesiology 1993a; 79: 1013–1021.

Sieber, F.E., Brown, P.R., Wu, Y., Koehler, R.C., & Traystman, R.J. Cerebral blood flow responsivity to CO_2 in anesthetized chronically diabetic dogs. Am.J.Physiol 1993b; 264: H1069–H1075.

Simons, P.C., Algra, A., Bots, M.L., Banga, J. D., Grobbee, D.E., & van der Graaf, Y. Common carotid intima-media thickness in patients with peripheral arterial disease or abdominal aortic aneurysm: the SMART study. Second Manifestations of ARTerial disease. Atherosclerosis 1999; 146: 243–248.

Singh, K.B. Persistent oestrus: an experimental model of the polycystic ovary syndrome. Obstet.Gynecol.Surv 1969; 24: 2–17.

Sloan, J.B. & Soltani, K. Iontophoresis in dermatology. J.Am.Acad.Dermatol 1986; 15: 671–684.

Sokoloff, L. The effect of carbon dioxide on the cerebral circulation. Anesthesiology 1960; 21: 664. Ref Type: Abstract

Sonesson, B., Hansen, F., Stale, H., & Lanne, T. Compliance and diameter in the human abdominal aorta - the influence of age and sex. Eur. J. Vasc. Surg. 1993; 7: 690–697.

St. John, S. Aortic stiffness: a predictor of acute coronary events? Eur. Heart. J. 2000; 21: 342-344.

Stacey-Clear, A., & Fish, P.J. Repeatability of blood flow measurement using multichannel pulsed Doppler ultrasound. Br.J.Radiol. 1984; 57: 419–420.

Stein I.F., & Leventhal M.L. Amenorrhoea associated with bilateral polycystic ovaries. Am. J. Obs.Gynae. 1935; 29: 181–191.

Steinberg, H.O., Ckaker, H., Leaming, R., Johnson, A., Brechtel, G., & Baron, A.D. Obesity/insulin resistance is associated with endothelial dysfunction. J. Clin. Invest. 1996; 97: 2601–2610.

Swanson, M., Sauerbrei, E.E., & Cooperberg, P.L. Medical implications of ultrasonically detected polycystic ovaries. J.Clin.Ultrasound 1981; 9: 219–222.

Talbott, E., Guzick, D., Clerici, A., Berga, S., Detre, K., Weimer, K., & Kuller, L. Coronary heart disease risk factors in women with polycystic ovary syndrome. Arterioscler.Thromb.Vasc.Biol. 1995; 15: 821–826.

Talbott, E., Clerici, A., Berga, S.L., Kuller, L., Guzick, D., Detre, K., Daniels, T., & Engberg, R.A. Adverse lipid and coronary heart disease risk profiles in young women with polycystic ovary syndrome: results of a case-control study. J.Clin.Epidemiol. 1998; 51: 415–422.

Talbott, E.O., Guzick, D.S., Sutton-Tyrrell, K., McHugh-Pemu, K.P., Zborowski, J.V., Remsberg, K.E., & Kuller, L.H. Evidence for association between polycystic ovary syndrome and premature carotid atherosclerosis in middle aged women. Arterioscler.Thromb.Vasc.Biol. 2000; 20: 2414–2421.

Tamura, K., Kanzaki, T., Tashiro, J., Yokote, K., Mori, S., Ueda, S., et.al. Increased atherogenesis in Otsuka Long-Evans Tokushima fatty rats before the onset of diabetes mellitus: association with overexpression of PDGF ß-receptors in aortic smooth muscle cells. Atherosclerosis 2000; 149: 351–358.

Tanner, F.M.C., Knoll, G., Boulanger, C.M., & Lüscher, T.F. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries: Role of scavenger receptor and endothelium-derived oxide. Circulation 1991; 83: 2012–2020.

Tardy, Y., Hayoz, D., Mignot, J.P., Richard, P., Brunner, H.R., & Meister, J.J. Dynamic non-invasive measurements of arterial diameter and wall thickness. J.Hypertens.Suppl. 1992; 10: S105–S109.

