POLYCYSTIC OVARY SYNDROME
COAGULATION AND METABOLIC STUDIES

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POLYCYSTIC OVARY SYNDROME
COAGULATION AND METABOLIC STUDIES

By

WILLIAM USINODE ATIOMO

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ABSTRACT

The polycystic ovary syndrome (PCOS) is a heterogeneous disorder in women characterised by chronic ovulatory failure, hyperandrogenaemia, and insulin resistance. Some women are completely asymptomatic and others present with extreme menstrual disturbance, severe hirsutism, infertility and recurrent miscarriage. The pathophysiology of PCOS is not completely understood, but it is thought that insulin resistance plays a central role.

In normal subjects, non-diabetic obese patients and patients with non-insulin dependent diabetes, insulin resistance is associated with elevated plasminogen activator inhibitor-1 (PAI-1) levels. PAI-1 is a glycoprotein, which inhibits the formation of plasmin (a proteolytic enzyme). Plasmin aids fibrinolysis and extracellular proteolysis. High PAI-1 and low plasmin levels increase the risk of thrombosis and impair extracellular proteolysis required in ovarian follicle growth, ovulation and embryo implantation.

This study was designed to determine whether elevated plasminogen activator inhibitor-1 (PAI-1) was associated with the insulin resistance present in PCOS, investigate its possible role in the causation of anovulation and recurrent pregnancy loss in these women and ascertain whether it was an additional thrombotic risk factor so that clinicians and patients could take appropriate measures to reduce this risk.

In a pilot study, systemic PAI-1 activity was significantly elevated in oligomenorrhoiec women with PCOS. A larger study supported these findings, but demonstrated that obesity was a significant confounding factor, as the increase in PAI-1 activity disappeared when standardised for weight. Activated Protein-C (APC) resistance was subsequently tested in these women because of the unexpected finding of an increased prevalence of a positive family history of thrombosis in women with PCOS compared with controls, but there was no increase in the prevalence of APC-resistance in PCOS.

In another project, the cellular distribution of PAI-1 protein in human ovaries was described for the first time using immunohistochemistry. It was localised to the granulosa and theca cell compartments in both polycystic and normal ovaries, however there was no significant difference in the intensity of PAI-1 staining between both groups on image analysis. PAI-1 messenger RNA expression was also evaluated in these biopsies by in-situ hybridisation, but no signal was detected suggesting that there was either a low overall RNA preservation in the tissues, or an insufficient sensitivity of the cocktail of oligonucleotide probes used.

This study did not support the hypothesis that elevated PAI-1 was a feature of PCOS, however the in-situ location of PAI-1 protein was demonstrated for the first time in the human ovary and consistent with a previously suspected role in ovulation. The results did not support a role for PAI-1 in anovulation, recurrent miscarriage or increased thrombosis in PCOS.
LIST OF CONTENTS

Copyright Statement 2
Title Page 3
Abstract 4
List Of Contents 5
List Of Tables And Illustrations 8
Acknowledgements 10
Authors Declaration 11

Chapter 1 Introduction 13
-Aims of Thesis 16
-Plan Of Thesis 16

Chapter 2 Literature Review 18

Chapter 3 Study Design 60
-Overall Study Design and Study Groups 60
-Patient Recruitment and Case Selection 62
-Data Collection, Assays and Laboratory Techniques 66
-Ultrasoundography 68
-Definitions 69

Chapter 4 The Plasminogen Activator System In Women With Polycystic Ovary Syndrome (A Pilot Study). 77
-Results 84
-Discussion 86
LIST OF CONTENTS (CONTINUED)

Chapter 5  Plasminogen Activator Inhibitor-1 And Metabolic Features Of Polycystic Ovary Syndrome. 88
-Results 96
-Discussion 99

Chapter 6  Immunohistochemical Localisation Of Plasminogen Activator-1 Protein In The Ovary. 108
-Results 117
-Discussion 124

Chapter 7  Plasminogen Activator- Inhibitor-1 Messenger RNA Expression In The Polycystic Ovary. 128
-Results 138
-Discussion 141

Chapter 8  Activated Protein-C Resistance And Polycystic Ovary Syndrome. 145
-Results 151
-Discussion 153

Chapter 9  Conclusions and further research questions. 156

 Appendices

 Proformas for study group 1 163
 PAI-1 assay 166
 Individual data for study group 1 168
 Proformas for study group 2 172
 Individual data for study group 2 176
 Proformas for study group 3 180
 Protocol for Immunohistochemistry 181
 Reagents & Protocols Used For In-Situ Hybridisation 187
<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Abbreviations</td>
<td>193</td>
</tr>
<tr>
<td>References</td>
<td>197</td>
</tr>
<tr>
<td>Publications And Presentations</td>
<td>239</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>3.1</td>
<td>General characteristics of the study groups used in the thesis</td>
</tr>
<tr>
<td>3.2</td>
<td>Sensitivities of some of the ovarian sonographic criteria used in the diagnosis of PCOS.</td>
</tr>
<tr>
<td>4.1</td>
<td>Clinical variables for patients with PCOS versus controls</td>
</tr>
<tr>
<td>4.2</td>
<td>Hormonal variables for patients with PCOS versus controls</td>
</tr>
<tr>
<td>4.3</td>
<td>Assays of the plasminogen activator system for patients with PCOS versus controls.</td>
</tr>
<tr>
<td>5.1</td>
<td>Power of a two-sample t-test to detect a 9au/ml difference of mean PAI-1 activity between women with PCOS and controls.</td>
</tr>
<tr>
<td>5.2</td>
<td>Clinical variables for patients with PCOS versus controls</td>
</tr>
<tr>
<td>5.3</td>
<td>Hormonal variables for patients with PCOS versus controls</td>
</tr>
<tr>
<td>5.4</td>
<td>PAI-1 and metabolic variables in patients with PCOS versus controls</td>
</tr>
<tr>
<td>6.1</td>
<td>Individual patient characteristics for immunohistochemical localisation of PAI-1 in polycystic ovaries.</td>
</tr>
<tr>
<td>8.1</td>
<td>General characteristics of women in the study with a positive family history of thrombosis.</td>
</tr>
<tr>
<td>8.2</td>
<td>APC ratios, protein S, protein C and antithrombin III levels in the women with a positive family history of thrombosis.</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

Figure 2.1  The plasminogen activator system ................................................. 32
Figure 2.2  Biosynthetic pathways for steroid hormones ................................. 50
Figure 3.1  Vaginal ultrasound appearance of a polycystic ovary ....................... 74
Figure 5.1  Scatter plot of PAI-1 for PCOS and controls. ................................. 99
Figure 6.1  Follicle from a polycystic ovary: immunostaining of granulosa cell layer and theca cell layer with anti PAI-1 monoclonal antibody .... 118
Figure 6.2  Edge of follicle from a polycystic ovary under high magnification: immunostaining of granulosa cell layer and theca cell layer with anti PAI-1 monoclonal antibody ........................................... 119
Figure 6.3  PAI-1 staining in the single layer of low columnar follicular cells surrounding the primary (pre-antral) follicle. .................................................... 120
Figure 6.4  PAI-1 staining in the granulosa and theca cell layers of the secondary (pre-antral) follicle. ................................................................. 121
Figure 6.5  PAI-1 staining in the theca cell layer of the tertiary (antral) follicle. ..... 122
Figure 7.1  Clear expression of mitochondrial ribosomal RNA in mucosa of inflamed appendix used as positive control for in situ hybridisation ................................................. 139
Figure 7.2  Absent signal for PAI-1 mRNA in polycystic ovaries. ....................... 140
Figure 7.3  Weak expression of mitochondrial ribosomal RNA in polycystic ovary ................................................................. 141
Figure 8.1  The coagulation cascade ................................................................. 146
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The majority of the work carried out in this study was performed by myself mainly during the period of registration, including the following: designing the protocols, obtaining ethical approval, patient recruitment, collection of clinical data (including venepunctures and pelvic ultrasound scans), separation and storage of the blood samples for coagulation assays, immunohistochemistry, in-situ hybridisation and the image analysis. I was also responsible for majority of the basic data analysis and all the word processing of the text.

Work not performed by me include; (i) The synthesis of oligonucleotide probes for in-situ hybridisation which was performed at the Department Of Pathology, University Of Bristol, (ii) The assays for PAI-1, Euglobulin clot lysis and APC-Resistance which were performed at the Coagulation Laboratory, Derriford Hospital, Plymouth (iii) Some statistical calculations including the analyses of variance and multiple regression equations, which were performed by Dr Steve Shaw, Department of Mathematics and Statistics, University of Plymouth.

Papers Published


Manuscripts Prepared For Publication And Presently Being Reviewed


AUTHORS DECLARATION (CONTINUED)

Presentations

The Missing Link In The Polycystic Ovary Syndrome - Is It Plasminogen Activator Inhibitor -1 ??

The in-situ location of Plasminogen activator inhibitor-1 in human polycystic and normal ovaries supports a role in ovulation.

Raised Plasminogen Activator Inhibitor 1 levels independent of body mass index in women with the polycystic ovary syndrome.
Venue: Copenhagen, Denmark, August 1997, at the XV FIGO world congress in obstetrics and gynaecology.

Impaired Fibrinolysis In Women With The Polycystic Ovary Syndrome.

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Mr Robert Fox MD MRCOG, Consultant In Obstetrics & Gynaecology, Musgrove Park Hospital, Somerset, Taunton.

Signed ..............................................................

Date ..............................................................
The polycystic ovary syndrome (PCOS) is a heterogeneous disorder of uncertain aetiology commonly found in women in the reproductive age group, and the many features of this syndrome can be broadly divided into three categories; clinical, endocrine and metabolic. The clinical features include anovulatory infertility, menstrual abnormalities, hirsutism, acne, alopecia, recurrent miscarriage and the presence of multiple small ovarian follicles on histology or ultrasound, while the endocrine features include elevated androgen, luteinizing hormone, oestrogen and prolactin levels (Franks 1995). Recently attention has been focused on the metabolic aspects of this syndrome, which include insulin resistance (or reduced insulin sensitivity), obesity, lipid abnormalities, an increased risk of non-insulin dependent diabetes and the possibility that insulin resistance may provide a unifying hypothesis for the hitherto elusive pathophysiology.

The exact mechanism of insulin resistance and its associated metabolic abnormalities in PCOS has not been fully explained. What is certain is that insulin resistance is present in obese and non-obese women with PCOS (Chang et al 1983, Dunaif et al 1989, Mahabeer et al 1989, Conway et al 1990, Dale et al 1992, Buyalos et al 1992), correlates positively with hyperandrogenaemia (Burghen et al 1980), and when treated with dietary restriction or metformin (Anderson et al 1995, Nestler & Jakubowicz 1996), there appears to be a lowering of androgen levels and an improvement in ovulation, and pregnancy rates. However the exact mechanisms of these inter-relationships are not fully understood.
The metabolic consequences of insulin resistance in general have been described as constituting an important metabolic syndrome (syndrome X) first described in 1988 (Reaven 1988). The features of this metabolic syndrome include hypertension, lipid abnormalities, an increased risk of coronary artery disease, raised uric acid levels and recently (Reaven 1994) the elevation of plasminogen activator inhibitor-1 (PAI-1).

PAI-1 is a glycoprotein whose main role is the regulation of plasmin formation in the plasminogen activator system (PAS) by inhibiting the conversion of plasminogen to plasmin by tissue plasminogen activator (Francis & Marder 1995). This system is not only involved in fibrinolysis, but many extracellular processes including the regulation of follicle growth (Peng et al 1993), ovulation (Beers 1975, Beers et al 1975, Piquette et al 1993) implantation (Sappino et al 1989) and tumour metastasis (Saskela 1985). It is therefore probable that elevated PAI-1 may link insulin resistance with reproductive failure in women with PCOS by limiting the plasmin formation required for follicle growth, ovulation and implantation of the embryo.

Unfortunately, the extent to which the metabolic features described as part of the insulin resistance in syndrome X overlap with the metabolic features in PCOS is unknown and as such whether elevated PAI-1 is also a feature of the metabolic aspects of PCOS is unknown. The studies in this thesis were therefore primarily designed to answer this question, but the other important clinical consideration was whether elevated PAI-1 was a hitherto unidentified risk factor for thromboembolic disease in women with PCOS.
Prior to the first study in this thesis, there were only two published studies on PAI-1 in women with PCOS. The first study (Dahlgren et al 1994) was a cross sectional study on 28 women with PCOS and 56 controls. In this study PCOS had been diagnosed histologically on wedge resection of the ovaries taken 25 to 34 years previously and the mean age of the participants at the time of the study was about 53 years. The results of the study showed that PAI-1 levels were not elevated in PCOS, but equally important was the fact that the other metabolic variables measured (fasting insulin, triglycerides and body mass index (BMI)) were not significantly different between the two groups. The authors concluded that their negative results might have been explained by the increased age of both groups as metabolic variables are increasingly affected by age. In the second paper (Anderson et al 1995), high PAI-1 levels in nine obese patients with PCOS were reduced after four weeks on a very low calorie diet. This reduction in PAI-1 concentration was associated with the return of regular menstruation and successful pregnancy in two women. However as there were no controls in this study, it did not answer the question of whether elevated PAI-1 levels were a unique feature of PCOS. This study was therefore designed with the following aims.
AIMS OF THESIS.

1. To determine whether elevated PAI-1 levels were associated with the metabolic aspect of PCOS and investigate the overall state of the plasminogen activator system in these women.

2. To investigate the expression of PAI-1 protein and mRNA in the polycystic ovary and compare it with normal ovaries.

3. Based on the above results, to consider whether PAI-1 and the PAS contributed to the pathophysiology of anovulation and pregnancy failure in women with PCOS, and whether they were at increased risk of thromboembolic disease from a prothrombotic PAS.

4. To determine in the light of the results whether altering PAI-1 levels in PCOS could affect fertility and risk of thromboembolism.

The null hypothesis was that the systemic and local (intra-ovarian) expression of PAI-1 in women with the PCOS was not different from the expression in normal controls.

ORGANISATION OF THESIS

In this thesis, the results of two clinical studies (a pilot and a definitive study) measuring systemic PAI-1 levels in the PCOS and of two laboratory-based studies on the localisation and quantification of PAI-1 protein and mRNA in polycystic ovaries, are presented. As a result of an unexpected finding of an increased prevalence of a positive family history of thrombosis in one of the clinical studies, the results of a further study measuring activated protein-C (APC) resistance (the commonest finding in familial thrombophilia) in women with PCOS compared with controls, are also presented. A detailed literature review of the current understanding of the PCOS can be found in Chapter 2. The overall study design is described in Chapter 3, and in chapter 4, a pilot study measuring systemic PAI-1 in the
PCOS is presented. This is followed by the results of a larger clinical study on systemic PAI-1 in chapter 5. Chapters 6 and 7 contain the results of the studies on the localisation of PAI-1 protein and mRNA and in chapter 8, the results of the study on APC-resistance are presented. The conclusions are outlined in chapter 9.
CHAPTER TWO

LITERATURE REVIEW

OUTLINE

In this chapter, an overview of the current understanding of the features, pathophysiology and management of PCOS are discussed. A general overview of insulin resistance and its metabolic consequences are discussed, and the possible role of PAI-1 in the pathophysiology of PCOS is discussed.

DEFINITION

The PCOS has three main clinical components (anovulation, hyperandrogenism and dysmetabolism) and a characteristic morphological appearance on ovarian ultrasound and histopathology. However there is still no consensus about which definition (clinical, morphological or endocrine) best characterises this syndrome as all these definitions have their own limitations because PCOS is multifaceted. Biological evidence of hyperandrogenism is generally accepted as a very sensitive marker of PCOS (Hopkinson et al 1998). However it is limited by the fact that in PCOS, levels of androstenedione and testosterone are modestly and inconstantly elevated in a non-specific manner (Dewailly 1997) and that the levels are dependent on the weight of the patient, with higher levels in more obese individuals.

The use of purely clinical signs of menstrual disturbance or androgen excess (hirsutism, acne or alopecia) in the diagnosis of PCOS is limited by the finding of polycystic ovaries on ultrasound in up to 20% of “normal” ovulatory women and in up to 50-87% of regularly menstruating women with hirsutism. In addition, because the degree of hirsutism
or alopecia found in a patient is partly related to varying degrees of end organ sensitivity (skin or hair follicle) to circulating androgens, this issue becomes more complicated, making it difficult to define clear diagnostic criteria for PCOS.

Although the first descriptions of the syndrome were based on the morphological appearances of the ovary (Stein & Leventhal 1935), this is not thought to be essential for the diagnosis. However with the recent development of high-resolution ultrasound, a more morphologically based diagnosis (Swanson et al 1981, Parisi et al 1984, Adams et al 1985, Yeh et al 1987, Pache et al 1991, Adams et al 1986) has been adopted and is believed by some authors to be a very sensitive marker of PCOS (Balen et al 1995). Unfortunately there is still no consensus about its use in the diagnosis of PCOS as the identification and quantification of the various parameters depend on the skill and dedication of the sonographer.

A consensus conference in 1990 achieved a definition of PCOS, stipulating that clinical symptoms such as anovulation and/or hyperandrogenism should serve as the selection criteria, with hormonal results used to exclude conditions that were not PCOS such as diseases of the adrenals or pituitary including adult-onset congenital adrenal hyperplasia, hyperprolactinaemia and androgen secreting neoplasms (Zawadzki & Dunaif 1992). However this definition was empirical, and although simple, economic and safe, it compromises clear cut and homogenous results in clinical research.

HISTORICAL LANDMARKS

There has always been a recognised correlation between obesity in women and amenorrhoea or menstrual irregularity, hirsutism and infertility, but it was only in 1935 that two American gynaecologists Irving Freiler Stein and Michael Leo Leventhal
associated these symptoms with a specific ovarian component (Stein & Leventhal 1935). They described seven women with amenorrhea and bilateral polycystic ovaries at laparotomy. They described a tendency to masculinizing changes in some of these women, and following the resection of one-half to three quarters of each ovary by wedge resection of the cortex containing these cysts, menstruation occurred monthly thereafter in every case. They suggested that a mechanical factor was the likely cause of the amenorrhea and sterility, and that it was possible that this factor impeded developing follicles in their pathway to the ovarian surface leading to them rupturing into cysts.

Keettel et al in 1957 were the first to document an endocrine abnormality in PCOS which was increased secretion of LH, and Kratochwil et al first described the ultrasound appearance of women with PCOS in 1972. The ovaries were defined as having a round shape with a symmetrical increase in volume. The association of PCOS with insulin resistance was an important landmark in the understanding of this syndrome. In 1976, Khan et al reported the finding of acanthosis nigricans and insulin resistance in six women, two of whom had polycystic ovaries. Prior to their publication, there had been two case reports in the literature linking polycystic ovaries with insulin resistance (Barnes et al 1974 & Givens et al 1974). In 1978, Cole et al described the spontaneous remission of insulin resistance in a patient with PCOS and acanthosis nigricans following the use of an oestrogen containing contraceptive and Burghen et al, in 1980, first investigated the relationship between plasma insulin levels and plasma testosterone and androstenedione in eight obese women with PCO, and six obese controls. They found a significant correlation between hyperinsulinism and hyperandrogenism. Although the exact mechanisms of insulin resistance in this syndrome are still not clear, recent studies have shown that treating it with oral hypoglycaemics such as metformin results in a lowering of androgen
levels (Nestler 1996) suggesting that insulin resistance is probably a key player in the pathogenesis of this syndrome.