Taylor, K.J.W. Doppler Ultrasound: Basic principles, instrumentation, and pitfalls. Radiology 1990; 174: 297–307.

Taylor, A.E., McCourt, B., Martin, K., Anderson, E.J., Adams, J.M, Schoenfeld, D & Hall, J. Determinants of abnormal gonadotrophin secretion in clinically defined women with PCOS. J. Clin. Endocrinol. Metab. 1997; 82: 2248–2256.

Taylor, A.E. Understanding the underlying metabolic abnormalities of polycystic ovary syndrome and their implications. Am.J.Obstet.Gynecol. 1998; 179: S94–S100.

Tébar, M., Ruiz, A., & Sánchez-Criado, J.E. Involvement of estrogen and follicle stimulating hormone on basal and luteinising hormone (LH) - releasing hormone-stimulated LH secretion in RU-486 induced 3-day estrous cycle in the rat. Biol.Reprod. 1998; 58: 615–619.

Teoh, H., Leung, S.W., Quan, A., Huang, M., Man, G.S., & Man, R.Y. Acetylcholine-mediated relaxation in rat thoracic aorta is enhanced following acute

exposure to physiological concentrations of 17-beta estradiol. Mol.Cell Biochem. 2000a; 207: 65–70.

Teoh, H., Quan, A., & Man, R.Y. Acute impairment of relaxation by low levels of testosterone in porcine coronary arteries. Cardiovasc.Res. 2000b; 45: 1010–1018.

Tep-areenan, P., Kendall, D.A., & Randall, M.D. Mechanisms of vasorelaxation to 17-beta-oestradiol in rat arteries. Eur. J. Pharmacol. 2003; 476: 139–149.

The Rotterdam ESHRE/ASRM-Sponsored Consensus Workshop Group Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil.Steril. 2004; 81: 19–25.

Timimi, F., Ting, H.H., Haley, E.A., Roddy, M.A., Ganz, P and Creager, M.A. Vitamin C improves endothelium dependent vasodilation in patients with insulin dependent diabetes mellitus. J. Am. Coll. Cardiol. 1998; 31: 552–557.

Urbinati, S., Di Pasquale, G., Androli, A., Lisa, A.M., Ruffini, M., lanzino, G & Pinelli, G. Frequency and prognostic significance of silent coronary artery disease in patients with cerebral ischaemia undergoing carotid endarterectomy. Vasc. Biol. 1992; 69: 1166–1170.

Urman, B., Sarac, E., Dogan, L., & Gurgan, T. Pregnancy in infertile PCOD patients. Complications and outcome. J.Reprod.Med. 1997; 42: 501–505.

Vaccarino, V., Holford, T.R., & Krumholz, H.M. Pulse pressure and risk of myocardial infarction and heart failure in the elderly. J.Am.Coll.Cardiol 2000; 130–138.

Van den Hoogen, P.C., Feskens, E.J., Nagelkerke, N.J., Menotti, A., Nissinen, A., & Kromhout, D. The relation between blood pressure and mortality due to coronary heart disease among men in different parts of the world. Seven Countries Study Research Group. N. Eng. J. Med. 2000; 342: 1–8.

Van den Schoot, P., Bakker, G.H., & Klijn, J.G.M. Effect of the progesterone antagonist RU486 on ovarian activity in the rat. Endocrinology 1987; 121: 1375–1382.

Van Popel, N.M., Westendorp, I.C., Bots, M.L., Reneman, R.S., Hoeks, A.P., Hofman, A., Grobbee, D.E., & Witteman, J.C. Variables of the insulin resistance syndrome are associated with reduced arterial distensibility in healthy non-diabetic middle-aged women. Diabetologia 2000; 43: 65–72.

Van Santbrink, E.J., Hipot, W.C., & Fauser, B.C. Classification of normogonadotrophin infertility: polycystic ovaries diagnosed by ultrasound versus endocrine characteristics of PCOS. Fertil.Steril. 1997; 67: 452–458.

Vermeulen, A., Verdonck, L., & Kaufman, M.J. A critical evaluation of simple methods for the estimation of free testosterone in serum. J. Clin. Endocrinol. Metab. 1999; 84: 3666–3672.

Vita, J.A., Treasure, C.B., Nable, E.G., McLenachan, J.M., Fish, R.D., Yeung, A.C., Vekshtein, V.I., Selwyn, A.P & Ganz, P. The coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation 1990; 81: 491–497

Vogel, R.A. Nitric oxide availability in hypercholesterolemia. In: Endothelium, Nitric Oxide, and Atherosclerosis. J.A. Panza & R.O. Cannon, editors. Futura Publishing Company, Inc, Armonk, NY. 1999: 163–183.

Wakisaka, M., Nagamachi, S., Inoue, K., Morotomi, Y., Nunoi, K., & Fujishima, M. Reduced regional cerebral blood flow in aged non-insulin dependent diabetic patients with no history of cerebrovascular disease: evaluation by N-isopropyl-123I-p-iodoamphetamine with single-photon emission computed tomography. J.Diabet.Complications 1990; 4: 170–174.

Wallengren, J., Ekman, R., & Sundler, F. Occurrence and distribution of neuropeptides in the human skin. Acta Dermato-Venereologia 1987; 67: 185–192.

Wallerath, T., Witte, K., Schafer, S.C., Schwarz, P.M., Prellwitz, W., & et.al. Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. Proc. Natl. Acad. Sci. USA 1999: 96: 13357–13362.

Walmsley, D. & Wiles, P.G. Assessment of the neurogenic flare response as a measure of nociceptor C fibre function. J.Med.Eng.Technol 1990; 14: 194–196.

Weiss, D. J., Richards., C, & Dunaif., A. Increased prevalence of polycystic ovaries in Hispanic women. Clin.Res. 1987; 35: 796. Ref Type: Abstract

Widder, B., Paulat, K., Hackspacher, J., & Mayr, E. Transcranial Doppler CO_2 test for the detection of hemodynamically critical carotid artery stenosis and occlusions. Eur. Arch. Psychiatr. Neurol. Sci. 1986; 236: 162–168.

Wild, R.A., Painter, P.C., Coulson, P.B., Carruth, K.B., & Ranney, G.B. Lipoprotein lipid concentrations and cardiovascular risk in women with polycystic ovary syndrome. J.Clin.Endocrinol. Metab. 1985; 61: 946–951.

Wild, R.A., Grubb, B., Hartz, A., Van Nort, J.J., Bachman, W., & Bartholomew, M. Clinical signs of androgen excess as risk factors for coronary artery disease. Fertil.Steril. 1990; 54: 255–259.

Wild, S., Pierpoint, T., Jacobs, H., & McKeigue, P. Long-term consequences of polycystic ovary syndrome: results of a 31 year follow-up study. Hum.Fertil.(Camb.) 2000; 3: 101–105.

Wild, R.A. Polycystic ovary syndrome: A risk for coronary artery disease? Am. J Obs. Gynae 2002; 186: 35–43.

Willekes, C., Hoeks, A.P., Bots, M.L., Brands, P.J., Willigers, J., & Reneman, R.S. Evaluation of off-line automated intima-media thickness detection of the common carotid artery based on M-line signal processing. Ultrasound Med.Biol.1999; 25: 57–64.

Willekes, C., Hoogland, H.G., Hoeks, A.P., & Reneman, R.S. Bladder filling reduces femoral artery wall distension and strain: beware of a full bladder! Ultrasound Med.Biol. 1998; 24: 803–807.

Willekes, C., Hoogland, H.G., Keizer, H.A., Hoeks, A.P., & Reneman, R.S. Three months use of third-generation oral contraceptives does not affect artery wall properties. Ultrasound Med.Biol. 1999; 25: 723–728.