**EPIDEMIOLOGY**

The prevalence of PCOS depends on the population studied, and the criteria used in the diagnosis. Irving Stein was only able to collect 90 cases with similar features to his originally described syndrome between 1935 and 1964 (Stein 1964). However the post-mortem studies by Sommers & Wadman in 1956 showed that some women did not have the hirsutism or amenorrhoea typically found in the Stein-Leventhal syndrome, and that the morphology of polycystic ovaries (PCO) was more common than previously thought. In a study of 257 "normal" volunteers who had not complained of any gynaecological symptoms, 22 percent were found to have ultrasound features of polycystic ovaries (Polson et al 1988). Most of these women had irregular menses compared to women without PCO (75% Vs <1%), and objective evidence of hirsutism was present in 45% of women with PCO compared with 7% without. Robert Fox however found ultrasound features of PCO in 8% of 50 women with normal ovulation and menstrual cycles who were partners of men with male infertility (Fox 1992a). In 173 anovulatory women evaluated in a reproductive endocrinology clinic, 30% of those with amenorrhoea and 75% of those with oligomenorrhoea had ultrasonographic features of PCO (Adams et al 1986). Overall, 57% of women with anovulation had ultrasound evidence of PCO. These results were consistent with the finding of PCO in 37% of women with amenorrhoea and 90% with oligomenorrhoea in a similar study in 1987 (Hull 1987). PCO was the commonest finding in women with anovulatory infertility in this study (73%).

In women with hirsutism and regular menstrual cycles, 50% to 92% have been found to have evidence of PCO on ultrasound (Adams et al 1986, Franks S 1989, O'Driscoll et al
and ultrasound evidence of PCO has been demonstrated in 92% of families of women with overt PCOS (Hague et al 1988).

HISTOLOGICAL FEATURES

Polycystic ovaries are typically enlarged and rounded with a thickened tunica albuginea. They contain multiple early forming and atretic cystic follicles usually arranged beneath the surface in a characteristic necklace pattern, but the follicles may also be scattered throughout an expanded ovarian stroma (White & Turner 1994). These follicles are lined by layers of granulosa cells of varying thickness and surrounded by a hyperplastic theca cell layer. There is usually little or no evidence of ovulation (corpora lutea or corpora albicantia). The numerous follicles lying in the ovarian cortex lead to a loss of the usual convolutions on the ovarian surface leaving a blue smooth mottled appearance. These findings may occur in only one ovary (Polson et al 1986).

The number of primordial follicles present in the polycystic ovary is similar to that in normal ovaries (Hughesdon 1982). However, the ovaries contain an increased number of primary, secondary and tertiary follicles, which rarely develop beyond a diameter of 6mm to 8mm (Fauser 1994).

Recent evidence from in-vitro studies suggests that the granulosa cells in PCO are not atretic, but viable and healthy. In one study (Almahbobi et al, 1996) up to 70% of the granulosa cells in PCO were found to be healthy and non-apoptotic on flow cytometric analysis. This finding was similar to the proportion of viable granulosa cells found in normal ovaries. In another study, there was no difference between polycystic and normal ovaries in the localisation of a cell cycle related nuclear antigen (Takayama et al 1996).
Women with PCO may also have endometrial hyperplasia, and endometrial carcinoma in premenopausal women is commonly associated with polycystic ovaries (Sherman & Brown 1979). These usually occur as a result of the unopposed high levels of oestrogens (as will be later discussed) on the endometrium.

ENDOCRINE FEATURES.

The endocrine features of this syndrome may include hyperandrogenaemia, elevated serum luteinizing hormone levels (LH), normal or low follicle stimulating hormone (FSH) levels resulting in a raised LH/FSH ratio, elevated circulating oestrone, normal oestradiol levels, a low sex hormone binding globulin (SHBG) concentration, hyperprolactinaemia, and insulin resistance.

Androgens

The androgens raised in PCOS include testosterone, dihydrotestosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), and androstenedione (Yen 1986). SHBG is a specific binding globulin for testosterone and oestradiol, and it is synthesised in the liver. In the globulin bound form, these steroids are not available for target tissue action. The free fraction of sex steroids is readily available for biologic action, thus the non-SHBG bound testosterone correlates with clinical androgenicity. Low SHBG levels in women with PCOS result in the increased availability of unbound testosterone which is raised in 59 to 90% of women with PCOS (Obhrai et al 1990, Eden et al 1988 & Fox 1992b), with the higher prevalence found in the studies that have only included women with oligomenorrhea. Raised total testosterone concentrations present in 50 to 55% of cases, androstenedione in 45 to 60% of cases (Duignan et al 1975 & Fox 1992b) and DHEAS in 13% to 50% (Hoffman et al 1984, Carmina et al 1986, Fox 1992c).
The ovaries and adrenal glands both contribute to hyperandrogenaemia in PCOS. The evidence for an ovarian contribution comes from studies showing that elevated DHEA and DHEAS levels in hirsutism are readily lowered by dexamethasone in PCOS patients whereas the elevated levels of testosterone and androstenedione are not (Yen 1986). Other studies show that ovarian suppression by gonadotrophin-releasing hormone analogues results in a decline in serum androstenedione and testosterone levels (Chang et al 1983a). Studies on ovarian and adrenal vein sampling have shown that the levels of DHEAS are higher in the adrenal vein compared to peripheral blood veins, and testosterone and androstenedione are higher in the ovarian veins compared to peripheral veins (Kirshner & Jacobs 1971), reflecting a contribution from both sources.

**Gonadotrophins**

In a paper presented at the 24th annual meeting of the Central Association of Obstetricians and Gynaecologists held in New Orleans in 1956, William Keettel, James Bradbury and Frederick Stoddard described their observation of hyperaemia and hypertrophy of the theca interna of immature rats following injection with extracts of the urine from women with Stein-Leventhal Syndrome (Keettel et al 1957). This response was judged to be primarily an LH response, based on evidence that LH acted primarily on the theca cells while FSH acted on granulosa cells (Gaarenstroom & de Jongh 1946). Subsequent studies have supported these findings and described raised LH levels in 48 to 100% of women with PCOS (McArthur et al 1958, Wentz et al 1975, Duignan et al 1975, Aono et al 1977, Conway et al 1989, Franks 1989, Obhrai et al 1990). LH secretion is pulsatile, and studies relying on single venepuncture assays of LH tend to give results of a lower frequency of LH hypersecretion than studies that rely on serial or 24 urinary estimations of LH secretion.
It is not clear whether this elevation in LH arises from a primary hypothalamo/pituitary defect or is a consequence of hyperandrogenism, elevated estrone levels, and or hyperinsulinaemia. Evidence for a primary hypothalamo/pituitary defect comes from studies showing an increased pulse frequency and amplitude of LH secretion from the pituitary (Kazar et al 1987, Waldstreicher et al 1988), and an abnormal forward shift in the LH surge in early pubertal girls with PCOS by eight hours (Zumoff et al 1983). On the other hand, hyperandrogenic states such as congenital adrenal hyperplasia may also exhibit elevated LH levels (Rosenwaks et al 1979), though administration of testosterone to eugonadal female-to-male transsexuals actually lowers plasma LH levels (Spinder et al 1989). To complicate the picture even further, the level of oestrone which is commonly raised in the PCOS can enhance the frequency of GnRH pulses (Urban et al 1991), and it has been shown that insulin may potentiate the stimulation of LH release by GnRH in cultured rat pituitary cells in-vitro (Adashi et al 1981). However, further in-vivo studies in rats have not confirmed these findings (Poretsky et al 1988).

In contrast to LH, FSH levels in the PCOS are normal or low when compared to controls (McArthur et al 1958, Baird et al 1977, Holte et al 1994). The reason for this discrepancy in pituitary gonadotrophin levels is not known, but possible explanations that have been put forward include a greater inhibitory effect of estrone and oestradiol on FSH than on LH, a decreased sensitivity of FSH release to GnRH stimulation, and secretion of follicular inhibin from polycystic ovaries resulting in the selective inhibition of FSH (Yen 1986).

**Oestrogens**

Evidence for oestrogenisation in women with PCOS has been adduced from the original observation of the return of uterine bleeding in the seven amenorrhoeic women described
by Stein and Leventhal in 1935. Subsequent studies have shown that serum oestrone levels are either raised or lie within the normal ranges for the early follicular and mid-follicular phases of the menstrual cycle in the PCOS (Baird et al 1977 & Polson et al 1987).

The serum oestradiol concentrations also appear to lie within the normal range for the early and mid-follicular phases of the menstrual cycle, but the pattern of secretion is different in that there is no mid luteal increase in oestradiol and there is less fluctuation than in the normal cycle (Polson et al 1987). Oestradiol in PCOS is mainly ovarian in origin, and although Short and London in 1961 found that ovarian cyst fluid contained no oestrone or oestradiol when compared with normal ovaries, on selective catheterisation of the ovarian veins, Wajchenburg et al in 1988 found that a large amount of oestradiol was generated suggesting that in combination, the numerous follicles in PCO produce a large amount of gonadal steroid.

There is no correlation between oestrone and oestradiol level in PCOS, which suggests a different mechanism of production. Evidence of a correlation between oestrone and androgens, is consistent with the peripheral conversion of androgens to oestrone in adipose tissue especially in obese women (Edman & MacDonald 1978). Oestrogen levels fluctuate widely within individuals (Backstrom et al 1982, Venturoli et al 1988), and it is not surprising that the ranges in oestradiol and oestrone concentrations in PCOS overlap too widely for oestrogens to be of clinical value in the diagnosis (Hull et al 1979). However, biological assessments of oestrogen states, such as the progesterone challenge test, appear to be better predictors of PCOS (Fox 1992d), and it is thought that these tests integrate the active and inactive components of the oestrogen pool.
**Prolactin**

Elevated prolactin levels are found in 4 to 30% of women with PCOS (Franks et al 1985, Conway et al 1989, Luciano et al 1984, Futterweit 1984), but their importance is unclear. Bromocriptine has been successfully used to treat anovulation in some women with PCOS (el Tabbakh et al 1988). However, the endocrine changes were otherwise similar in women with either normal or raised prolactin levels, which suggests that bromocriptine acted through a mechanism other than its effect on the dopamine pathway.

The observed wide variation in the prevalence of hyperprolactinaemia in PCOS may arise because of the failure of some studies to take into account the skewed distribution of prolactin with a long upper tail to at least 800μg/L (Jeffcoate, 1978) and it may well be that hyperprolactinaemia and PCOS co-exist in most cases by chance. On the other hand, ultrasound evidence of polycystic ovaries has been found in up to 50% of women with hyperprolactinaemic amenorrhoea (Abdel Gadir et al 1992), and polycystic ovaries may be found in women with prolactin secreting tumours and acromegaly (Futterweit & Kreiger 1979).

**Sex Hormone Binding Globulin**

SHBG has a molecular weight of 80,000 to 90,000. Its levels are influenced by sex steroids, obesity and insulin. Androgens inhibit SHBG, while oestrogens and obesity increase SHBG levels (Anderson 1974). In the PCOS, SHBG levels are lower than in controls (Plymate et al 1981, Suikkari et al 1989). Traditionally, sex steroids were thought to be the main regulators of SHBG in PCOS. However, recent evidence suggests that insulin is a more important regulator of SHBG in PCOS (Buyalos et al 1993, Hamilton-Fairley et al 1995). Hepatic production of SHBG is inhibited by insulin (Singh et al 1990, Plymate et al 1988, Conover & Lee 1990), and an improvement in hirsutism is
associated with calorie restriction and a corresponding increase in SHBG with decrease in insulin levels (Kiddy et al 1992).

INSULIN RESISTANCE & METABOLIC FEATURES

Overview of the functions, secretion, synthesis and mode of action of insulin

Insulin is a hormone with a variety of biologic functions, the most important of which is the regulation of glucose transport across the cell membrane. Other important functions of insulin include the stimulation of glycogen synthesis and lipogenesis and the inhibition of lipolysis, glycogenolysis and gluconeogenesis (Madison 1969). Insulin stimulates glucose uptake into muscle and adipose tissue through specific insulin sensitive glucose transporters (James et al 1989) but it has very little effect on hepatic glucose uptake, which is more dependent on glucose concentration in the portal vein.

Insulin is secreted by the beta cells in the pancreas in response to glucose, glyceraldehyde, amino-acids and certain hormones. Glucose is the main physiological secretagogue, and the insulin response to glucose is biphasic. A rapid first phase insulin response occurs between 5 to 10 minutes and is followed by a continuous second phase which is sustained for the duration of the glucose stimulus (Howell and Bird 1989). Insulin is synthesized in the pancreatic β-cells as pre-proinsulin, a larger precursor hormone which is encoded on the short arm of chromosome 11 (Howell and Bird 1989). Pre-proinsulin is cleaved to form pro-insulin, which is subsequently processed by two endopeptidases to form insulin, C-peptide and intermediary fragments of proinsulin. In a mature pancreatic β-cell, more than 95% of the hormone released is insulin, and less than 5% proinsulin.

Insulin initiates its metabolic actions by binding to a specific trans-membrane glycoprotein receptor (Khan 1985, Haring 1991), which results in a chain of intracellular events leading
to the biological effects attributed to it. This receptor has two extracellular \( \alpha \)-subunits and two \( \beta \)-subunits that transverse the cell membrane (Jacobs and Cuatrecasas 1981, Ullrich et al 1985). The \( \alpha \)-subunits initially bind the insulin to the receptor and the two \( \beta \)-subunits are insulin sensitive protein kinases that are activated following insulin binding. Activation of tyrosine kinase leads to the autophosphorylation of the receptor itself. The phosphorylation cascade generates one or more second messengers which mediate many of the actions of insulin.

**General aspects of insulin resistance**

Insulin resistance is defined as a subnormal biologic response to a given concentration of insulin and it is often used to signify a reduced response of glucose transport to a given concentration of insulin. It is related to physiological states such as pregnancy and puberty (Amiel et al 1986, Buchanan et al 1990), central adiposity, level of physical conditioning and skeletal muscle mass. The mechanism of the association between central obesity and insulin resistance is unknown, but proposed mechanisms include a release of non-esterified fatty acids from intra-abdominal fat cells into the portal circulation affecting hepatic insulin metabolism and peripheral glucose uptake (Randle et al 1963, Kissebah et al 1982), an impairment in the physiological haemodynamic response following glucose ingestion with a reduction in muscle blood flow instead of the usual increase (Baron et al 1990), down regulation of the insulin receptor (Seely & Olefsky 1993) and recently alterations in sensitivity to leptin (Segal et al 1996, Sorensen et al 1996).

Insulin resistance also features in many aetiologically diverse pathological conditions by a variety of mechanisms. These include mutations of the insulin receptor gene (Moller et al 1991, Krook et al 1996), raised concentrations of circulating hormones which antagonise the metabolic actions of insulin (glucagon, cortisol, catecholamines and growth hormone),
drug treatment, a defect in the function of an insulin sensitive glucose transporter protein (GLUT 4), abnormal endogenous insulin pulsatility (Bell 1996) and anti-insulin receptor antibodies. Ethnic differences in insulin sensitivity have also been observed; Mexican-Americans, Pima Indians and people from the Indian subcontinent are relatively more hyperinsulinaemic and less insulin sensitive than Caucasians (Haffner et al 1986, Lillioja et al 1987, Ferrannini et al 1991, McKeigue et al 1991).

Insulin sensitivity can be assessed in-vivo by any of the following tests; insulin/glucose ratios, the oral glucose tolerance test, insulin suppression test, glucose clamp, intravenous glucose tolerance test or the insulin tolerance test. These methods all rely on the measurement of insulin except the insulin tolerance test. They all have limitations, and none is suitable for routine clinical use.

In most cases of insulin resistance there is a compensatory hyperinsulinaemia secondary to insulin resistance. Glucose tolerance and fasting glucose may therefore be normal. Impaired glucose tolerance and diabetes develops only when compensatory insulin secretion fails to meet the degree of resistance.

**Metabolic Features Of Insulin Resistance**

In 1988, Gerald Reaven of Stanford University suggested that a combination of insulin resistance and compensatory hyperinsulinaemia predisposed individuals to develop a high plasma triglyceride (TG) and low high-density lipoprotein (HDL) cholesterol concentration, high blood pressure, and coronary heart disease. He suggested that this cluster of abnormalities constituted an important clinical syndrome which he designated syndrome X (Reaven 1988). Subsequently, several other metabolic abnormalities such as abnormalities of fibrinolysis (specifically raised plasminogen activator inhibitor-1 (PAI-1)
1) and elevated uric acid levels have been identified as belonging to this syndrome. These features can develop independently of obesity, are not dependent on a sedentary lifestyle (Reaven 1994) but are all related to insulin resistance.

The exact role of insulin resistance in the aetiology of these metabolic and clinical features has not been well established. Hyperinsulinaemia has been shown to enhance renal sodium retention and to increase sympathetic nervous system activity, events which would certainly tend to increase blood pressure (Smith, 1993). However acute hyperinsulinaemia in humans leads to vasodilatation and blood pressure does not increase (Anderson et al, 1993).

Hypertriglyceridaemia and low HDL are risk factors for coronary heart disease (Manninen, et al 1992; Assmann et al, 1992) and insulin stimulates proliferation of smooth muscle cells and augments lipid synthesis in vascular smooth muscle cells, which may cause atheroclerosis.

PAI-1 is a glycoprotein present in plasma, platelets, endothelial cells and in the extracellular matrix where it may inhibit local proteolysis (Salonen et al, 1989, Schleef et al, 1990). It inhibits the activation of plasminogen to plasmin by tissue plasminogen activator (figure 2.1) which is important in fibrinolysis (Francis & Marder 1995), ovulation (Piquette et al, 1993) and implantation (Sappino et al, 1989). Elevated levels of PAI-1 are found in patients with coronary heart disease (Hamsten et al, 1985 Paramo et al, 1985, Metha et al, 1987) and high PAI-1 levels are also a significant risk factor in the development of fatal ischaemic heart disease in women (Meade et al 1996). PAI-1 correlates positively with insulin resistance in diabetics (Juhau-Vague et al 1989) and factors such as exercise (Speiser et al 1988), low calorie diets (Sundell et al 1989,
Mehrabian et al 1990, Huisveld et al 1990) and metformin (Vague et al 1987) which improve insulin sensitivity also lower PAI-1 levels. It has therefore been suggested that elevated PAI-1 levels may link insulin resistance and atherothrombosis (Juhan-Vague et al 1991), although the mechanisms by which insulin increases the levels of PAI-1 are yet to be completely elucidated.

Figure 2.1  The plasminogen activator system.

\[
\begin{align*}
\text{u-PA} & \quad \text{t-PA} \\
\downarrow & \quad \downarrow \\
\text{PLASMINOGEN} & \rightarrow \rightarrow \text{PLASMIN} \leftarrow \alpha_2\text{-antiplasmin} \\
\downarrow & \\
\text{FIBRIN} & \rightarrow \rightarrow \text{FIBRIN DEGRADATION PRODUCTS}
\end{align*}
\]

\(u\text{-PA} = \) urokinase type plasminogen activator

\(t\text{-PA} = \) tissue type plasminogen activator

\(\Rightarrow = \) Inhibition

**Insulin resistance in PCOS**

The first link between a hyperandrogenic state and an abnormality in carbohydrate metabolism was established in 1921 when Achard and Thiers described "diabetes of bearded women". More interest was aroused in this correlation when Khan et al in 1976 described the syndromes of extreme insulin resistance in six young women with acanthosis nigricans. Two of these women were found to have polycystic ovaries, diagnosed by gynaecography in one case and laparoscopy in the other. They also had hirsutism, primary amenorrhoea, elevated testosterone levels and virilization in one case though neither of
them was obese. However, these syndromes are rare and they represent extreme cases of insulin resistance and hyperandrogenaemia. Insulin resistance was described in the more commonly encountered form of PCOS (Burghen et al 1980) following a case report in which insulin resistance was reversed by oestrogen which resulted in a concomitant reduction of androgens (Cole & Kitabchi 1978). Basal insulin levels were higher in the eight obese women with PCOS compared with six obese controls and, following an oral glucose tolerance test, the glucose sums were significantly higher in the PCOS group. They also found a significant correlation between insulin and testosterone levels. In 1983, Chang et al reported similar findings in the PCOS in the absence of obesity and acanthosis nigricans. Looking at ten non-obese women with the PCOS and ten non-obese controls, Chang found a mean basal insulin level that was significantly higher in the PCOS group and, following an oral glucose tolerance test, the mean rise in insulin was also greater. Subsequently, other researchers have confirmed that insulin resistance is present in non-obese women with PCOS (Dunaif et al, 1989, Mahabeer et al 1989, Conway et al, 1990, Dale et al 1992, Buyalos et al 1992).

Insulin resistance in PCOS is characterised by a decreased sensitivity to insulin in peripheral tissues (muscle and fat) but not in the liver in contrast to the insulin resistance of type II diabetes (Dunaif et al 1993, Peiris et al 1989). Furthermore it appears to reflect an increased secretion of insulin itself rather than of proinsulin and its split products (Conway et al 1993).

The mechanisms of insulin resistance in PCOS are poorly understood, and the presence of insulin resistance in non-obese women with PCOS suggests that the defect in insulin action is clearly not due to obesity alone. The mechanisms proposed include a defective degradation of insulin, anti-insulin receptor antibodies, elevated counter-regulatory
hormones of glucose homeostasis, hyperandrogenism, obesity, insulin receptor defects and post-binding defects in the insulin receptor pathway. A defect in insulin degradation was ruled out by the finding of hyperinsulinaemia without any insulin degrading activity in the study by Burghen et al in 1980. In addition, none of the patients in this study had insulin antibodies and the titre of insulin receptor antibody was measured in one patient and found to be low. Counter-regulatory hormones such as cortisol and growth hormone were also found to be low, and not related to the degree of insulin response to the oral glucose tolerance test (OGTT). It has been shown that androgen administration can induce insulin resistance (Billiar et al 1987). However, studies in which the hyperandrogenism in PCOS has been eliminated by the use of a GnRH agonist have demonstrated that insulin resistance can persist after androgen levels normalise, and the severity may not be diminished (Geffner et al 1986).

A reduced binding of insulin to its receptor was suggested by studies in blood cells (Flier et al 1985, Jialal et al 1987), though normal insulin receptor binding has been shown in adipocytes (Ciraldi et al, 1992). In the latter study, an eight-fold increase in insulin concentration was required to attain comparable glucose transport rates in adipocytes from eight women with PCOS compared with eight controls suggesting a post-receptor defect in the insulin transduction pathway. However, the exact mechanism of this defect is not certain. Recently, a decreased expression of an insulin dependent glucose-transporter protein GLUT-4 has been reported (Rosenbaum et al 1993), and a decrease in insulin receptor autophosphorylation has also been described in women with PCOS (Dunaif et al 1994).

Insulin resistance in PCOS seems to be a feature of the subgroup of women with menstrual irregularity, but not those with regular menses even where hyperandrogenism is present.
(Conway et al 1990, Robinson et al 1993). This implies that insulin resistance somehow contributes to the mechanism of anovulation in this syndrome, which is supported by in-vitro studies, which show that insulin stimulates the synthesis of androstenedione and testosterone in the theca and stromal cells from human ovaries (Barbieri et al 1986). This would suggest that although patients with PCOS are insulin resistant, their ovaries are insulin sensitive. However the exact mechanisms of this observation are unknown.

**Metabolic aspects of PCOS.**

As discussed above, insulin resistance is associated with glucose intolerance, hypertension, lipid abnormalities, elevated PAI-1 levels and an increased risk of coronary artery disease. But studies looking at the prevalence of these features in women with the PCOS provide either conflicting or inconclusive results.

**Diabetes:** Although most studies show that, overall, young women with PCOS maintain normal glucose tolerance despite being insulin resistant, there is evidence of an increased tendency to non insulin dependent diabetes (NIDDM) in PCOS. Gjonnaes in 1989 found an increase in the incidence of gestational diabetes in 89 women with PCOS who conceived following ovarian diathermy, and Dahlgren et al in 1992 found that 15% of 33 women aged 40 to 59 years with a histological diagnosis of PCOS made 22 to 31 years previously had diabetes mellitus, compared to 2.3% in the control group. A similar trend was found in a different study using the same subjects but a different control group published two years later (Dahlgren et al 1994). In a recent study, the incidence of gestational diabetes was higher in the pregnancies of 47 in women with PCOS compared with 100 healthy pregnant controls (Urman et al 1997). NIDDM in PCOS women tends to develop at a substantially earlier age (third to fourth decade) than in the general population (sixth to seventh decade) (Harris et al 1987).
Hypertension: Most cross-sectional studies in young women using single blood pressure measurements do not show an increased prevalence of hypertension in women with PCOS independent of the effects of obesity. However, two recent studies using 24-hour ambulatory blood pressure measurements in such women gave conflicting results. In the first study, daytime systolic and mean arterial blood pressures were higher in 36 women with PCOS compared with 55 controls (Holte et al 1996). Although the women with PCOS had increased truncal obesity, these findings persisted after this was taken into account. However, in the second study, there was no difference in blood pressure between non-obese women with PCOS and controls of a similar weight and age (Sampson et al 1996). Hypertension may only become manifest after many years of insulin resistance, which may explain these findings. There appears to be no prospective epidemiological study looking at this issue in women with PCOS, although in a transectional retrospective cohort follow-up of patients with PCOS aged 40 to 59 years, women with PCOS had a four-fold increase in the prevalence of hypertension (Dahlgren et al 1992). Women with PCOS are at increased risk of pregnancy induced-hypertension (Gjonnaess et al 1989, Urman et al 1997).

Lipid abnormalities: The results from studies on lipid states in women with PCOS are inconsistent, which is not surprising, as lipid levels may be influenced by body weight, steroid hormones and insulin states which are not consistent in these women. Some studies have found lower high-density lipoproteins (HDL) in women with PCOS compared with controls (Wild et al 1988, Conway et al 1992 & Robinson et al 1996). These results do not agree however with those of a recent study, which found no difference in HDL levels between lean women with PCOS and weight-matched controls (Sampson et al 1996). Very-low-density lipoprotein cholesterol (VLDL-C) has been shown to be higher
in women with PCOS compared with weight-matched controls (Wild et al 1988), but studies on triglycerides are conflicting. Some show an increased concentration in women with PCOS (Mattsson et al 1984, Wild et al 1985) but, where obesity has been taken into account, no difference has been found (Conway et al 1992, Robinson et al 1996, Sampson et al 1996). No study of women with PCOS has shown a higher mean serum cholesterol level independently of obesity.

**PAI-1 and possible role in cardiovascular disease and reproductive failure:** Insulin resistance in the PCOS (Dunaif et al in 1989, Mahabeer et al 1989, Conway et al in 1990, Dale et al 1992, Buyalos et al 1992) may cause elevated PAI-1. Because of the role of PAI-1 in fibrinolysis, in locally directed proteolysis during ovarian follicle development in rats (Peng et al 1993) and in mouse embryo implantation (Sappino et al 1989), high PAI-1 in PCOS may increase the risk of thromboembolic disease and poor reproductive performance. Although there is no direct evidence of an increased risk of thromboembolic disease in PCOS, indirect evidence suggests that this may be the case, as women with clinical signs of androgen excess often have abnormal coronary angiograms (Wild et al 1990). Using a risk factor model derived from an earlier prospective population study in which myocardial infarction was predicted by serum triglycerides, waist/hip ratio, diabetes and hypertension, it has been estimated that women with PCOS would be 7 times more likely to develop myocardial infarction compared to age matched controls (Dahlgren et al 1992). Women with PCOS have also been shown to have more extensive coronary artery disease than women with normal ovaries in a study in which the extent of coronary artery disease was assessed by quantitative angiography and compared with the presence or absence of polycystic ovaries on ultrasound in 143 women who had undergone a coronary angiography (Birdsall et al 1997).
However a recent prospective epidemiological study (HS Jacobs 1996) in which a cohort of 842 women with a mean age of 53.8 years in whom an ovarian diagnosis of polycystic ovaries was made before 1970 showed that there were 10 deaths from coronary heart disease, yielding a standardised mortality ratio of 1.35 (95% confidence interval 0.65-2.48). There were two deaths from other circulatory diseases (standardised mortality ratio 0.27; 95% confidence interval 0.03-0.96; p=0.04. This study therefore showed that women with PCOS did not have an increased risk of coronary mortality. It may well be that the increased degree of oestrogenisation in PCOS offers a cardioprotective effect, which compensates for their cardiovascular risk factors.

A speculative role for high PAI-1 levels in anovulatory infertility PCOS is supported by studies which show that components of the PAS are present in follicular fluid (Andolf et al 1995) and granulosa cells of human ovaries (Piquette et al 1993) and that plasmin weakens the follicular wall in-vitro (Beers 1975). Plasmin also activates the conversion of procollagenase to collagenase present in the basement membrane around the graffian follicle at ovulation (Stetler -Stevenson 1990). In a previous study on rat ovaries (Peng et al 1993), using in-situ hybridisation, PAI-1 mRNA was mainly found in the cell types located in the outer layer of follicles including the theca interna, theca externa, interstitial cells and ovarian stroma. Following HCG stimulation, the dominant pre-ovulatory follicles which protrude onto the surface of the ovary were surrounded by less interstitial and stromal tissue that expressed PAI-1 compared to the smaller non-ovulatory follicles that were in the interior part of the ovary which were surrounded by layers of PAI-1 expressing tissue. Thus insulin driven elevation in ovarian PAI-1 may contribute to limited follicle growth and anovulation by limiting the production of plasmin in the granulosa and theca cells of women with PCOS. This hypothesis is consistent with the observation that anovulatory women with PCOS are significantly more insulin resistant compared with
controls in this study, it was not possible to determine whether elevated PAI-1 was a unique feature of PCOS. These two studies and other subsequent studies on PAI-1 in PCOS are discussed in more detail in chapter 5.

CLINICAL FEATURES


Menstrual irregularity usually dates back to puberty and may take the form of oligomenorrhoea, amenorrhoea or frequent periods. However, menstrual cycles may be normal. Studies show that oligomenorrhoea is present in 29 to 47% of women with PCOS, amenorrhoea in 19 to 51% cases (Goldzieher and Green, 1962; Franks, 1989; Conway et al, 1989) and frequent periods in 3% of cases (Balen et al 1995). Normal cycles are present in 15 to 30% of cases.

Anovulatory infertility affects 20% of all couples with infertility (Hull et al 1985), but it is a feature of 30 to 74% of women with PCOS (Goldzieher and Green 1962, Franks 1989, Conway et al 1989). The exact mechanisms remain largely unexplained, but may include a negative effect of LH stimulated androgen production on follicle development (Louvert et al 1975), subnormal local FSH levels (Erickson et al 1979), impaired actions of insulin like growth factor on granulosa cell aromatase activity (Giudice et al 1995), aberrations in the intra-ovarian activin/inhibin system (Roberts et al 1994) and an imbalance in the intra-ovarian plasminogen-plasmin pathway (Sampson et al 1996, Atiomo et al 1998).
Miscarriage is another cause of childlessness in women with PCOS and in a study by Sagle et al in 1988, 46 (82%) out of 56 regularly menstruating women with recurrent miscarriage were found to have ultrasound evidence of polycystic ovaries. A high prevalence (56%) of polycystic ovaries was also found in another study of 500 women with recurrent miscarriage (Clifford et al 1994). It has been suggested that miscarriage occurs in women with polycystic ovaries because of hypersecretion of LH, as this appears to be a marker of subfertility and early pregnancy loss (Regan et al 1990). This hypothesis was supported by the finding of raised LH in women with recurrent miscarriage in association with polycystic ovaries in the study by Clifford et al in 1994. However a subsequent study in which LH levels were suppressed before pregnancy in a randomised controlled trial of 106 women with a history of three or more consecutive first trimester miscarriages, polycystic ovaries and hypersecretion of LH did not find an improvement in pregnancy outcome (Clifford et al 1996). The link between PCO and miscarriage thus remains unexplained.

Hirsutism and acne are the manifestations of hyperandrogenaemia in this syndrome. The hirsutism associated with PCOS usually dates back to menarche, is gradual in onset and is rarely accompanied by other signs of virilization such as clitoromegaly and temporal hair loss. In a study of 1741 women with ultrasound detected polycystic ovaries, hirsutism was present in 66.2% of women with PCOS and 34.7% had acne (Balen et al 1995). Other studies also show a similar trend with a reported prevalence of hirsutism in 34 to 69% of cases and acne in 9 to 24% of cases (Goldzieher and Green 1962, Franks 1989, Conway et al 1989).

Testosterone and androstenedione levels in anovulatory but non-hirsute women with PCO are similar to the levels found in women with hirsutism (Franks 1989, Conway et al 1989),
while women with hirsutism and PCO may have normal serum androgen concentrations (Franks 1989, Conway et al 1989, Lobo 1991). However, the daily production rate of testosterone is elevated in virtually all women with PCO (Yen 1986), and the normal serum concentrations of testosterone in women with hirsutism may be a reflection of increased clearance by peripheral tissues.

**Obesity** in women with PCOS is usually truncal, and they tend to have a high waist hip ratio. It is associated with more severe forms of the disease including extreme anovulation, oligomenorrhea and increased hirsutism, but non-obese women with PCOS may also present with these features. Weight reduction is associated with the return of regular menstruation, fertility, a reduction in hirsutism, an improvement in insulin sensitivity, an increase in SHBG levels and a correction of gonadotrophin and sex steroid abnormalities (Kopelman et al 1981, Harlass et al 1984, Pasquali et al 1989, Kiddy et al 1992, Anderson et al 1995).

**ULTRASOUND FINDINGS**

With successive advances in ultrasound technology, the criteria used in the diagnosis of PCO have evolved from an overall increase in ovarian size (Kratchowil 1972) to the recognition of characteristic follicular patterns of distribution and specific features in the ovarian stroma (Swanson 1981, Adams 1985). In a study of 76 women with menstrual disturbance and hirsutism, Adams et al in 1985 defined PCO as the presence of 10 or more small follicular cysts 2-8mm in size distributed evenly around the ovarian periphery with an increased amount of stroma and women with ultrasound features of PCO had higher testosterone and LH levels, and an increased ovarian volume, compared with women with normal or multifollicular ovaries (14.6 cm³, 6.4 cm³ and 8.0 cm³ respectively). Although they did not state how these ultrasound criteria were set, most subsequent clinical studies
on PCOS use them. These features contrast with multifollicular ovaries, where the follicles are fewer in number and there is minimal stromal tissue.

Not all women with the clinical features of PCOS will have the characteristic ultrasound features (Hann et al 1984), but there is a close correlation between these ultrasound criteria and histological features of polycystic ovaries (Saxton et al 1990). Women with PCOS may also have an increased endometrial thickness from unopposed oestrogenisation, and have recently been shown to have an increased ovarian stromal blood flow velocity on doppler ultrasound (Zaidi et al 1995).

AETIOLOGY & PATHOPHYSIOLOGY

With the heterogeneous nature of the PCOS, it is not surprising that there is no consensus about which factors explain its aetiology and/or pathophysiology. Instead, several factors have been shown to play a role. Traditionally, endocrine abnormalities arising from the hypothalamo-pituitary-ovarian (HPO) axis, or the adrenals, were thought to explain many of the aspects of this syndrome, but recent research has focused on the role of insulin resistance, intra-ovarian paracrine factors, dysregulation of cytochrome P450c17α, leptin, genetics and intrauterine programming.

Hypothalamo-Pituitary mechanisms

The concept of "inappropriate feedback" as the basis of anovulation and the endocrine abnormalities in women with PCOS (Yen et al 1976) has for a long time been the favoured hypothesis. In this model, it was suggested that the outpouring of oestrogen from extra-ovarian sources marred the cyclic changes in oestrogen production normally responsible for appropriate feedback regulation of the mid-cycle gonadotrophin surge. This resulted in an increased pituitary sensitivity to LHRH, an increased pulsatile LH release and a reduced...
FSH release. This chronically elevated LH level in turn caused increased ovarian androgen production by the theca cells, which was not converted to oestrogens because of a defective FSH dependent aromatase system in the ovary. The extraglandular aromatisation of these androgens (mainly in adipose tissue) to oestrogens, which occurred independently of FSH thus, results in the completion of a self-perpetuating cycle.

In support of this hypothesis, oral administration of oestrone was shown to enhance the disparity between LH and FSH found in PCOS, but was not found in normal controls (Chang et al 1982). Against this hypothesis, however, is the observation that not all women with PCOS have raised LH levels (Givens et al 1976) (up to 10% of women with PCOS have basal levels and a pulsatile pattern of LH release that are indistinguishable from normal cycles). In addition, oestrone rises very rapidly in response to gonadotrophins in women with PCOS excluding the possibility of primarily peripheral conversion. The underlying mechanism for the augmented pituitary sensitivity to LHRH by oestrogens remains to be defined.

It has also been suggested that in the hypothalamus a dissociation of dopaminergic and opioid inhibition of GnRH release may result in increased GnRH secretion (Yen 1986) and evidence in support of this comes from studies which show that the elevated LH levels in PCOS are reduced by exogenous dopamine inhibition (Quigley et al 1981). However the reversal of the cyclic release of gonadotrophins in response to several types of treatment is not in favour of a primary hypothalamic defect as this cyclicity is programmed in the hypothalamus.
**Adrenal Factors**

Adrenal hyperandrogenaemia is a well recognised feature of the PCOS (Lobo 1984, Hoffman et al 1984, Carmina et al 1986, Obhrai et al 1990, Fox 1992c), but levels of adrenocorticotropic hormone (ACTH) and cortisol are normal (Lachelin et al 1979). Although the cause is unknown, adrenal hyperandrogenaemia has been proposed as a possible aetiological factor, based on studies which show that women with congenital adrenal hyperplasia (CAH) have an increased prevalence of PCO (Hague et al 1990), and on studies which show that synthetic androgen administration to transexuals undergoing female to male gender transfer develop ovaries which look polycystic (Amirikia et al 1986). In addition, suppression of adrenal androgen synthesis in women with congenital adrenal hyperplasia (CAH) causes the return of ovulation (Chrousos et al 1982), although this effect can also be achieved with low dose corticosteroids which casts doubt on the role of adrenal hyperandrogenaemia alone in the aetiology of anovulation. A physiological rise in adrenal androgen secretion occurs at puberty. It has been suggested that this process may be accentuated and prolonged in the PCOS, which might affect normal hypothalamic maturation prior to the menarche causing persistent anovulatory cycles without a persistent adrenal abnormality (White & Turner 1994). Because the exaggerated rise of androgen levels in response to ACTH is similar to the amplification of LH-dependent androgen production by insulin, Fox speculated that insulin might regulate adrenal function in PCOS (Fox 1992c). However this hypothesis was not supported by the lack of a correlation between insulin and DHEAS levels in his study of oligomenorrhoeic women with PCO.