Williams, S.B., Cusco, J .A, Roddy, M.A., Johnstone, M.T & Creager, M.A. Impaired nitric oxide mediated vasodilation in patients with non-insulin dependent diabetes mellitus. J. Am. Coll. Cardiol. 1996; 27, 567–574.

Wilson, K.A., Hoskins, P.R., Lee, A.J., Fowkes, F.G., Ruckley, C.V., & Bradbury, A.W. Ultrasonic measurement of abdominal aortic aneurysm wall compliance: a reproducibility study. J.Vasc.Surg. 2000; 31, 507–513.

Winer, N., Sowers, J.R., & Weber, M.A. Gender differences in vascular compliance in young, healthy subjects assessed by pulse contour analysis. J.Clin.Hypertens. 2001; 145–152.

Winkles, J.A., Alberts, G.F., Brog, E., & Libby, P. Endothelin-1 and endothelin receptor mRNA expression in normal and atherosclerotic human arteries. Biochem.Biophys.Res.Commun. 1993; 191: 1081–1088.

Wong, M., Edelstein, N., Wollman, J., & Bond, M.G. Ultrasonic-pathological comparison of the human arterial wall. Verification of intima-media thickness. Arterioscler.Thromb. 1993; 482–486.

Wulffele MG, Kooy A, de Zeeuw D, Stehouwer CD, Gansevoort RT. The effect of metformin on blood pressure, plasma cholesterol and triglycerides in type 2 diabetes mellitus: a systematic review. J. Intern. Med. 2004; 256:1–14.

Yamaguchi, T., Ohnaka, K., Takayanagi, R., Umeda, F., & Nawata, H. Enhanced secretion of endothelin 1 by elevated glucose levels from cultured bovine endothelial cells. FEBS. Lett. 1990; 267: 16–18.

Yamashita, T., Sasahara, T., Pomeroy, S.E., & Collier, G.N.P.J. Arterial compliance, blood pressure, plasma leptin, and plasma lipids in women are improved with weight reduction equally with a meat-based diet and a plant-based diet. Metab.Clin.Exp. 1998; 47: 1308–1314.

Yang, R., Thomas, G., Bunting, S., Ko, A., Ferrara, N., Keyt, B., Ross, J., and Jin, H. Effects of vascular endothelial growth factor on hemodynamic and cardiac performance. J. Cardiovasc. Pharmacol. 1996; 27: 838–844. Ref Type: Abstract

Yang, W.H.T., Thompson, C.S., Mikhailidis, D.P., Davidson, B.R., Winslet, M.C., & Seifalian, A.M. Direct measurement of hepatic tissue hypoxia by using a novel $tcpO_2/pCO_2$ monitoring system in comparison with near-infrared spectroscopy. Liver International 2003; 23: 163–170.

Yeh, H.C., Futterweit, W., & Thornton, J.C. Polycystic ovarian disease: US features in 104 patients. Radiology 1987; 163: 111–116.

Yen, S.S.C., Vela, P., & Rankin, J. Inappropriate secretion of follicle stimulating hormone and luteinising hormone in polycystic ovarian disease. J. Clin. Endocrinol. Metab. 1970; 30: 435–442.

Yildiz, B.O & Gedik, O. Assessment of glucose intolerance and insulin sensitivity in polycystic ovary syndrome. Reprod. BioMed. 2004; 8:649–656.

Zawadzki, J. & Dunaif, A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Polycystic Ovary Syndrome. A.Dunaif, J.R. Givens, F. Haseltine, & G.R. Merriam, editors. Blackwell Scientific, Boston. 1992: 377–384.

Zanchetti, A., Bond, M.G., Hennig, M., Neiss, A., Mancia, G., Dal Palu, C., Hansson, L., Magnani, B., Rahn, K.H., Reid, J., Rodicio, J., Safar, M., Eckes, L., & Ravinetto, R. Risk factors associated with alterations in carotid intima-media thickness in hypertension: baseline data from the European Lacidipine Study on Atherosclerosis. J.Hypertens. 1998; 16: 949–961.