**Ovarian Factors**

Gonadotrophins, steroids, insulin-like growth factors and their binding proteins, activins/inhibins, the renin-angiotensin system and the plasminogen activator system have all been proposed as factors which play a role in the intra-ovarian pathogenic mechanisms...
resulting in disordered folliculogenesis in the PCOS. In the “Two-cell, two-gonadotrophin principle” (Erickson 1992), the theca cells synthesise and secrete androstenedione in response to LH, which diffuses across the basal lamina into the granulosa cells where it is aromatised to oestriol and subsequently oestradiol in response to FSH stimulation. Oestradiol is released into the circulation where it modulates the release of gonadotrophins from the pituitary resulting in ovulation. Short and London showed that follicular fluid from polycystic ovaries had very high concentrations of androstenedione, but no oestrone or 17β-oestradiol and concluded on the basis of this finding that polycystic ovaries had a defect in the aromatase system that prevented the conversion of C19 steroids to C18 oestrogens (Short & London 1961, Short 1962). It is thought that this high androstenedione/oestrone ratio results in disordered folliculogenesis. Although the mechanism of this biochemical defect remains unknown, the suggestions include a reduction in bioactive FSH available in the follicles for induction of aromatisation, an absent signal transduction system for FSH receptors in the granulosa cells, the presence of an FSH inhibitor, and the presence of an aromatase inhibitor. Follicular fluid from women with PCOS has however been shown to contain as much FSH as the normal dominant follicle (Erickson 1992). It has also been shown that granulosa cells from women with PCOS have an inherent ability to respond to FSH induction of aromatase activity, but that the graffian follicles never develop to a size where the aromatase enzyme is normally expressed (Erickson 1979). It has been suggested that the inhibitor of FSH may be insulin like growth factor binding protein (IGFBP), epidermal growth factor or inhibins (Redmond 1995).

The insulin like growth factors (IGFs) are polypeptides which modulate cell proliferation and differentiation, and are thought to be important in the growth and differentiation of ovarian follicles. They are structurally similar to pro-insulin, and although they are mainly
produced in the liver, ovarian production also occurs. They have their own receptors, and also bind weakly to insulin receptors at the end organ. In the circulation, they are bound by binding proteins (IGFBPs) which influence the amount of free IGF available to bind to receptors. There are two subclasses of IGFs depending on whether or not they are growth hormone dependent (IGF-I is and IGF-II is not). IGFBP1 is found mainly in amniotic fluid, IGFBP2 in the fetus and neonate, but IGFBP3 is the major binding protein in adult serum and is produced in the liver (Suikarri A-M et al 1988).

In women with PCOS a dysfunctional IGF system may impair follicular function. Although IGF-1 is found in normal concentrations in PCOS follicles at a concentration similar to that found in serum (Eden et al 1990), women with PCOS have been shown to have an 80% decrease in serum IGFBP-I (Pekonen et al 1989, Suikkari et al 1989) as well as reduced levels in the follicular fluid. The resulting increased IGF-I/IGFBP-1 ratio results in an increased bioavailable IGF-I that probably stimulates the theca interstitial cells to produce androgens, as well as affect granulosa cell function. However, the cellular distribution of IGFBP mRNA and protein in PCOS follicles is indistinguishable from that in small follicles of normal ovaries (el-Roeiy et al 1994), and in women with and without PCOS, IGF-1 has also been shown to accentuate the stimulatory effects of FSH on ovarian aromatase activity and oestradiol production equally (Mason et al 1993). It is therefore clear from these conflicting findings that the exact role of the IGF/IGFBP system in the pathogenesis of PCOS is not fully understood.

Inhibin and activin are dimeric glycoproteins. Inhibin was first described in 1932 as a substance that inhibited anterior pituitary secretions (McCullough 1932). It was extracted from follicular fluid in 1985 and found to inhibit FSH synthesis and release in animals (Robertson 1985). Inhibin is thought to play a complementary role in the negative
feedback effect of oestradiol on FSH secretion during the follicular cycle. Activin stimulates FSH secretion (Ling et al 1986), but it may also inhibit FSH release in the periovulatory period (Di Simone et al 1994). Activin and inhibin also have autocrine and paracrine activity in the human ovary. Inhibin inhibits aromatization while activin stimulates it (Di Simone et al 1994, Ying et al 1986). Activin also promotes mitogenesis in granulosa cells (Rabinovici et al 1990). In polycystic ovaries, the cellular localisation of inhibin and activin has been shown in one study to be similar to that found in normal ovaries (Jaatinen et al 1994) while in another study the granulosa cells from small antral follicles were less active in polycystic than normal ovaries with respect to inhibin alpha-subunit mRNA expression. Systemic levels of immunoactive inhibin in PCOS are similar to those found in normal women (Buckler et al 1988, Reddi et al 1989), but when measured in a recently developed ELISA assay, inhibin A and B concentrations were raised in PCOS (Anderson et al 1998) which was thought to be a reflection of the increased number of small follicles in PCOS. It is therefore not possible to confirm the speculative role of these peptide hormones in the pathophysiology of PCOS.

The possible role of the renin-angiotensin system in disordered folliculogenesis in the PCOS has also been evaluated in a study in which intense staining for renin and angiotensin was found in multiple cystic follicles and the stroma of ovarian biopsies from women with PCOS (Palumbo et al 1993). The staining was seen in only a limited number of follicles in normal control ovaries, suggesting that this system may underlie the basic cellular mechanism, which leads to arrest of follicle development in PCO. However research evidence for this hypothesis is scanty.

The plasminogen activator system is a cascade of enzymes and proenzymes, which lead to plasmin formation. Plasmin is a proteolytic enzyme, which plays a role in animal
ovulation, and it is possible that an insulin-mediated inhibition of plasmin release, by causing an elevation in PAI-1 levels, may disturb follicle growth and release in PCOS. This issue is fully addressed in four of the studies in this thesis.

Cytochrome P450c17α
Recent evidence suggests that PCOS may be influenced by a hyperfunction of the androgen-forming enzyme, cytochrome P-450c17α, within ovarian theca cells. In steroid biosynthesis, this enzyme binds both pregnenolone and progesterone, and converts them sequentially to 17-hydroxyprogesterone/pregnenolone by 17α-hydroxylation and androstenedione/ dehydroepiandrosterone by 17,20-Lyase activity (Fig 2.2 below). Evidence of cytochrome P-450c17α hyperfunction comes from studies which show a greater 17-hydroxyprogesterone, androestenedione and oestrone response to the administration of a gonadotrophin (Barnes et al 1989) in women with PCOS compared with controls. This hyper-responsiveness resembled those of males and was not lowered following the suppression of adrenal function by dexamethasone implying that the adrenals were not the main source of these hormones.

The cause of cytochrome P450c17α over-expression in these women is unknown, but it has been suggested that it may be explained variously by a direct stimulatory effect of LH on the ovarian theca cells, amplification of the effect of LH on theca cells through synergy with insulin and insulin-like growth factor-1 (without a rise in systemic LH secretion), an escape of P450c17α from desensitisation to LH, and most recently as a direct consequence of hyperinsulinaemic insulin resistance (Rosenfield et al 1990, Nestler & Jakubowicz 1996).
In the adrenal gland P450c17α also forms the 17-ketosteroids, but more dehydroepiandrosterone is formed than androstenedione (figure 2.2). The same gene codes for P450c17α in the adrenal gland and ovary (Miller 1988), and an increased adrenal 17-ketosteroid response to ACTH is common in PCOS (Lachelin et al 1979). Although it was previously thought that mild homozygous or heterozygous congenital adrenal hyperplasia was responsible, it may be that this response is due to an abnormal regulation of P450c17α in the adrenal cortex. The hypothesis of a dysregulation in P450c17α activity may therefore explain the finding of PCOS in association with normal or raised serum LH, insulin resistance and adrenal hyperandrogenaemia.

Figure 2.2 Biosynthetic pathways for steroid hormones.

<table>
<thead>
<tr>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOLESTEROL</td>
</tr>
<tr>
<td>↓ P450 scc</td>
</tr>
<tr>
<td>PREGNENOLONE</td>
</tr>
<tr>
<td>→ 3β</td>
</tr>
<tr>
<td>PROGESTERONE → ALDOSTERONE</td>
</tr>
<tr>
<td>↓ P450c17α</td>
</tr>
<tr>
<td>17-HYDROXY-</td>
</tr>
<tr>
<td>→ 17-HYDROXY P450 e21,c11,18 → CORTISOL</td>
</tr>
<tr>
<td>PREGNENOLONE</td>
</tr>
<tr>
<td>→ P450c17α</td>
</tr>
<tr>
<td>DEHYDRO -</td>
</tr>
<tr>
<td>→ ANDROSTENEDIONIOLE 17β → TESTOSTERONE</td>
</tr>
<tr>
<td>EPIANDROSTERONE</td>
</tr>
<tr>
<td>P450Arom ↓</td>
</tr>
<tr>
<td>OESTRONE</td>
</tr>
<tr>
<td>↑ 17β → OESTRADIOL</td>
</tr>
</tbody>
</table>

P450 = cytochrome P450, 3β = 3β-hydroxysteroid-dehydrogenase, scc = side chain cleavage, 7β = 17β-reductase, c17α, c21, c11, 18, arom = site of action of specific P450 enzymes, Arom = aromatase
Obesity

Approximately 50% of women with PCOS are obese, and obesity may also be involved in the pathophysiology of PCOS. Generally, a significant correlation exists between obesity and infertility (Rogers & Mitchell 1952, Hartz et al 1979), and obesity and miscarriage (Hamilton-Fairley et al 1992). There is also a correlation between the age of onset of oligomenorrhoea or amenorrhoea and the age of onset of obesity in PCOS (Pasquali et al 1985) and fertility, hyperandrogenaemia, hyperinsulinaemia and menstruation improve with weight reduction in these women. The links between obesity and reproductive function are not clear. However the possible mechanisms include increased insulin resistance which leads to elevated basal insulin levels, reduced synthesis of SHBG and consequently increased free testosterone and a reduced IGFBP1 concentration, abdominal distribution of fat which is associated with increased insulin resistance, lower SHBG concentrations and elevated free testosterone levels in premenopausal women (Kissebah et al 1982, Kissebah et al 1988, Freedman et al 1990, Kirschner & Bardin 1972), and recently leptin resistance. Leptin is a protein secreted by white fat cells, which is thought to send a signal to the satiety receptors in the hypothalamus. It is a product of the obesity gene and mice that have a deficiency of leptin have a voracious appetite, obesity, and infertility and have low gonadotrophin levels. This infertility appears to be reversed on treatment with leptin but not dietary restriction (Chehab et al 1996). Serum levels of leptin correlate positively with obesity and BMI and fat secretion of leptin is stimulated by insulin. Fat cells may also have the defect of tyrosine autophosphorylation, which is present in PCOS. It has therefore been hypothesised that in women with PCOS, insulin resistance in fat cells impairs the leptin response to obesity. Thus excessive eating continues despite increased body weight which aggravates the insulin resistance (Jacobs 1996). However most studies on leptin in women with PCOS show that the baseline levels are not different from

**Fetal Programming Hypothesis.**

Recently, there has been evidence to suggest that PCOS may originate during intrauterine development. Insulin resistance and lipid abnormalities in adult life are associated not only with acquired obesity, but with low birth weight and low rates of infant growth, and could be a consequence of foetal metabolic adaptations to under-nutrition (Phillips 1996). Women with high growth rates in infancy have an increased risk of ovarian cancer, which may be a reflection of long term disturbances in the pattern of gonadotrophin release that are established in-utero (Barker et al 1995).

Because of the above observations, Creswell and colleagues in 1997 examined 235 women aged 40 to 42 years born in Sheffield whose birth records were available and related the prevalence of polycystic ovaries and plasma concentrations of gonadotrophins and androgens to the women's birth weight and length of gestation. Their results showed that obese, hirsute, hyperandrogenic women with polycystic ovaries had a high birth weight, while thin women with polycystic ovaries, high LH and normal testosterone had prolonged gestation. These findings suggest that PCOS may be programmed from birth, but the findings are preliminary and the exact mechanism by which these intrauterine events produce these effects is unknown.

**Genetic Theories**

Although there is evidence that the PCOS is familial, its genetic basis is controversial. A family history is usually present for hirsutism, menstrual dysfunction and cystic ovaries (Givens et al 1971). Determination of the mode of inheritance has been difficult because
there has been no clearly defined male phenotype. Three studies have however
demonstrated an association between PCOS and premature male pattern baldness (MPB)
in male relatives of women with PCOS (Ferriman et al 1979, Lunde et al 1989, Carey et al
1993). The incidence of MPB was 44% compared to 7% in the non-PCOS related
families. These observations are consistent with an autosomal dominant mode of
inheritance.

In an attempt to define the gene responsible for PCOS, Carey and colleagues in 1993
identified a base change in the promoter region of the gene encoding P450c17α which was
associated with PCO, but using polymorphic markers this allele did not segregate with
PCO or MPB within families, thereby excluding it as the major genetic abnormality.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSES

In practice, a combination of clinical, ultrasound and biochemical features are used in the
diagnosis of PCOS, as the presentation is variable. A diagnosis of PCOS is likely if there
is a history of menstrual disturbance and hirsutism, with ultrasound features of PCO as
earlier described, in association with raised total or free testosterone, elevated LH (or
LH/FSH ratio) and hyperoestrogenaemia.

Women with PCOS may, however, have regular menstrual cycles and their only outward
manifestation may be recurrent miscarriage or hirsutism. Using ultrasound as a reference
method, Fox et al in 1991 suggested that a combination of the free androgen index
(calculated as testosterone (nmol/litre) x 100/ SHBG) and an elevated LH level were the
most accurate predictors of PCOS, but this was as good as combinations incorporating the
progesterone challenge test.
In women with suspected PCOS, it is important to rule out other endocrine causes of oligomenorrhoea/amenorrhoea. These include hypothalamic amenorrhoea (associated with weight loss, exercise, anxiety and chronic illness), pituitary adenomas, hyperthyroidism, hypothyroidism, late onset congenital adrenal hyperplasia (LOCAH), androgen secreting tumours, premature ovarian failure and Cushings syndrome (White & Turner 1994). Hypothalamic amenorrhoea is usually obvious from the history and is associated with low LH and FSH levels, pituitary adenomas are also associated with low levels of gonadotrophins and may be picked up on radiological tests of the pituitary fossa, thyroid disorders may be detected clinically or on thyroid function tests, and LOCAH although rare by measuring the 17α-hydroxyprogesterone response to corticotrophin. Androgen secreting tumours may be present if there is a short history of severe hirsutism and a testosterone concentration of more than 7 nmol per litre.

**TREATMENT**

**Anovulation:** The main principles of treating anovulatory infertility are to rule out other possible factors which may contribute to infertility such as tubal disease, to correct any underlying disorder (e.g. obesity), to induce regular unifollicular ovulation, and to achieve a high conception rate but with a low incidence of multiple pregnancy and miscarriage.

Anti-oestrogens (clomiphene citrate 50 - 100mg or tamoxifen 20 - 40 mg) are usually the first line therapy in inducing ovulation. Clomiphene may cause thickening of the cervical mucus, and when an ovulatory dose is reached, assessment of the cervical mucus at the time of ovulation is helpful. Anti-oestrogens are usually commenced on day two of the cycle and given for five days. If the patient has oligo or amenorrhoea, it is important to rule out pregnancy and induce a withdrawal bleed with a five-day course of a progestogen. This improves the response rate (Homburg 1988) and sheds any sub-optimal endometrium.
(Franks et al 1985). The usual starting dose is 50mg, but could be increased to 100mg if no response is achieved after three cycles. If a follicular response but no LH surge occurs, human chorionic gonadotrophin may be added in mid-cycle. A dose of more than 100mg rarely offers any benefit, and may be associated with side effects. Ovulation rates are high with rates up to 80%, however the pregnancy rates are only about 40%. Clomiphene may be associated with a risk of ovarian cancer after prolonged use (Rossing et al 1994) and the Committee of Safety of Medicines recommend that it should not normally be used for more than 6 cycles (CSM 1995).

For women who do not respond to anti-oestrogens, the therapeutic options are either parenteral gonadotrophin therapy or laparoscopic ovarian diathermy. Exogenous gonadotrophins may be either human menopausal gonadotrophin (HMG), or pure human FSH. The use of gonadotrophins is associated with the ovarian hyperstimulation syndrome and a multiple pregnancy rate of up to 20% (Duncan et al 1994). In one study of HMG in PCOS, the cumulative conception rate was 62% and the live birth rate 54% at 6 months (Balen et al 1994) and 73% and 62% respectively at 12 months. The multiple pregnancy rate in that study was 19%, but fell to 7% after the introduction of real time ultrasound for cycle monitoring and there were three cases of ovarian hyperstimulation. The risk of ovarian hyperstimulation and multiple pregnancy can be reduced by starting with very low doses of gonadotrophin and monitoring follicular development with ultrasound scans.

Women with PCOS have a higher risk of developing ovarian hyperstimulation syndrome (OHSS) with ovulation induction (Wang and Gemzell 1980), but the exact mechanism is unknown. This occurs when too many ovarian follicles are stimulated and results in abdominal distension, discomfort, nausea, and vomiting and occasional breathlessness. The mechanism may involve activation of the ovarian renin-angiotensin pathway and
excessive secretion of vascular epidermal growth factor. Ascites, pleural and pericardial effusions worsen the condition and the resultant haemoconcentration may lead to thromboembolism. The situation worsens if pregnancy occurs. Treatment usually involves the prevention of dehydration, pain relief and the prevention of thromboembolism.

For women who fail to respond to the above measures, laparoscopic ovarian diathermy may be used. Gjonnaes first described it in 1984, but its mechanism of action is unknown. It appears to be as effective as routine gonadotrophins in the treatment of clomiphene resistant anovulation, and it is less likely to result in multiple pregnancy and hyperstimulated ovaries compared with gonadotrophin therapy (Abdel Gadir et al 1990). The main complication of this procedure is peri-ovarian adhesions, although treating one ovary may reduce this, as ovulation can be triggered from both ovaries after the treatment of one ovary (Balen & Jacobs 1994). Various forms of laser have also been used.

The pregnancy rates after these procedures (diathermy and laser) vary from 20 to 88% (Donesky & Adashi 1995) with an average of 55%. The reason for the variations in reported pregnancy rate is unclear. The use of diathermy (rather than laser), younger age, a shorter duration of infertility and a higher pre-operative LH level were associated with a higher success rate (Li et al 1998).

Miscarriage rates are about 13% after ovarian diathermy (Li et al 1998) compared to over 20% after medical treatment (Abdel Gadir et al 1990). There may therefore be a case for laparoscopic ovarian drilling replacing gonadotrophins as the second line treatment for anovulation in PCOS.
Hirsutism

In addition to the use of local measures for hair removal, the principles of management of hirsutism include the determination of the type and source of hyperandrogenaemia (if present), exclusion of androgen secreting tumours, the use of androgen suppressive therapy if any androgens are significantly elevated and the use of an anti-androgen if the levels are normal or close to normal.

The common anti-androgens include cyproterone acetate (CPA), spironolactone, and flutamide. Dexamethasone may be used for suppression of adrenal androgens and the oral contraceptive pill or GnRH analogues for the suppression of ovarian androgens and to elevate SHBG levels.

Cyproterone acetate is a potent progestogen with anti-androgenic activity. It is usually given in combination with ethinyl oestradiol where hirsutism and menstrual disturbance co-exist. High doses may be associated with fluid retention weight gain, liver damage and mood changes. Spironolactone the aldosterone antagonist binds oestrogen and progesterone receptors and inhibits cytochrome P45017c. The side effects include lethargy, dizziness and light-headedness. Some women develop polymenorrhoea and the oral contraceptive pill may be prescribed if no improvement occurs after a few cycles. Flutamide is a pure anti-androgen (Cusan et al 1990). Its main drawbacks are its hepatotoxicity and cost (Wysowski et al 1993, Wallace et al 1993). It is usually used as a last resort. Anti-androgens may be feminising to male foetuses, and it is important that pregnancy is avoided while on them, hence their formulation as oral contraceptives when oestradiol is added such as in Diannette. Dexamethasone is a useful adjunct to anti-androgens when there are elevated androgens of adrenal origin. Where the combined oral contraceptive (COC) is thought necessary, progestogens with androgenic activity are best
avoided. Although there is evidence of a reduction in hair growth with the COCs, it is often not enough to satisfy the patient. They are therefore best used in combination with an anti-androgen.

Local measures, such as electrolysis and waxing, are useful while waiting for drug therapy to work. Response to treatment is usually slow and women are usually advised to stay on treatment for 1-2 years before tapering off.

**Acne** may be treated with broad-spectrum antibiotics, the combined pill, retinoic acid derivatives or anti-androgens. **Alopecia** may be treated with anti-androgens, but generally takes a long time to respond to treatment.

**Menstrual irregularity** may be controlled by the COCs or cyclical progestogens. Women with oligo or amenorrhoea in whom fertility is not a problem may prefer not to be treated. They are however at increased risk of sudden vaginal bleeding and may be at increased risk of endometrial cancer (Sherman & Brown 1979). It is therefore important to assess the endometrial thickness by ultrasound in these women especially when they are over the age of 40 years when this should be done annually.

**Metabolic problems** also need consideration in the treatment of these women. The best treatment for these conditions is weight reduction. It is also advisable to perform an oral glucose tolerance test and a lipid profile in obese young women with PCOS. Usually the diabetes in PCOS is non-insulin dependent and oral agents are the treatment of choice. Where hypertension is present, it is important to avoid a potassium sparing diuretic and angiotensin converting enzyme inhibitors if the patient is already on spironolactone.
because of the risk of hyperkalemia. Calcium channel blockers and thiazide diuretics are the most useful antihypertensives in these women.

In addition to improving insulin sensitivity, metformin, the oral hypoglycaemic has recently been shown to result in lower androgen levels, lower PAI-1 levels (Nestler & Jakubowicz 1996, Velaquez et al 1997) and may be a useful adjunct in the treatment of anovulation in these women. However larger clinical trials are required to test this hypothesis.

**SUMMARY**

PCOS is a common endocrine abnormality in women of the reproductive age group. The clinical manifestations are variable, and the aetiology and pathogenesis are not fully understood. Insulin resistance, a genetic defect inherited in an autosomal dominant pattern affecting the regulation of enzymes responsible for ovarian androgen production and factors originating in the intrauterine period may all play a role. Treatment is symptomatic and usually directed at the presenting clinical problem. The metabolic aspects are increasingly recognised as an area requiring careful attention because of the possible long-term sequelae. A better understanding of the underlying pathophysiology of this syndrome is the key to developing more logical and effective treatment.
CHAPTER THREE

STUDY DESIGN

OUTLINE

A detailed account of the sources and selection of subjects (or material) used in this thesis is presented. The definitions, ultrasound methods, data collection and methods of data analysis are described.

OVERALL STUDY DESIGN

The primary aim of this study was to evaluate systemic plasminogen activation, PAI-1 and the ovarian expression of PAI-1 protein and mRNA in women with PCOS and controls. A series of clinical and laboratory based cross sectional studies were performed in which these variables were measured in the setting of a district general hospital in the United Kingdom (Derriford Hospital, Plymouth). For the clinical studies, suitable patients and controls were recruited from the infertility, endocrinology and gynaecology clinics, local general practitioners and from amongst female members of staff. For the laboratory based studies, suitable paraffin embedded ovarian biopsies taken from women with and without a diagnosis of PCOS who had undergone ovarian biopsies between 1982 and 1985 were obtained from the archives of the histology department.

STUDY GROUPS

Three study groups were used and they were recruited between December 1995 and November 1997 (see table 3.1).
Table 3.1 - General Characteristics Of The Three Study Groups In This Thesis

<table>
<thead>
<tr>
<th></th>
<th>GROUP 1</th>
<th></th>
<th>GROUP II</th>
<th></th>
<th>GROUP III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOS n=11</td>
<td>Controls n=12</td>
<td>PCOS n=41</td>
<td>Controls n=25</td>
<td>PCOS n=5</td>
</tr>
<tr>
<td>Mean Age (years)</td>
<td>27</td>
<td>31.8</td>
<td>28.4</td>
<td>32</td>
<td>27.8</td>
</tr>
<tr>
<td>Mean Weight (Kg)</td>
<td>86.6</td>
<td>68.6</td>
<td>80.6</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td>Mean BMI (W/H²)</td>
<td>31</td>
<td>26</td>
<td>29.5</td>
<td>28.5</td>
<td>N/A</td>
</tr>
<tr>
<td>Menstrual Cycles</td>
<td>Oligo/A menorrh</td>
<td>Regular</td>
<td>Oligo/A menorrh</td>
<td>Regular</td>
<td>Oligo/A menorrh</td>
</tr>
<tr>
<td>Hirsutism Score**</td>
<td>Raised</td>
<td>Normal</td>
<td>Raised</td>
<td>Normal</td>
<td>N/A</td>
</tr>
<tr>
<td>Ovarian Morphology</td>
<td>PCOS</td>
<td>Normal</td>
<td>PCOS</td>
<td>Normal</td>
<td>PCOS</td>
</tr>
<tr>
<td>Free Androgen Index</td>
<td>Raised</td>
<td>Normal</td>
<td>Raised</td>
<td>Normal</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A = not measured

** = Hirsutism as assessed by the Ferriman Gallwey score

Amenorrh = Amenorrhoea

Group 1 was subjects investigated in a pilot study evaluating systemic levels of components of the plasminogen activator system (PAS) including PAI-1. It consisted of 11 women with PCOS and 12 controls recruited from the subfertility and gynaecology clinics and from amongst female members of staff at the hospital and the infertility clinic.

Group II were investigated in a larger clinical study of systemic levels of components of the PAS, and metabolic, coagulation and hormonal variables. This consisted of 83 women recruited from the endocrinology, infertility and gynaecology clinics, from local general...
practitioners and from amongst the female staff at the hospital out of which 41 women with PCOS and 25 controls were eligible for the study.

Group III consisted of archival blocks of stored ovarian biopsies taken from five women with PCOS and six controls analysed by immunohistochemistry and in-situ hybridisation for PAI-1 antigen and mRNA.

All the subjects in the three study groups were Caucasian.

ETHICAL APPROVAL

These studies were carried out following approval by the Ethical Committee of the South and West Devon Health Authority. The aims and nature of the investigations were explained and subjects were free to decline if they so wished without any prejudice on their clinical management. Informed consent was obtained in writing from all the participants in the clinical studies.

PATIENT RECRUITMENT AND CASE SELECTION

For the clinical studies, all the members of staff in the department of obstetrics and gynaecology, the department of endocrinology and local General Practitioners were informed of the aims and nature of the studies, by a letter and posters placed in the hospital wards and clinics. They were requested to make contact if they saw any patients with suspected PCOS who would be interested in participating in the study. A search of the hospital records, of the endocrinology clinic database from 1995 onwards and a review of all ultrasound reports with a diagnosis of polycystic ovaries from 1994 onwards was also performed. Women who were considered suitable were approached in writing or by telephone asking if they were interested in participating in the study following a full
explanation of the aims and methods of the project supplemented with a patient information sheet. General practitioner information sheets were also sent out. Interested participants were invited to the hospital and prospectively evaluated for the diagnosis or exclusion of PCOS by history, biochemical tests, and transvaginal or abdominal ultrasound scans. In a few cases a previous ultrasound report of polycystic ovaries as defined below was accepted. The following inclusion and exclusion criteria were used for the clinical studies.

INCLUSION CRITERIA USED IN CLINICAL STUDIES OF METABOLIC AND THROMBOTIC FEATURES IN PCOS (Study groups I & II)

<table>
<thead>
<tr>
<th>PCOS</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic oligomenorrhoea or amenorrhoea</td>
<td>Regular menstrual cycles</td>
</tr>
<tr>
<td>Ultrasound features of polycystic ovaries</td>
<td>Normal ovaries on ultrasound scan.</td>
</tr>
</tbody>
</table>

EXCLUSION CRITERIA

- Current pregnancy
- Delivery or miscarriage occurring within the preceding 3 months
- Recent surgery (within 3 months)
- History of myocardial infarction
- Use of aspirin or heparin
- Sex steroid therapy
- A history of haematological disease, malignancy or liver disease
- Hyperprolactinaemia
- A positive synacthen test following raised 17-hydroxyprogesterone levels
- A history of thrombosis

The last three criteria were not used in the pilot clinical study.
Stage in menstrual cycle during which samples for PAI-1 were obtained.

The samples for PAI-1, taken from the women in study groups I and II were obtained at random stages of their menstrual cycles. This was because, the only study to my knowledge in the literature which had addressed this issue, had shown that systemic PAI-1 levels did not fluctuate during different stages of the menstrual cycle (Dorr et al 1993). It is important to note however that, because by definition women with PCOS have erratic periods and may be amenorrhoeic for years, it would have been impractical to insist that all the samples for PAI-1 were taken from them at a specified day or stage in the menstrual cycle.

The exact day of the cycle when these samples were obtained can be found in the data section of the appendix. Briefly, in study group I, in the control group, three women were menstruating at the time the samples were obtained, four were pre-ovulatory and five post-ovulatory. In the PCOS group, one woman was menstruating, two had completed their menses but were 14 days or less from the first day of their LMP (last menstrual period) and eight women had the samples taken 15 days or more from their LMP.

In study group II, in the control group, two women were menstruating at the time the samples were obtained, nine were pre-ovulatory and 14 post-ovulatory. In the PCOS group, five women were menstruating, five had completed their menses but were 14 days or less from the first day of their LMP (last menstrual period) and 31 women had the samples taken 15 days or more from their LMP.

The selection of suitable material for study group III was as follows. The histological reports of women with PCOS who had undergone a wedge resection for the treatment of infertility between 1982 and 1996 were retrieved from the records of our hospital's
Histopathology department. Control normal ovaries were selected from women of a similar age who had either undergone an oophorectomy or ovarian biopsy during the same period. Only cases where the initial histopathology report unequivocally diagnosed polycystic ovaries were selected and cases where the histology report specifically described the ovaries as "normal" or containing "follicle cysts" (but without any of the other stigmata of PCO) were selected. The hospital records of these women were reviewed and the details noted included their age, menstrual histories, indication for surgery, medication at surgery, weights and results of any endocrine tests if present. This information was recorded on a standard proforma (appendix 3) and the histological slides from the women where this information was available from their records were reviewed by an independent histopathologist blind to the original diagnoses. Cases were defined as PCOS if there was in addition to a histology report of PCO, a documented history of oligo or amenorrhoea, and a complete agreement with a histological diagnosis of PCO on review of the slides by a histologist blind to the original diagnosis. Biochemical evidence of PCOS (raised androgens or a raised LH/FSH ratio) was considered a desirable additional criterion, but this was not made an absolute requirement. Control ovaries were defined as normal, if the initial histology report stated that the ovaries were "normal" or had follicle cysts less than 2cm in diameter (without the other stigmata of PCO) and if the women had documented evidence of regular menstrual cycles at the time of surgery. These slides were also verified by the histopathologist. Ovaries with malignancy, endometriosis, large ovarian cysts or from women on sex steroids at the time of their surgery were excluded from the study.
INCLUSION CRITERIA USED FOR LABORATORY STUDIES OF PAI-1 PROTEIN AND mRNA IN PCOS (Study Group III)

<table>
<thead>
<tr>
<th>PCOS</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documented history of oligomenorrhoea or</td>
<td>Regular menstrual cycles</td>
</tr>
<tr>
<td>amenorrhoea.</td>
<td></td>
</tr>
<tr>
<td>Histological features of polycystic ovaries</td>
<td>Normal ovaries on histology.</td>
</tr>
<tr>
<td>+/- Documented biochemical evidence of PCOS</td>
<td>or ovaries with follicular cysts</td>
</tr>
<tr>
<td>(raised androgens and raised LH/FSH ratio)</td>
<td>without other features of PCOS</td>
</tr>
</tbody>
</table>

EXCLUSION CRITERIA FOR STUDY GROUP III

Benign ovarian cysts > 2cm
Malignant ovarian neoplasia
Endometriosis

DATA COLLECTION, ASSAYS AND LABORATORY TECHNIQUES

Details were obtained from the subjects recruited into the clinical studies by direct interviews and recorded on standard proformas (appendices 1&2). The information obtained included their age, menstrual, obstetric, medical, family and social histories and details about their smoking and alcohol intake. Hirsutism was assessed with a Ferriman Gallwey chart, and their heights, weights, blood pressures and waist and hip circumferences were measured. Fasting blood samples were taken for PAI-1 and other components of the PAS, testosterone, SHBG, LH, FSH, oestradiol and DHEAS in the pilot study (Group I) and in addition to these, prolactin, 17-hydroxyprogesterone, insulin, glucose and lipids in the main clinical study (group II).
Insulin sensitivity (the degree of insulin resistance) was assessed using a Homeostatic Model Assessment (HOMA) computer programme obtained from Dr Jonathan Levy at the University of Oxford department of Clinical Medicine. This model is a structural computer model of the glucose/insulin feedback system. It incorporates mathematical descriptions of the functions of the various organs involved in plasma glucose control and their interactions, based on empirical data of fasting insulins and glucose from subjects in the Oxford population. It provides a model of physiological reality in a "reference individual" in that it will find the equilibrium point of plasma glucose, insulin, C-peptide and proinsulin in the fasting state. It has two key parameters, which are pancreatic beta cell function (%B) and peripheral and hepatic insulin sensitivity (%S). The model can predict the plasma glucose, insulin, C-peptide and proinsulin for any possible combination of these two parameters in the fasting state. Since any one combination of a particular %B and %S is associated with only one combination of glucose, insulin, C-peptide and proinsulin, it is possible to determine from an individual’s plasma concentrations glucose and either insulin or C-peptide the particular combination of %B and %S that would have produced them. Insulin sensitivities were thus calculated for the subjects in this study using their fasting glucose and insulin levels. The insulin results were first converted from their original units in mU/L to pmol/L before entry into the model and the conversion factor used was X 7.24.

More details about the exact assays performed and the laboratory techniques can be found in the relevant data chapter. Ultrasound scans were performed as detailed below either on the same day or at a later date. Following this the participants were thanked and allowed home. Any abnormal findings were discussed with the patient, and their General Practitioner was informed.
Ovarian biopsies from the women in study group III were tested for PAI-1 protein with the steptavidin-biotin method using a monoclonal antibody to PAI-1 and PAI-1mRNA was evaluated by in-situ hybridisation using a cocktail of digoxigenin labelled oligonucleotide probes. More details about these techniques can be found in the relevant data chapters.

ULTRASONOGRAPHY

Transvaginal or abdominal ultrasound scans were performed according to the patient’s preference, but they were informed that visualisation of the ovaries was easier using the vaginal route. 7.0 MHz and 3.5MHz transducers were used for the vaginal and abdominal ultrasound scans respectively. For the vaginal ultrasonography, the women were requested to empty their bladders before the procedures, and asked to lie supine on a couch with a wedge to support the pelvis. The transducer was lubricated with KY jelly, protected in a sheath and re-lubricated with KY jelly, following which it was introduced into the vagina. The time-gain compensatory curve controls were adjusted on the console to improve the image quality, the uterus was first examined in the sagittal and coronal plains and the maximum endometrial thickness was measured. Next the ovaries were examined. The maximum diameter of the ovary was identified, and measured to provide the length. The probe was then turned through an angle of 90 degrees to provide measurements of the ovarian width and thickness. The numbers of follicles in each ovary were counted and measurements of the diameters of the largest and smallest follicles were taken in the vertical plane. The distribution of the follicles was noted, and the stromal echogenicity was defined subjectively by comparing it to the echogenicity of the uterine wall. Any other abnormalities in the pelvis were also noted.

At the end of the procedure, the probe was removed from the vagina, the sheath removed and discarded and the probe cleaned with methylated spirit.
For the abdominal ultrasound scans, patients were requested to arrive with a full bladder for adequate visualisation of the pelvic organs. The women were asked to lie supine on a couch with the suprapubic region exposed. Paper towels were used to protect garments. Coupling gel was spread over the transducer that was then placed over the lower abdomen in the mid-line just above the pubic hairline. The time-gain compensatory curve controls were adjusted on the console to improve the image quality, and the pelvis was scanned in longitudinal and transverse sector planes. The uterus was identified and measurements made of the endometrial thickness. The ovaries were then visualised in turn using the ovarian vessels as reference points and measured as described above. The pelvis was also inspected for other abnormalities. During the examination, the ultrasound findings were discussed with the patients and any questions answered. At the end of the examination they were allowed to empty their bladders and any further questions were answered.

**DEFINITIONS**

**General Considerations**

The selection criteria for the definition of PCOS for this thesis had to be decided against the background controversies in the definition outlined in chapter 2. The main considerations were

1) Choosing selection criteria with good sensitivity for the diagnosis of PCOS

2) The ability to compare results with previously published data on similar studies on women with PCOS.

3) The need to use evidence of anovulation (from a history of oligo or amenorrhoea) in the inclusion criteria because one of the aims of the studies in this thesis was to consider the role of PAI-1 in anovulation in PCOS.
Two cross sectional studies were published before the onset of recruitment for the large clinical study (Dahlgren et al 1994, Sampson et al 1996). In both cases, the diagnosis of PCOS was made on a combination of ovarian morphological appearance of polycystic ovaries (on histology in one case and ultrasound in the other) and menstrual disturbance. Hyperandrogenaemia was not specifically used as a selection criterion.