Zhang, M., Wang, G.J., Benishin, C.G., & Pang, P.K.T. Rapid effect of progesterone on the contraction of rat aorta in-vitro. J Ph. Pharm. 2002; 54: 1534.

Zimmermann, S., Phillips, R.A., Dunaif, A., Finegood, D.T., Wilkenfeld, C., Ardeljan, M., Gorlin, R., & Krakoff, L.R. Polycystic ovary syndrome: lack of hypertension despite profound insulin resistance. J. Clin. Endocrinol. Metab. 1992; 75: 508–513.

APPENDICES

1. 0   **Abbreviations used in Excel spread sheets**

2.0   **Attached CD with Excel spread sheets of:**

2.1   **Raw data for measurements in Chapter 5**

2.2   **Raw data for measurements in Chapters 6 and 7 A& B**

2.3   **Raw data for measurements in Chapter 8**

2.4   **Raw data for measurements in Chapter 9 A & B**

ABBREVIATIONS

ACCB	Average common carotid artery back pressure
ACCPI	Average common carotid artery pulsatility index
ACCRI	Average common carotid artery resistance index
ACCV	Average common carotid artery velocity
ACh	Acetylcholine
AICB	Average internal carotid artery back pressure
AICPI	Average internal carotid artery pulsatility index
AICRI	Average internal carotid artery resistance index
AICV	Average internal carotid artery velocity
BACKPRCC	Back pressure right common carotid artery
BACKPRIC	Back pressure right internal carotid artery
BMI	Body mass index
CHOL	Total cholesterol
DACCPI	Difference average common carotid artery pulsatility index
DACCRI	Difference average common carotid artery resistance index
DACCV	Difference average common carotid artery velocity
DAICPI	Difference average internal carotid artery pulsatility index
DAICRI	Difference average internal carotid artery resistance index
DAICV	Difference average internal carotid artery velocity
DBP	Diastolic blood pressure
DDBP	Difference diastolic blood pressure
DDCA	Diastolic diameter common carotid artery in PCOS women
DDCCA–N	Diastolic diameter common carotid artery in control women

257

DDCCA–P	Diastolic diameter common carotid artery in PCO women
DDCFA	Diastolic diameter common femoral artery in PCOS women
DDCFA–N	Diastolic diameter common femoral artery in control women
DDCFA–P	Diastolic diameter common femoral artery in PCO women
DDIA	Diastolic diameter internal carotid artery in PCOS women
DDIA–N	Diastolic diameter internal carotid artery in control women
DDIA–P	Diastolic diameter internal carotid artery in PCO women
DLC	Diastolic diameter left common carotid artery
DLI	Diastolic diameter left internal carotid artery
DRC	Diastolic diameter right common carotid artery
DRI	Diastolic diameter right internal carotid artery
DSBP	Difference systolic blood pressure
FAI	Free androgen index
FASTG	Fasting glucose
FASTI	Fasting insulin
FSH	Follicle stimulating hormone
GACCPI	Gas average common carotid artery pulsatility index
GACCRI	Gas average common carotid artery resistance index
GACCV	Gas average common carotid artery velocity
GAICPI	Gas average internal carotid artery pulsatility index
GAICRI	Gas average internal carotid artery resistance index
GAICV	Gas average internal carotid artery velocity
GDBP	Gas diastolic blood pressure
GLCCPI	Gas left common carotid artery pulsatility index
GLCCRI	Gas left common carotid artery resistance index