Ultrasound Definition of Polycystic and Normal Ovaries

The diagnostic value of each ultrasound parameter has been difficult to assess since there is no gold standard for the diagnosis of the polycystic ovary syndrome phenotype. Although polycystic ovaries were initially defined as the presence of 10 or more 2-8mm follicles distributed around the periphery or all over the ovary associated with the presence of an increased and highly echogenic ovarian stroma on abdominal ultrasound (Adams et al 1985), it is not clear how these parameters were derived. Ovarian enlargement was also included as one of the features of PCO in this study as the mean ovarian volume was 14.6cm$^3$ in the PCO group compared to 8 and 6.4 cm$^3$, in women with multifollicular and normal ovaries respectively, but the upper limit of "normal" was not defined. Subsequently, a diversity of sonographic criteria has been used for the diagnosis of polycystic ovaries (Yeh et al 1987, Pache et al 1992, Robert et al 1995), with various sensitivities for the detection of the PCOS phenotype (table 3.2). This is not surprising given the heterogeneity of the PCOS phenotype. Although the original description of PCO was made following abdominal ultrasound scan, many studies have since used transvaginal ultrasound for the diagnosis of PCOS. There have been attempts to objectively define the vaginal ultrasound features of PCOS and in one such study, Fox evaluated 10 women with a PCOS phenotype defined as a combination of hirsutism and abnormal menstruation and 20 non-hirsute women with normal cycle length (Fox R 1992e). He found that the women with normal menstrual cycles had up to 12 small follicles (<10mm) in each ovary with
little stroma and all but one of the hirsute patients had increased numbers of small follicle with abundant stroma. Because of this diversity in the ultrasound diagnosis of PCOS, Dewailly and colleagues in 1993 evaluated the sensitivity and specificity of various ultrasound criteria. They concluded that increased ovarian stroma (assessed by computerised quantification) was the most valuable diagnostic probe for PCOS. However in a further study, 45% of patients with the clinical phenotype of PCOS, had an ovarian area which overlapped the range found in controls and patients with hypothalamic anovulation (Dewailly et al 1994). This suggests that although ovarian hypertrophy at ultrasound is suggestive of PCO, its absence does not exclude the diagnosis. This statement is supported by the finding that in the only study using histological correlation’s to assess the accuracy of the ultrasound diagnosis of PCO (Saxton et al 1990), using the criteria originally defined by Adams et al in 1985, there was a perfect correlation between the number and distribution of cysts on ultrasound and on histology. However, stromal hyperplasia was only present in 61% of the polycystic ovaries.
Table 3.2 Sensitivities Of Some Of The Ovarian Sonographic Criteria Used In The Diagnosis Of PCOS.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Ultrasound Route</th>
<th>Ultrasound Parameter</th>
<th>Criteria indicative of PCO</th>
<th>Sensitivity for PCOS phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeh et al 1987</td>
<td>Abdominal</td>
<td>Number of follicles 5-8mm size</td>
<td>&gt; 5</td>
<td>74%</td>
</tr>
<tr>
<td>Pache et al 1992</td>
<td>Vaginal</td>
<td>Ovarian volume</td>
<td>&gt; 8cm³</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of follicles &lt; 6mm</td>
<td>&gt;11</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean Follicular size</td>
<td>&lt; 4mm</td>
<td>70%</td>
</tr>
<tr>
<td>Robert et al 1995</td>
<td>Vaginal</td>
<td>Increased stromal echogenicity</td>
<td>Present</td>
<td>94%</td>
</tr>
<tr>
<td>Robert et al 1995</td>
<td>Vaginal</td>
<td>Increased stromal area</td>
<td>&gt; 7.6cm³</td>
<td>61%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased maximum ovarian area</td>
<td>&gt; 10.8 cm³</td>
<td>55%</td>
</tr>
</tbody>
</table>
Against the background of these controversies in the morphological diagnosis of PCO, polycystic ovary for these studies was defined as the presence of 10 or more 2 to 8mm follicles distributed around the periphery or all over the ovary associated with the presence of a bright ovarian stroma (See figure 3.1). In the presence of a stroma of equivocal echogenicity, either the presence of an enlarged ovary (volume of > 9cm$^3$) or a peripheral distribution of ovarian follicles, in addition to the other criteria was taken as indicative of polycystic ovaries. These criteria were chosen because they were consistent with the criteria stipulated by Adams and colleagues in their original definition of PCOS. Stromal echogenicity was not made a stringent criterion because of the subjectivity of its evaluation. Normal Ovaries were defined as those with less than 10 follicles and with at least one follicle of a maximum diameter greater than 8mm.

**Menstrual Pattern**

Generally, there is no widely accepted definition of a normal menstrual cycle in terms of cycle length, and there do not appear to be any prospective studies looking at fertility rates in women with extreme degrees of cycle length and variability. In this thesis, oligomenorrhoea was defined by the presence of a history of an average cycle length of 35 days or more with variability between cycles of 10 days or more of at least 6 months.
Figure 3.1  Vaginal Ultrasound Scan Of A Polycystic Ovary

Figure shows a polycystic ovary in longitudinal section. Note the numerous numbers of small follicles distributed around the periphery of the ovary, the increased stromal echogenicity and ovarian enlargement.
duration. This limit was set because the upper limit of normal for a menstrual cycle length is arbitrarily defined as 5 weeks (Hull & Abuzeid 1992) and this definition is consistent with the only cross-sectional study of PCOS and PAI-1 where menstrual pattern had specifically been incorporated in the definition of PCOS (Sampson et al 1996). Amenorrhoea was defined as the absence of periods for six months or more. For study group III, where this information was derived from the hospital records (see case selection below) a documentation of a history of oligomenorrhoea or amenorrhoea was accepted regardless of the duration. Normal menstrual cycles were defined as spontaneous menstrual cycles with a duration of 21-35 days with less than a 10-day variability between cycles.

**Histological evidence of Polycystic ovaries**

This was defined as ovaries containing multiple cystic follicles of approximately equal size situated superficially beneath a thickened outer ovarian cortex with little or no evidence of ovulation as determined by the presence of corpora lutea or corpora albicantia. These criteria were consistent with those used in the first study looking at some components of the PAS (PAI-1 and fibrinogen) in women with PCOS (Dahlgren et al 1994). In that study, PCOS was histopathologically defined from ovarian biopsies from women who underwent wedge biopsies 25-34 years before the study.

**Polycystic Ovary Syndrome**

The definition of PCOS in this thesis was based on the presence of a combination of a morphological appearance of polycystic ovaries as defined above (on ultrasound for the clinical studies, and on histology for the laboratory based studies) and a clinical finding of oligo or amenorrhoea, with other possible causes of oligo or amenorrhoea ruled out by specific endocrine tests. Despite the non-inclusion of hyperandrogenaemia in the
definition, women with PCOS in the clinical studies had significantly higher free androgens (measured by the free androgen index), and Ferriman Gallwey scores than the control groups providing reassuring evidence that the inclusion criteria used for this study were satisfactory for the diagnosis of PCOS (see tables 4.1 and 5.1).

In this thesis, the terms "insulin resistance" and "reduced insulin sensitivity" are synonymous. Thus low insulin sensitivity implies increased insulin resistance.

**DATA STORAGE AND GENERAL STATISTICAL METHODOLOGY**

The data from each subject was stored on individual proformas (see appendix 1, 2 & 3) and subsequently transferred into a spreadsheet (microsoft excel) for basic data analysis after the exclusion of identifying data. Sample size calculations were performed prior to the large clinical study. The distribution of continuous variables was identified using a normal probability plot. Parametric data (normally distributed) were described by their means and standard deviations (SD), and the probability of differences between groups of normally distributed data being the result of chance was tested with an unpaired student's t test. Non-parametric data were described by their median and range, and the difference between groups of non-parametric data was tested with a Mann-Whitney U test. Some of the measured variables had a very limited range of values and for these neither a t-test nor Mann Whitney test was appropriate. In these cases, Fisher's exact test was used to compare the proportions of 'high' values for the groups. An analysis of covariance was used to evaluate the effects of possible confounding variables on dependent variables. A Pearson's correlation coefficient was used to test the relationship between groups of normally distributed data. A multiple regression analysis was used where indicated to evaluate the effects of potential independent variables on a dependent variable. A p value of ≤ 0.05 was accepted as statistically significant.
CHAPTER FOUR

THE PLASMINOGEN ACTIVATOR SYSTEM IN WOMEN WITH POLYCYSTIC OVARY SYNDROME (PILOT STUDY).

OUTLINE

A pilot study is described in which plasminogen activator inhibitor-1 (PAI-1), plasminogen, fibrinogen and α-2 antiplasmin concentrations and euglobulin clot lysis times (ECLT) were determined in a group of oligomenorrhoeic women with polycystic ovaries and compared with a control group of women who had regular menstrual cycles and normal ovaries on ultrasound.

INTRODUCTION

As discussed in chapter 2, insulin resistance and a corresponding elevation in PAI-1 in the PCOS may contribute to an increased risk of thromboembolic disease, anovulatory infertility and miscarriage. Prior to this pilot study, there was only one published cross-sectional study on PAI-1 in the PCOS (Dahlgren et al 1994), and in the study, 28 peri-menopausal women with PCOS and 56 age and weight matched controls had metabolic and haemostatic variables evaluated. No difference was found in systemic PAI-1 between 28 women with PCOS and 56 controls, and there was also no difference in the fasting insulin levels between both groups in the study. This may have been partly explained by the fact that 20% of the control population had hypertension suggesting that there was a high prevalence of metabolic disorders possibly because most women in the study were in their fifties when the prevalence of these metabolic disorders rise.
This pilot study was performed to determine whether elevated PAI-1 levels were present in PCOS, determine the feasibility of a larger definitive study and provide baseline data for sample size calculations prior to it.

SUBJECTS AND METHODS

Eleven women with the PCOS and 12 controls took part in this study (group 1 as described in chapter 3). The study design and patient recruitment are described in chapter 3. These women were requested to come up to the hospital for the study between 8 and 10 am in the morning because of the diurnal variation in PAI-1 (Juhan-Vague et al 1992) and participants were requested to fast overnight and refrain from smoking from midnight. On arrival at the hospital, their weights and heights were recorded and they were then sat upright or in a supine position for 15 minutes during which clinical details were obtained (age and menstrual, obstetric, medical, family and social histories). They were given a Ferriman Gallwey chart (Ferriman D & Gallwey J 1961) to score their perceived hirsutism.

Then, 18 to 20 ml of blood were taken from an antecubital vein on the left forearm with minimal or no venous occlusion into four to five citrate tubes for ECLT, PAI-1, fibrinogen, plasminogen and α-2-antiplasmin (prolonged venous occlusion can stimulate the production of tissue plasminogen activator (tPA) and PAI-1 from the endothelial cells). Five mL of blood were collected into a separate tube for testosterone, SHBG, LH, FSH, oestradiol and DHEAS assays. Blood pressure was then checked on the right arm and the sphygmomanometer cuff was inflated midway between systolic and diastolic pressures for 10 minutes. After this test, another 18 to 20 mL of blood were collected from below the cuff for post-venous occlusion samples. Tables 4.1 and 4.2 show the clinical and biochemical features of both groups and provide confirmation that the recruited subjects
had PCOS.

Table 4.1 - Clinical Variables For Patients With PCOS Versus Controls.

<table>
<thead>
<tr>
<th>Clinical Variable</th>
<th>PCOS (n = 11)</th>
<th>Controls (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age ± SD (y)</td>
<td>27 ± 3.6</td>
<td>31.8 ± 7.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Mean BMI ± SD (kg/m²)</td>
<td>31 ± 5.3</td>
<td>25.9 ± 2.6</td>
<td>0.003</td>
</tr>
<tr>
<td>Median hirsutism score (range)</td>
<td>8 (1-22)</td>
<td>1 (0-8)</td>
<td>0.003*</td>
</tr>
<tr>
<td>Median cycle length in weeks (range)</td>
<td>20.75 (7.5-104)</td>
<td>4 (3.7-5.0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Number with history of Subfertility (%)</td>
<td>7 (64%)</td>
<td>4 (33%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Number of nulliparas (%)</td>
<td>7 (64%)</td>
<td>7 (58%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Number with previous miscarriage(%)</td>
<td>2 (18%)</td>
<td>3 (25%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Number of smokers (%)</td>
<td>4 (36%)</td>
<td>4 (33%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Number of alcohol units/wk (range)</td>
<td>2 (0-8)</td>
<td>3 (0-14)</td>
<td>0.49*</td>
</tr>
<tr>
<td>Mean systolic blood pressure ± SD (mmHg)</td>
<td>123 ± 14</td>
<td>114 ± 15</td>
<td>0.14</td>
</tr>
<tr>
<td>Mean diastolic blood pressure ± SD (mmHg)</td>
<td>82.7 ± 6</td>
<td>72.7 ± 9.9</td>
<td>0.008</td>
</tr>
</tbody>
</table>

NOTE. BMI = body mass index, * = Determined by the Mann Whitney U test.
Table 4.2 - Hormonal variables for patients with PCOS versus controls.

<table>
<thead>
<tr>
<th>Hormonal Variable</th>
<th>PCOS (n = 11)</th>
<th>Controls (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean FAI ± SD</td>
<td>11.02 ± 8.21</td>
<td>2.2 ± 0.9</td>
<td>0.0053</td>
</tr>
<tr>
<td>Mean SHBG ± SD (nmol/L)</td>
<td>30.4 ± 17</td>
<td>54.5 ± 27</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean Testosterone ± SD (nmol/L)</td>
<td>2.67 ±1.1</td>
<td>1.19 ± 0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Mean DHEAS ± SD (µmol/L)</td>
<td>10.8 ± 4.5</td>
<td>7.4 ± 2.7</td>
<td>0.04</td>
</tr>
<tr>
<td>Median oestradiol (pmol/L) (range)</td>
<td>82 (46-463)</td>
<td>135.5 (20-353)</td>
<td>0.93*</td>
</tr>
<tr>
<td>Median FSH (U/L) (range)</td>
<td>4.6 (1.8-5.2)</td>
<td>4.55 (2.9-23.1)</td>
<td>0.44*</td>
</tr>
<tr>
<td>Median LH (U/L) (range)</td>
<td>7.0 (1.1-13.5)</td>
<td>4.05 (1.6-36.4)</td>
<td>0.23*</td>
</tr>
<tr>
<td>Mean LH/FSH ratio ± SD</td>
<td>1.7 ± 0.9</td>
<td>1 ± 0.43</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Determined by the Mann Whitney U test.

FAI = Free androgen index. (testosterone / SHBG x 100)

SHBG = sex hormone binding globulin

DHEAS = Dehydroepiandrosterone sulphate

FSH = Follicle stimulating hormone

LH = Luteinizing hormone

The samples for ECLT, PAI-1, fibrinogen, plasminogen and α-2-antiplasmin were immediately placed on ice and centrifuged within 30 minutes of collection at 2000g for 15 minutes at 4 °C. Hormonal assays were performed in the combined laboratory at Derriford Hospital in Plymouth. Plasma was frozen at -70 °C and tested within three months for PAI-1 activity, alpha-2 anti-plasmin and plasminogen concentrations. ECLT were measured immediately on fresh plasma.
Laboratory Assays

The PAS is assessed using tests that measure a combined effect of plasminogen activators and inhibitors such as the euglobulin clot lysis time (ECLT), or fibrin plate lysis and specific assays of the activators and inhibitors of this system and the change in the values of these factors in response to venous occlusion which stimulates fibrinolysis.

ECLT was performed as previously described (Dacie & Lewis 1995). The principle of this assay is that plasma is diluted and acidified forming a precipitate (euglobulin) which contains plasminogen activator, plasminogen and fibrinogen. Most of the inhibitors are left in the solution. The precipitate is re-dissolved, the fibrinogen clotted with thrombin and the time for clot lysis measured. The normal range for this assay is 90 - 240 minutes.

PAI-1 activity was tested using a commercially available chromogenic assay (Chromogenix, Sweden). The principle of this assay is that a fixed amount of t-PA is added in excess to undiluted plasma, where part of it rapidly forms an inactive complex with PAI-1. In the presence of a stimulator, the residual t-PA activates plasminogen to plasmin. The amount of plasmin formed is inversely proportional to the PAI-1 activity in the sample. The amount of plasmin is determined by measuring the amidolytic activity of plasmin on a chromogenic substrate S-2403. The release of p-nitroaniline (pNA) is determined at 405nm. The range of normal values for PAI-1 is less than 15 arbitrary units per millilitre (au/ml). The intra-assay coefficient of variation for this assay was 2.4% at a level of 12 au/ml, and 0.4% at 23 au/mL while the inter-assay coefficient of variation 7.6% and 1.6% at 12au/mL and 23 au/mL respectively.

Plasminogen and α-2 antiplasmin levels were also tested using chromogenic assays (Instrumentation Laboratory, Milano Italy). The principle of the plasminogen assay is that
an excess of streptokinase is added to diluted plasma. The plasminogen present will combine with streptokinase forming a complex with plasmin-like activity. This acts on a chromogenic substrate releasing pNA, which is monitored at 405nm and is directly proportional to the plasminogen level. The normal range for this assay is 80 to 120% and the sensitivity 15%. In the α-2 antiplasmin assay, diluted plasma samples are incubated with an excess of plasmin. α-2 antiplasmin has a powerful and rapid plasmin - inhibitory potential, and the residual plasmin is measured by its activity on a chromogenic substrate. The amount of the pNA released is inversely proportional to the α-2 antiplasmin level. The normal range of this assay is 80 to 120% and the sensitivity 15%.

Fibrinogen concentration was tested using an automated coagulation laboratory analyser (Instrumentation Laboratory, Milano, Italy) which measures the intensity of light scattered by a plasma sample before during and after clot formation. The light scatter reached at equilibrium is proportional to the fibrin concentration and therefore the total clottable fibrinogen. The normal range is 2 to 6 g/l.

FSH and LH concentrations were tested using the Amerlite enhanced luminesence method (Johnson and Johnson, New Brunswick, NJ, USA).

Serum DHEAS, serum testosterone, oestradiol and SHBG levels were tested using radioimmunoassay kits (Johnson and Johnson, New Brunswick, NJ, USA; Medgenix, Cheveus, Belgium; Sorin diagnostics, Salluggia, Vercelli, Italy and Farmos diagnostics, Espoo, Finland respectively).

Free androgen index was calculated using the formula (Testosterone/ SHBG x 100).
The inter and intra-assay coefficients of variation for all these assays were less than 9% and the sensitivities for testosterone, DHEAS, SHBG, oestradiol, LH and FSH were 0.2 nmol/L, 0.4 μmol/L, 8 nmol/L, 16.5 ± 2 pmol/L, 0.2 IU/L, and 0.5 IU/L respectively.

All of these assays were subjected to regular external quality assessment schemes apart from ECLT and PAI-1, which were not routinely performed in the coagulation laboratory.

**Statistical analysis**

Means and standard deviations were calculated for the clinical and biochemical variables, and for the assays of the PAS (tables 4.1-4.3). The analysis used to compare the two groups depended on the distribution of the variables. For those variables where probability plots indicated a normal distribution, a student’s t-test was used to compare the means. Two other variables, oestradiol and LH (U/L) were ‘normalised’ by the removal of an outlier. Where appropriate, t-tests took account of any differences in standard deviations between the two groups. For variables which were clearly not normally distributed according to a normal probability plot (hirsutism score, cycle length, alcohol intake, oestradiol, FSH, LH, basal ECLT, post venous occlusion ECLT, and post venous occlusion PAI-1 activity), a Mann-Whitney U test was appropriate for comparing the two groups. Some of the measured variables had a very limited range of values and for these neither a t-test nor Mann Whitney test was appropriate. In these cases, Fisher’s exact test was used to compare the proportions of ‘high’ values for the two groups. An analysis of covariance was used for plasminogen activator inhibitor-1 to take account of the possible effect of BMI and testosterone as confounding variables. A p value ≤ 0.05 was accepted as significant.
RESULTS

Women with PCOS were significantly younger and had a greater BMI compared with the controls (Table 4.1). They also had significantly higher Ferriman Gallwey scores, cycle lengths, free androgen index, testosterone, DHEAS, LH/FSH ratios and lower SHBG levels (Table 4.1 & 4.2) and the diastolic blood pressures were significantly higher in the PCOS group. Table 4.3 shows the results of assays of the PAS (see appendix 1 for all the individual results). The mean basal PAI-1 activity and the mean basal fibrinogen concentrations were significantly higher in the women with PCOS than in controls (p=0.049 and 0.004 respectively). The mean pre-venous occlusion ECLT was longer in the women with PCOS (p=0.016). 45% of the women with PCOS had a blunt response to the venous occlusion test as compared to 9% in the control group (p = 0.06). A blunt response to the venous occlusion test was defined as less than a 28% reduction in ECLT. This was based on data from a previous study (Gris et al 1993) in which a control population of 90 apparently healthy women was used to define a normal response to the venous occlusion test. A reduction in ECLT of less than 28% was below the first percentile. There was a very close positive correlation between ECLT and PAI-1 levels in both groups (r = 0.88 in controls and r = 0.90 in women with PCOS).

The effect of possible confounding variables (body mass index, and testosterone) on PAI-1 was assessed by applying an analysis of co-variance to PAI-1 with body mass index and testosterone as co-variates. In all cases no significant difference between the control and polycystic ovary syndrome groups was found when either one or both of these variables were included as a co-variate.
Table 4.3 - Assays of the plasminogen activator system for patients with PCOS versus controls.

<table>
<thead>
<tr>
<th>Assay Parameter</th>
<th>PCOS (n = 11)</th>
<th>Controls (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median basal ECLT (range) in minutes</td>
<td>412.5 (120-577)</td>
<td>187.5 (105-420)</td>
<td>0.016*</td>
</tr>
<tr>
<td>Median post VO ECLT (range) in minutes</td>
<td>150 (45-562.5)</td>
<td>82.5 (45-405)</td>
<td>0.055*</td>
</tr>
<tr>
<td>Mean basal fibrinogen concentration ± SD (g/L)</td>
<td>4.02 ± 0.64</td>
<td>3.18 ± 0.6</td>
<td>0.004</td>
</tr>
<tr>
<td>Mean post VO fibrinogen concentration ± SD (g/L)</td>
<td>4.66 ± 0.7</td>
<td>3.8 ± 0.6</td>
<td>0.007</td>
</tr>
<tr>
<td>Mean basal PAI-1 activity ± SD (au/ml)</td>
<td>19.7 ± 12</td>
<td>10.9 ± 7.9</td>
<td>0.049</td>
</tr>
<tr>
<td>Median post VO PAI-1 activity ± SD (au/ml)</td>
<td>16 (4-37.5)</td>
<td>8 (1.5-26)</td>
<td>0.116*</td>
</tr>
<tr>
<td>Mean basal plasminogen level ± SD (%)</td>
<td>96 ± 21</td>
<td>95 ± 8</td>
<td>0.095</td>
</tr>
<tr>
<td>Mean basal alpha 2 a-plasmin level ± SD (%)</td>
<td>103 ± 6.7%</td>
<td>100 ± 8.8%</td>
<td>0.38</td>
</tr>
</tbody>
</table>

VO = Venous occlusion
ECLT = Euglobulin clot lysis time
PAI-1 = Plasminogen activator inhibitor-1
A-plasmin = antiplasmin
* = Determined by the Mann Whitney U test
DISCUSSION

The cross sectional design of this pilot study was chosen to determine if elevated PAI-1 was present in PCOS compared with a similar control group and the results showed that women with PCOS had a longer baseline ECLT, a blunt response of the ECLT to the venous occlusion test, and a higher PAI-1 activity which implied that women in the PCOS group had an impaired plasmin producing capacity compared to the controls. The ECLT is a reflection of all components of the fibrinolytic system and the very close correlation between PAI-1 and ECLT on linear regression does suggest that the ECLTs were largely determined by PAI-1 levels. However it is important to note that tissue plasminogen activator (tPA) was not measured in this study and the other measured components of the PAS were not evaluated in a multiple regression analysis to determine which of them best predicted the ECLT. These were not thought to be necessary in this pilot study.

The selection of the study group may be criticised on the grounds that most of the ultrasound scan procedures for the diagnosis of polycystic ovaries had been transvaginal while the criteria used in the selection of the PCOS group were those described by Adams and colleagues in 1985, using abdominal ultrasound scans and that the definition of PCOS should have incorporated biochemical evidence of hyperandrogenaemia. However the endocrine characteristics of the PCOS group included an elevated free androgen index and a raised LH/FSH ratio which were consistent with the diagnosis of PCOS (Franks 1995). Although the women with PCOS were younger than the controls, it is unlikely that this 4-year difference in mean age could have been responsible for the observed results in this relatively young age group. On the other hand, as demonstrated by the analysis of covariance, the results may have been explained by the greater BMI in the PCOS group, as obesity is associated with impaired fibrinolysis and elevated PAI-1 levels (Ogston et al 1964, Vague et al 1989). However in a paper subsequently published just after the
completion of data collection for this study (Sampson et al 1996), PAI-1 was raised in 24 non-obese women with PCOS and extreme menstrual disturbance compared with 10 age and weight-matched controls with regular menstrual cycles suggesting that elevated PAI-1 was present in PCOS independent of BMI. This issue is addressed in the next chapter where controls of a similar BMI to women with PCOS were recruited.

It would have been ideal to measure insulin resistance in this pilot study so as to evaluate the relationships between PAI-1 and insulin in these women. However a positive correlation between fasting insulin concentrations and PAI-1 was shown in the 2 papers published on PAI-1 and PCOS before this study (Dahlgren et al 1994, Anderson et al 1995) and this was not thought to be necessary for the purposes of this pilot study. The elevated diastolic blood pressure found in the PCOS group is consistent with the metabolic syndrome X, but in this instance it may have been a reflection of the increased BMI in the PCOS group, as obesity is a risk factor for hypertension.

In summary, this pilot study found a prolonged ECLT and elevated PAI-1 levels in oligomenorrheic women with PCOS compared to controls with regular menstrual cycles, although women with PCOS were more obese which may have biased the results. These results not only implied that women with PCOS were at increased risk of thromboembolic disease, but it also suggested an alternative explanation for the pathophysiology of anovulation and miscarriage in them, in which the balance in the plasminogen activator system from elevated PAI-1 levels results in the deficient formation of plasmin. As plasmin is required for extracellular proteolysis this would lead to impaired follicular rupture, anovulation, failed implantation and miscarriage. It was then clearly important to establish whether elevated PAI-1 was present in PCOS independent of BMI and this issue is addressed in the next chapter.
CHAPTER FIVE

PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) AND METABOLIC FEATURES OF POLYCYSTIC OVARY SYNDROME (PCOS).

OUTLINE

The results of a definitive study measuring systemic PAI-1 activity and other factors in the plasminogen activator system, are presented and discussed. The inter-relationships between PAI-1, and metabolic aspects of the insulin resistance syndrome in women with PCOS and controls are also evaluated.

INTRODUCTION

In the previous chapter, a pilot study showed that women with PCOS had a significant elevation in PAI-1 activity and a prolongation of euglobulin clot lysis times (ECLTs) compared to a control group of women without PCOS. The results were consistent with the hypothesis that an insulin mediated elevation in PAI-1 may increase the risk of atherothrombosis in women with PCOS and contribute to their anovulatory infertility and recurrent pregnancy loss by limiting the plasmin mediated proteolysis required for these processes (Beers 1975, Sappino et al 1989, Gris et al 1993, Patrassi et al 1994, Tsafiri et al 1989). Because the results of the pilot study may have been biased by a significant increase in body mass index (BMI) in the PCOS group and because obesity is related to PAI-1 and impaired fibrinolysis ((Ogston et al 1964, Vague et al 1989), this study was performed to measure systemic PAI-1 activity in a larger number of women with and without PCOS, and to determine whether elevated PAI-1 levels were present in oligomenorrhoeic women with PCOS independently of BMI. In addition, the relationship between metabolic aspects of the insulin resistance syndrome (syndrome X) such as
obesity, insulin resistance and lipids and PAI-1 activity were assessed. The null hypothesis was that the mean PAI-1 activity in oligomenorrheic women with PCOS was not different from regularly menstruating controls of a similar BMI.

SUBJECTS AND METHODS

This was a cross sectional study comparing women with PCOS to controls of a similar BMI. Prior to the study, a sample size calculation was performed based on the following factors.

1. $\delta$, the minimum detectable difference between population means.
2. $\sigma^2$, the population variance expected from the data.
3. $\alpha$, the significance level at which the probability of a type I error (i.e. falsely rejecting the null hypothesis) was acceptable, and
4. $1-\beta$, the power of the test to detect a difference between population means (where $\beta$ is the probability of committing a type II error of falsely accepting the null hypothesis).

In considering what minimum detectable difference between population means ($\delta$) to set for our study, it was felt that a difference associated with a clinical finding of PCOS was desirable. Prior to the pilot study, there had been no study showing a difference in mean PAI-1 activity between women with PCOS and controls. The pilot study showed a difference of mean PAI-1 activity of 9au/ml between women with PCOS and controls. The pilot study showed a difference of mean PAI-1 activity of 9au/ml between women with PCOS and controls, therefore the $\delta$ was set at this level. The standard deviations of 7.91 in the control group and 12.05 in the PCOS group obtained in the pilot study were used to calculate the variance ($\sigma^2$) expected from the data. The $\alpha$ was set at 0.05 (5%) based on the usual convention (Zar J 1996), and calculations were performed using a statistical computer programme for studies with powers ($1-\beta$) ranging from 84-99.9% (see table 5.1).
Table 5.1: Power of a two-sample t-test to detect a 9au/ml difference of mean PAI-1 activity between women with PCOS and controls.

<table>
<thead>
<tr>
<th>Sample Sizes</th>
<th>Power (1-β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n₁=15, n₂=30</td>
<td>84</td>
</tr>
<tr>
<td>n₁=25, n₂=40</td>
<td>95</td>
</tr>
<tr>
<td>n₁=30, n₂=50</td>
<td>98</td>
</tr>
<tr>
<td>n₁=40, n₂=60</td>
<td>99.5</td>
</tr>
<tr>
<td>n₁=50, n₂=75</td>
<td>99.9</td>
</tr>
</tbody>
</table>

n₁=controls, n₂ = PCOS.

By convention, the β in sample size calculations is usually set at 10 or 20% (Swinscow TDV 1996). It was therefore decided desirable to recruit 30 to 40 women with the PCOS and 15 to 25 controls for this study as this was expected to provide a study with at least 84% power to detect a difference of 9 au/ml in PAI-1 activity between the two groups with a 5% chance of a type I error and less than a 20% chance of a type II error.

**Data collection.**

The patient recruitment, inclusion and exclusion criteria for PCOS and controls and the method of data collection are as described briefly in chapter 3. During the recruitment process, controls of an increased BMI were specifically sought from the local dieticians clinic and from amongst female members of staff to ensure that the BMI in both groups were similar, as it was expected that women with PCOS would have an above average BMI as was the case in the pilot study.

Tables 5.2 and 5.3 show the results of the clinical and endocrine features of both groups. The findings in the PCOS group were consistent with the diagnosis including higher
Ferriman Gallwey scores, free androgens index, LH levels, and they were more likely to have a positive history of infertility. Interested participants were requested to come in to the hospital fasted, having refrained from smoking from 12.00 midnight the day before the tests. All the tests were carried out between 7.30 and 10.00 am in the morning to minimise the diurnal variation in PAI-1 levels (Juhan-Vague et al 1992). On arrival at the hospital, written consent was obtained, and the patients were rested sitting for 15 minutes during which clinical details were obtained (age, menstrual and obstetric histories, medical and drug history, family history and a Ferriman Gallwey score for hirsutism performed).

Blood was then taken from an antecubital vein of either forearm with minimal or no venous occlusion for PAI-1, tPA, plasminogen, fibrinogen, α-2 antiplasmin, glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, insulin, testosterone, LH, FSH, SHBG, prolactin and 17 hydroxy progesterone. The samples for PAI-1, tPA, plasminogen, fibrinogen and alpha-2 antiplasmin were placed immediately in an ice tray, following which their blood pressures were checked on the opposite arm using the appropriate size cuff according to the mid arm circumference and using Korotkoff IV sounds as the diastolic blood pressure. The mid arm circumference was taken as a point midway between the acromion and the elbow, and a large blood pressure cuff was used if it was greater than 35cm. The blood pressure cuff was left inflated midway between systolic and diastolic pressures for 10 minutes or however long the women could tolerate it for. Following this, post-venous occlusion samples were taken mainly for tPA and kept immediately on ice.

The blood pressure cuff was then deflated and their heights, weights, waist circumference and hip circumference were checked. The waist and hip circumferences were measured with the patients wearing indoor clothes. Height was measured in metres using a soft tape, body weight was measured in kilograms to the nearest 0.1 kg and the BMI thus calculated
as weight / height$^2$. Waist circumference was measured with the women wearing indoor clothes at the level of the umbilicus, and the maximum hip measurement at the level of the greater trochanters was used as the hip circumference. The waist/hip ratio was thus derived from these measurements. Following this a separate appointment was made for the ultrasound scan where this was not possible on the same day. The blood samples were then taken to the laboratory for separation, storage and analysis.

Table 5.2 - Clinical variables for patients with PCOS versus controls.

<table>
<thead>
<tr>
<th>Clinical Variable</th>
<th>PCOS (n = 41)</th>
<th>Controls (n = 25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age ± SD (y)</td>
<td>28.4 ± 5.9</td>
<td>32 ± 5</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean BMI ± SD (kg/m$^2$)</td>
<td>29.5 ± 5.6</td>
<td>28.4 ± 6.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Mean Waist-Hip ratio ± SD</td>
<td>0.90 ± 0.055</td>
<td>0.90 ± 0.053</td>
<td>0.94</td>
</tr>
<tr>
<td>Mean hirsutism score ± SD</td>
<td>11.3 ± 7.02</td>
<td>6.5 ± 5.16</td>
<td>0.002</td>
</tr>
<tr>
<td>Median No. of periods in previous 12 months</td>
<td>6 (0-11)</td>
<td>12 (6-14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number with history of Subfertility (%)</td>
<td>17 (41%)</td>
<td>5 (20%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Number with family history of thrombosis(%)</td>
<td>12 (29%)</td>
<td>2 (8%)</td>
<td>0.051</td>
</tr>
<tr>
<td>Number of smokers (%)</td>
<td>10 (24%)</td>
<td>6 (24%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Number of alcohol units/wk (range)</td>
<td>0.5 (0-30)</td>
<td>0.5 (0-15)</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean systolic blood pressure ± SD (mmHg)</td>
<td>117.3 ± 13.6</td>
<td>111.4 ± 13.2</td>
<td>0.083</td>
</tr>
<tr>
<td>Mean diastolic blood pressure ± SD (mmHg)</td>
<td>78.3 ± 10.9</td>
<td>73.4 ± 10.9</td>
<td>0.08</td>
</tr>
</tbody>
</table>

NOTE. BMI = body mass index
Table 5.3 - Hormonal variables for patients with PCOS versus controls.

<table>
<thead>
<tr>
<th>Hormonal Variable</th>
<th>PCOS (n = 41)</th>
<th>Controls (n = 25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median FAI (range)</td>
<td>6.4 (0.6-21.2)</td>
<td>3.2 (0.5-10.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median SHBG (range) n mol/L</td>
<td>34.75 (16.4-129)</td>
<td>55 (23-131)</td>
<td>0.001</td>
</tr>
<tr>
<td>Median Testosterone (range) n mol/L</td>
<td>2.5 (0.3-5.2)</td>
<td>1.5 (0.6-5.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Median FSH (U/L) (range)</td>
<td>5.9 (2-18.1)</td>
<td>5.5 (2.5-34.1)</td>
<td>0.42</td>
</tr>
<tr>
<td>Median LH (U/L) (range)</td>
<td>12.3 (4.4-44.2)</td>
<td>7 (2.3-63.7)</td>
<td>0.041</td>
</tr>
<tr>
<td>Median LH/FSH ratio (range)</td>
<td>2.35 (0.34-4.89)</td>
<td>1.27 (0.27-6.77)</td>
<td>0.06</td>
</tr>
<tr>
<td>Median Prolactin (range)</td>
<td>231.5 (76-784)</td>
<td>217.5 (88-646)</td>
<td>0.67</td>
</tr>
<tr>
<td>Median 17-OHP (range)</td>
<td>5.4 (0.4-22.8)</td>
<td>8.2 (1-18.6)</td>
<td>0.036</td>
</tr>
</tbody>
</table>

FAI = Free androgen index. (testosterone / SHBG x 100)

SHBG = Sex hormone binding globulin

FSH = Follicle stimulating hormone

LH = Luteinizing hormone

17-OHP = 17 Hydroxyprogesterone

Assays

The blood samples for PAI-1, tPA, plasminogen and alpha-2 anti plasmin and fibrinogen were centrifuged within 30 minutes of collection at 4°C for 20 minutes at 3000g. Next, the platelet free plasma was separated into microcentrifuge tubes (Camlab limited, cambridge ref QP/519), changing pipettes between pre and post occlusion samples. A small amount was tested for fibrinogen. They were then stored at -70°C for later analysis of PAI-1, tPA, plasminogen and α-2 anti plasmin. These assays were all subsequently performed within 8 months of collection except tPA, which was not performed because of limited financial resources. The assays for glucose, hormones, and lipids were performed on the fresh samples in the Combined Laboratory at Derriford Hospital Plymouth. Serum was stored at -20°C, and analysed for insulin within 12 months of collection.
The methods used for PAI-1, plasminogen, α-2 anti plasmin and fibrinogen were the same as those used in the pilot study. However we did not repeat the ECLT as we had found a very close positive correlation between ECLT and PAI-1 levels in our pilot study \( (r = 0.88 \) in controls and \( r = 0.90 \) in women with PCOS). The post venous occlusion samples that were taken mainly for the measurement of tPA were not tested because of limited financial resources as previously stated. The methods, intra and inter-assay coefficient of variation, and sensitivities for the assays for FSH, LH, testosterone and SHBG were the same as in the pilot study. Free androgen index was calculated using the formula \( \text{Testosterone/SHBG levels x 100} \).

**Glucose** was tested using the hexokinase method on a Hitachi 911 automated analyser. The sensitivity of the assay was 2mmol/L and the intra and inter assay coefficients of variability 2.2% and 2.3% respectively.

**Cholesterol** was tested using an enzymatic endpoint method on a Hitachi 747 automated analyser. The sensitivity of the assay was 1mmol/L and the intra and inter assay coefficients of variability 1.8% and 2.4% respectively. **Triglycerides** were tested on a Hitachi 747 automated analyser. The sensitivity of the assay was 0.1mmol/L and the intra and inter assay coefficients of variability 1.3% and 2.4% respectively. **HDL Cholesterol** was tested on the Beckman CX7 automated analyser. The sensitivity of the assay was 0.2 mmol/L and the intra and inter assay coefficients of variability 2.6% and 5.6% respectively. **LDL Cholesterol** was derived by calculation using the Friedwald formula.

**Prolactin** was tested using the automated chemiluminescence assay on the chiron ACS:180 analyser standardised against the WHO 3rd international reference preparation.
The sensitivity was 6.4 mIU/L the intra assay coefficients of variation were 2.5%, 2.8% and 3.8% at 57, 721 and 2565 IU/L respectively and the inter assay coefficients of variation were 3.6%, 4.0% and 4.5% at 57, 721 and 2565 IU/L respectively.

The assays for insulin and 17-hydroxyprogesterone were performed in external laboratories. Insulin assays were done at the biochemistry laboratory at the Heath hospital Cardiff using a radioimmunoassay (Medgenix, Chevus, Belgium) with a sensitivity of 4mU/L and an inter assay coefficient of variation of 10.9% and 15.6% at 17.4 and 129 mU/L respectively. This assay was specific for insulin and did not cross-react with proinsulin. Assays for 17-hydroxyprogesterone were done at the immunoassay laboratory at St. Bartholomew's hospital in London. Insulin sensitivity (the degree of insulin resistance) was assessed using a Homeostatic Model Assessment (HOMA) computer programme as discussed in chapter three.

**Statistical Analysis**

Summary statistics for the clinical, biochemical and haematological data were presented as means and standard deviations if normally distributed, medians and ranges if the distribution was not normal, and as proportions for categorical variables. For quantitative variables, assessment for the normality of the spread was carried out using a normal probability plot. For those variables that were normally distributed, a student's t-test was used to compare the means in the PCOS and control groups. For those variables where there was clear evidence of non-normality, a Mann-Whitney test was used. Other variables, which were either categorical or which had a small range of values, were analysed where possible using a chi-squared test. In all cases, a two-tailed test was applied.
An analysis of co-variance was performed to compare the pre-venous occlusion PAI-1 levels of the PCOS and control groups using age and BMI as co-variates as these variables may be related to PAI-1 levels. A linear regression analysis was used to evaluate the overall individual relationships between BMI, triglyceride, insulin, and HDL cholesterol on the one hand and PAI-1 levels on the other. A multiple regression analysis was performed with pre-venous occlusion PAI-1 as the dependent variable and insulin, triglycerides, BMI, HDL cholesterol, free androgen index, family history of thrombosis and group (whether PCO or control) as independent variables. A “p” value of < 0.05 was accepted as statistically significant.