GLCCV	Gas left common carotid artery velocity
GLICPI	Gas left internal carotid artery pulsatility index
GLICRI	Gas left internal carotid artery resistance index
GLICV	Gas left internal carotid artery velocity
GRCCPI	Gas right common carotid artery pulsatility index
GRCCRI	Gas right common carotid artery resistance index
GRCCV	Gas right common carotid artery velocity
GRICPI	Gas right internal carotid artery pulsatility index
GRICRI	Gas right internal carotid artery resistance index
GRICV	Gas right internal carotid artery velocity
GSBP	Gas systolic blood pressure
HDL	High density lipoprotein
HT	Height
INSRES	Insulin resistance index
LC–C	Left common carotid artery compliance
LC–S	Left common carotid artery stiffness index
LCCB	Left common carotid artery back pressure
LCCPI	Left common carotid artery pulsatility index
LCCRI	Left common carotid artery resistance index
LCCV	Left common carotid artery velocity
LI–C	Left internal carotid artery compliance
LI-S	Left internal carotid artery stiffness index
LDL	Low density lipoprotein
LICB	Left internal carotid artery back pressure
LICPI	Left internal carotid artery pulsatility index

LICRI	Left internal carotid artery resistance index
LICV	Left internal carotid artery velocity
LH	Luteinising hormone
L–NAME	N^G – nitro – L-arginine methyl ester
MEANBP	Mean blood pressure
N–C–C	Common carotid artery compliance in control women
N–C–CF	Common carotid artery compliance in control women
N–C–IC	Internal carotid artery compliance in control women
N–IMT–BULB	Intima media thickness of carotid bulb in control women
N–IMT–C	Intima media thickness of common carotid artery in control omen
N–IMT–IC	Intima media thickness of common femoral artery in control women
OEST	Oestradiol
PCO	Polycystic ovary syndrome
PCO–C–C	Common carotid artery compliance in PCO women
PCO–C–CF	Common carotid artery compliance in PCO women
PCO–C–IC	Internal carotid artery compliance in PCO women
PCO–IMT–BULB	Intima media thickness of carotid bulb in PCO women
PCO–IMT–C	Intima media thickness of common carotid artery in PCO women
PCO–IMT–CFA	Intima media thickness of common femoral artery in PCO women
PCOS	Polycystic ovary syndrome
PCOS–C–C	Common carotid artery compliance in PCOS women
PCOS–C–CF	Common femoral artery compliance in PCOS women
PCOS–C–IC	Internal carotid artery compliance in PCOS women
PCOS–IMT–BULB	Intima media thickness of carotid bulb in PCOS women

PCOS–IMT–C	Intima media thickness of common carotid artery in PCOS women
PCOS–IMT–CF	Intima media thickness of common femoral artery in PCOS women
PE	Phenylephrine
PROLAC	Prolactin
RCCB	Right common carotid artery back pressure
RC–C	Right common carotid artery compliance
RI–C	Right internal carotid artery compliance
RCCPI	Right common carotid artery pulsatility index
RCCRI	Right common carotid artery resistance index
RCCV	Right common carotid artery velocity
RC–S	Right common carotid artery stiffness index
RI–S	Right internal carotid artery stiffness index
RICB	Right internal carotid artery back pressure
RICPI	Right internal carotid artery pulsatility index
RICRI	Right internal carotid artery resistance index
RICV	Right internal carotid artery velocity
SBP	Systolic blood pressure
SD	Standard deviation
SDCA	Systolic diameter common carotid artery in PCOS women
SDCCA–N	Systolic diameter common carotid artery in control women
SDCCA–P	Systolic diameter common carotid artery in PCO women
SDCFA	Systolic diameter common femoral artery in PCOS women
SDCFA–N	Systolic diameter common femoral artery in control women
SDCFA-P	Systolic diameter common femoral artery in PCO women
SDIA	Systolic diameter internal carotid artery in PCOS women

SDIA–N	Systolic diameter internal carotid artery in control women
SDIA–P	Systolic diameter internal carotid artery in PCO women
SHBG	Sex hormone binding globulin
SNP	Sodium nitropruside
SLC	Systolic diameter left common carotid artery
SLI	Systolic diameter left internal carotid arterty
SRC	Systolic diameter right common carotid artery
SRI	Systolic diameter right internal carotid artery
TRIGL	Triglyceride
WHR	Waist to hip ratio
WT	Weight