RESULTS
Eighty-three women were recruited into the study from which 66 were suitable for inclusion based on the entry criteria. Forty-one of these women had PCOS as defined by the presence of a combination of oligo or amenorrhoea and ultrasound features of polycystic ovaries, and 25 were regularly menstruating controls. The individual results from all these women can be found in appendix 2 and the scatter plot of PAI-1 for both PCOS and controls is found in figure 5.1. There was no significant difference found in the mean BMI ($P = 0.25$) and waist hip ratio of both groups ($P = 0.94$), but the mean age in the control group was significantly higher than in the PCOS group (32 ± 5 vs 28.4 ± 6 years, $P = 0.01$).

A normal probability plot showed that PAI-1 activity was not normally distributed in the overall population and a Mann Whitney test was therefore used to compare the median levels in the PCOS and control groups. This showed that PAI-1 activity was not significantly increased in the PCOS group compared with the control group {12 (2-38.5) vs 8 (0-36) au/ml respectively, $p=0.17$ } as shown in table 5.4. The normal range for PAI-
1 activity with the assay used in this study was up to 15au/ml. 43% of the women in the PCOS group had a result above this level compared to 28% in the control group, but this difference was not statistically significant (p=0.29). An analysis of covariance with age and BMI as co-variates found no evidence of an effect of either covariate (BMI, p=0.53 & age p=0.24) on PAI-1 activity. 12 women with PCOS had a positive family history of thrombosis (6 in first degree relatives) compared to 2 women in the control group (29% vs 8%, P = 0.051). As a family history of strokes and myocardial infarction were also elicited separately, with no difference found in both groups, this family history of thrombosis was assumed to refer to venous thrombosis. There was no difference between groups in the prevalence of a family history of type II diabetes or hypertension.

Women with PCOS had significantly higher fasting insulin levels, lower insulin sensitivity and a lower HDL cholesterol ratio compared with controls (P< 0.01). But there was no significant difference in the serum concentrations of plasminogen, α-2 antiplasmin, fibrinogen, glucose, triglycerides, total cholesterol and LDL cholesterol ratio (Table 5.4). A linear regression analysis for all patients showed a significant positive correlation between BMI, triglycerides, fasting insulin and PAI-1 activity ( r=0.65 & p < 0.001, r=0.57 & p < 0.001 and r = 0.57 & p < 0.001) and a negative correlation between HDL cholesterol and PAI-1 (r = -0.41 & p < 0.01). Multiple regression analysis found that triglycerides were the most significant predictor of PAI-1 activity (p < 0.0001). However fasting insulin levels, a positive family history of thrombosis and a low HDL cholesterol ratio (p=0.0005, p=0.013 & p=0.026 respectively) were also significant predictors of PAI-1 activity. The fitted prediction model was: Predicted PAI-1 = 9.942 + 6.412 Triglycerides + 0.2245 Insulin + 6.457 FH thrombosis - 5.311 HDL cholesterol. Which means that for example for every increase of 1 in the triglyceride value, the expected increase in PAI-1
activity is 6.412. The value of $R^2$ was 63.9% suggesting that the above model explained 63.9% of the variability in PAI-1 values.

Table 5.4 - PAI-1 and metabolic variables in patients with PCOS versus controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS (n = 41)</th>
<th>Controls (n = 25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median PAI-1 activity (range) au/ml</td>
<td>12 (2-40.5)</td>
<td>8 (0-36)</td>
<td>0.17</td>
</tr>
<tr>
<td>Mean plasminogen level ± SD (%)</td>
<td>97.7 ± 18.8</td>
<td>98.7 ± 19.8</td>
<td>0.84</td>
</tr>
<tr>
<td>Median fibrinogen (range) g/L</td>
<td>3.31 (2.2-5.31)</td>
<td>3.23 (2.36-5.62)</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean alpha 2 a-plasmin level ± SD (%)</td>
<td>99.1 ± 11.5%</td>
<td>97.8 ± 10.4%</td>
<td>0.65</td>
</tr>
<tr>
<td>Mean fasting glucose level ± SD mmol/L</td>
<td>4.94 ± 0.48</td>
<td>4.84 ± 0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>Median fasting Insulin level ± SD mu/L</td>
<td>17 (4.6-134.5)</td>
<td>9.6 (3.7-41.5)</td>
<td>0.009</td>
</tr>
<tr>
<td>Median Insulin Sensitivity (range) %S</td>
<td>43.17% (5.48 - 160)</td>
<td>82.8% (21.8-193)</td>
<td>0.005</td>
</tr>
<tr>
<td>Median HDL cholesterol ratio (range)</td>
<td>1.46 (0.65-2.67)</td>
<td>1.93 (0.96-2.51)</td>
<td>0.007</td>
</tr>
<tr>
<td>Median Triglycerides (range) mmol/l</td>
<td>0.94 (0.46-4.91)</td>
<td>1.05 (0.37-3.56)</td>
<td>0.92</td>
</tr>
<tr>
<td>Mean fasting total cholesterol ± SD mmol/L</td>
<td>5.28 ± 1.17</td>
<td>5.74 ± 1.20</td>
<td>0.15</td>
</tr>
<tr>
<td>Mean LDL cholesterol ratio ± SD</td>
<td>3.11 ± 0.92</td>
<td>3.18 ± 0.96</td>
<td>0.77</td>
</tr>
</tbody>
</table>
DISCUSSION

The key findings from this study were that systemic PAI-1 activity was not significantly raised in oligomenorrheic women with PCOS compared to regularly menstruating controls of a similar BMI and that the only significant metabolic features in the PCOS group were a higher fasting insulin level, reduced insulin sensitivity and lower HDL cholesterol ratios. There was no significant difference in fasting blood glucose, triglycerides, total cholesterol, LDL cholesterol and blood pressure in both groups. PAI-1
activity was also found to be significantly related to BMI, triglycerides, fasting insulin, HDL cholesterol levels, and a positive family history of thrombosis.

The definition of PCOS used in this study may be criticised because evidence of hyperandrogenaemia was not made an absolute requirement. However as discussed in chapters 2 & 3, there is no gold standard for the definition or diagnosis of the PCOS and the finding of a higher median free androgen index, fasting insulin and LH/FSH ratio in the PCOS group provided reassuring evidence that the inclusion criteria used for this study were satisfactory for the diagnosis of PCOS. In addition, none of the other published cross-sectional studies on PAI-1 in PCOS (Dahlgren et al 1994, Sampson et al 1996, Velaquez et al 1997) included hyperandrogenaemia in the entry criteria which makes it easier to compare the results from our study with these studies. Another potential criticism of this study is that the PAI-1 and lipid measurements were taken at random with respect to ovarian activity. Although the amount of PAI-1 expressed in ovarian tissue has been shown to vary with gonadotrophin levels (Peng et al 1993), studies on systemic PAI-1 have shown that there are no significant fluctuations during the menstrual cycle (Dorr et al 1993) and there are also no significant fluctuations in lipid levels (Lebech et al 1990).

The mathematical model used to evaluate insulin resistance in this study was practical and simple compared to some of the other methods available for evaluating insulin resistance such as the glucose clamp tests. However it is important that certain points are made about this model. Firstly, it does not differentiate between hepatic or peripheral insulin sensitivity (Rudenski et al 1991), as it assumes that changes in insulin sensitivity at the periphery and at the liver are parallel. Secondly, the ideal experimental determination of data for fasting glucose and insulin is best done taking average concentrations of glucose and insulin over three five minute samples which reduces assay noise and compensates for
the innate pulsatility of insulin secretion. This was not done in this study, which may have affected the validity of the results of the mathematical model. The reason for this was that it was anticipated that using only one glucose and insulin sample in this model would reduce the financial costs from performing three assays on the three samples obtained from each subject. However, in retrospect, by pooling the three five minute plasma samples for insulin or glucose, only one assay would still have been required. Nevertheless, the model served its purpose for this study which was primarily to compare insulin sensitivity in both study groups and not to determine the mechanisms of insulin resistance (or reduced insulin sensitivity) in both groups. Thus although only one blood sample was taken for glucose and insulin estimation, it did not appear to matter much as the results showed that insulin sensitivity was still significantly lower in the PCOS group as expected.

The PAI-1 results from this study were consistent with the study carried out by Dahlgren et al (1994) in which 28 peri-menopausal women with PCOS and 56 age and weight matched controls had metabolic and haemostatic variables evaluated. Except that in their study no difference was found in the fasting insulin levels between the two groups. However this may have been partly explained by the fact that 20% of their control population had hypertension suggesting that there was a high prevalence of metabolic disorders possibly related to most women in the study being in their fifties. On the other hand, the results conflict with the conclusions drawn from 2 other cross-sectional studies on PAI-1 (Sampson et al 1996, Velaquez et al 1997) and the results of the pilot study in chapter 4 (Atiomo et al 1998) all of which found raised PAI-1 in women with PCOS. However all these studies had drawbacks which made their conclusions debatable.

In the study by Sampson et al (1996), looking at PAI-1 in 24 non-obese women with ultrasound evidence of PCOS and menstrual disturbance (group 1) compared with 26
women with ultrasound evidence of PCOS and regular cycles (group 2) and 10 age and weight matched controls with normal menstrual cycles and normal ovarian ultrasound (group 3), mean PAI-1 activity was elevated in group 1 compared to the other groups. These authors concluded that PAI-1 was higher in women with PCOS. However the difference in mean PAI-1 levels between groups 1 and 3 (which were similar to the 2 groups in this chapter) was not statistically significant (10.0 vs 5.1 au/ml p=0.059). It may be argued that this occurred because of their relatively small control group, and that larger numbers might have shown a significant difference. But in this study with more than double their control populations we did not find a significant difference in PAI-1 activity. It is important to note that a student’s t test was used in their study to compare the differences in mean PAI-1 between groups because the data was normally distributed, as compared to a Mann Whitney test in ours. The drawback of the Mann Whitney test is that although it is the appropriate test to compare data derived from populations when the data are non-parametric, it is also more likely that a type II error (risk of falsely accepting the null hypothesis) can occur. This was the ideal test to use given that the PAI-1 results from our study were not normally distributed. However the fact that the proportion of women in our study with PAI-1 results within the normal range was similar in both the PCOS and control group does suggest that our results were reliable. In the study by Velaquez et al (1997), basal PAI-1 levels were significantly higher in the PCOS group (p<0.01) as found in the pilot study and a significant reduction in PAI-1 activity was found after treatment with an 8 week course of metformin in 16 non-diabetic women with PCOS and 6 normal controls. In both these studies women with PCOS had a significantly greater BMI which was not present in the study reported in this chapter where the control population had a similar BMI to the PCOS group.
Although the mean age of the control group was 3.6 years more than the PCOS group, and PAI-1 activity reduces with increasing age, it is unlikely that such a small difference in ages would have caused a bias in the PAI-1 results. This is supported by the fact that the analysis of co-variance did not find any evidence of an effect of age on PAI-1 activity.

The finding of a higher fasting insulin concentration independent of BMI in women with PCOS in this study is consistent with many previous studies which have demonstrated insulin resistance and fasting hyperinsulinaemia in both obese and non-obese women with PCOS. It has been speculated that this insulin resistance causes a metabolic risk profile associated with an increased risk of cardiovascular disease (Jacobs 1996) as found in Syndrome X (Reaven 1994). However the degree to which the features of insulin resistance in syndrome X overlaps with that found in PCOS is uncertain. In this study, women with PCOS had significantly higher fasting insulin levels, lower insulin sensitivity and lower HDL cholesterol ratios, but none of the other features of the metabolic syndrome X which also include high blood pressure, hypertriglyceridaemia, elevated PAI-1, and elevated uric acid levels. Although uric acid was not measured in this study, normal serum concentrations have been found in women with PCOS (Anttila et al 1996). It may be argued that single blood pressure measurements as used in our study are not as sensitive as 24-hour ambulatory blood pressure profiles for the detection of hypertension, and that this was the reason there was no difference in blood pressure in this study, however in a recent study where blood pressure was measured in women with PCOS and controls, using 24-hour ambulatory profiles, no significant difference in any measured blood pressure variable was found (Sampson et al 1996). Similarly, studies on lipid states in women with PCOS are not consistent, for example the lower HDL cholesterol ratios found in women with PCOS in the study in this chapter have been found in some (Mattesson et al 1984,

The similar waist-hip ratios in both study groups was unexpected as the PCOS is usually associated with central obesity. However these results may have arisen because of a measurement error as the subjects in this study were measured dressed.

It is clear that the results of some of the studies of the metabolic profile of women with PCOS conflict with the results of this study because some studies have included women of higher BMI in the PCOS group compared to the control population. The discrepancy between the lipid, blood pressure and PAI-1 values in found in this study, where the controls have been matched for BMI and that described in the syndrome X may represent a distinction between the mechanism of insulin resistance in women with PCOS and that related to simple obesity. This hypothesis is supported by the findings of a study by Dunaif et al (1992) in which insulin action in vivo was determined with euglycaemic clamp tests and in vitro in isolated abdominal adipocytes in 16 obese and 14 non obese women with PCOS compared with 15 obese and 17 non-obese age and weight matched non-diabetic ovulatory women. They found that the $ED_{50}$ insulin for hepatic glucose production was significantly increased only in the obese PCO women compared with obese controls suggesting an interaction between obesity and PCOS. These findings are consistent with that of this study where despite both the PCO and control groups being obese (mean BMIs of 29.5 and 28.4 respectively), fasting insulin levels were significantly higher in the PCOS group who were also significantly less insulin sensitive.

The finding of a significant overall correlation between BMI, triglycerides, fasting insulin, and PAI-1 activity on linear and multiple regression analysis, is consistent with the
findings of previous studies on women with and without PCOS (Shaw et al 1963, Ogston et al 1964, Vague et al 1986, Vague et al 1989, Juhan-Vague et al 1989, Dahlgren et al 1994, Anderson et al 1995, Sampson et al 1996, Velaquez et al 1997). However the exact mechanism responsible for these correlations are unknown. Insulin has been shown to stimulate PAI-1 synthesis and release in porcine endothelial cells and in the hepatoma cell line in-vitro. These correlations may be a reflection of insulin resistance, which is associated with obesity and raised triglycerides. This is supported by the results of multiple regression analysis in some studies showing that in most cases these correlations disappear after adjustment for insulin (Vague et al 1986, Vague et al 1989, Juhan-Vague et al 1991). However this hypothesis is not supported by the finding of normal PAI-1 levels in non-diabetic patients with Cushing’s disease or acromegaly in spite of hyperinsulinaemia and insulin resistance (Scelles et al 1992). Some studies have also shown that acute elevation of endogenous insulin with an oral glucose load (Juhan-Vague et al 1991), the acute administration of exogenous insulin (Potter Van Loon et al 1990) or the infusion of insulin for 24 hours (Juhan-Vague P et al 1991), does not affect PAI-1 activity. However it has been suggested that triglycerides could be prime candidates for triggering excessive secretion of PAI-1 (Stiko-Rham et al 1990). This is supported by the finding in this study on multiple regression analysis that triglycerides were a significant independent predictor of PAI-1 activity, that there were similar triglyceride levels in both groups and that there was also no significant difference in PAI-1. The relationship between triglycerides and PAI-1 has also been shown not to be consistent as a study in which PAI-1 levels were compared in three groups of hypertriglyceridaemic patients: one with obesity, one of normal body weight and one with alcohol induced hypertriglyceridaemia showed a weak overall correlation between PAI-1 and triglyceride levels (Raccah et al 1996).
The finding of a three-fold increase in the prevalence of a positive family history of thrombosis in women with PCOS in this study was an unexpected but interesting finding as this has never been previously described in the literature as far as I know. It was not mirrored by prothrombotic changes in PAI-1, fibrinogen, plasminogen and α-2 antiplasmin levels which are hypothetical causes of thrombophilia. This finding led to the testing of the stored platelet free plasma obtained from these women for the more common causes of familial thrombophilia such as activated protein C (APC) resistance, antithrombin III, protein C and protein S. The results of this analysis are presented as a separate chapter (8), but in summary it showed that there was not an increased prevalence in any of these factors in the PCOS group. However it is interesting to note that there was an independent correlation between the presence of a positive family history of thrombosis and PAI-1 on the multiple regression analysis as PAI-1 is not a common cause of familial thrombophilia. One would have expected that as there were more people with a familial history of thrombophilia in the PCOS group, PAI-1 activity would have been significantly raised.

In summary, this cross-sectional study showed that systemic PAI-1 activity was not raised in 41 oligomenorrhoeic women with PCOS compared with 25 regularly menstruating weight matched controls. The only significant metabolic features of the PCOS group were insulin resistance, hyperinsulinaemia and lower HDL cholesterol ratios, but not the hypertriglyceridaemia, hypertension and raised PAI-1 described as part of the metabolic syndrome X. An interesting but unexplained finding was an increase in the presence of a positive family history of thrombosis in women with PCOS. These findings were not consistent with the hypothesis that elevated PAI-1 levels put women with PCOS at increased risk of thromboembolic disease or that it contributed to their anovulatory infertility and miscarriage, but it raised a few questions about the exact mechanism of the interaction between the insulin resistance of PCOS and the metabolic syndrome X and the
link between PCOS and thrombosis. It is however important to note that this study addresses statistical differences, which may not be the same as differences, that are biologically important, and differences that are not apparent at a systemic level may be found at the end organ. Therefore only experimental work on the effect of different tissue levels of PAI-1 on various biological processes of interest will provide that information.

The next 2 chapters contain the results of studies where some basic descriptive information on the tissue expression of PAI-1 in the human ovaries were obtained as a preliminary step to dynamic studies on the effect of various tissue levels of PAI-1 on the ovulatory process.
CHAPTER SIX

IMMUNOHISTOCHEMICAL LOCALISATION OF PLASMINOGEN ACTIVATOR-1 PROTEIN IN POLYCYSTIC OVARIES.

OUTLINE
A study on the immunohistochemical localisation and quantitation of PAI-1 in ovarian biopsies from women with PCOS and normal ovaries is described. Detailed accounts of the general principles of immunohistochemistry and the experimental procedures used in this study are presented.

INTRODUCTION
The histologic make up of the mammalian ovary consists of several tissue types, which secrete a variety of factors that play a vital role in follicle growth, development, and ovulation. These factors include, sex steroids, peptides and growth factors (Taymor 1996), but how these highly integrated processes result in the full development of one follicle at ovulation is not fully understood.

In women with polycystic ovaries, the number of primordial follicles present are similar to those found in normal ovaries (Hughesdon 1982), but they contain an increased amount of primary, secondary and tertiary follicles which rarely develop beyond a diameter of 6 to 8mm diameter (Fauser 1994). The factors leading to this are still poorly understood, but proposed mechanisms include a negative effect of LH-stimulated androgen production on follicle development (Louvert et al 1975), subnormal FSH levels (Erickson et al 1979); impairment of the synergistic actions between FSH and insulin like growth factor on granulosa cell aromatase activity (Giudice et al 1995), aberrations in the intraovarian activin
/ inhibin system (Roberts et al 1994), and an alteration in the ovarian renin angiotensin system (Palumbo et al 1993).

PAI-1 and tissue plasminogen activator are present in follicular fluid (Beers 1975), and granulosa cells from human ovaries undergoing assisted conception (Piquette et al 1993). As ovulation approaches, an increase in plasminogen activator secretion by rat granulosa cells has been observed (Beers et al 1975). Plasmin which activates the conversion of procollagenase to collagenase, is thought to be necessary for the breakdown of the type IV collagen present in the basement membrane around the graafian follicles at ovulation (Stetler Stevenson 1990), and a suppression of ovulation rates has been demonstrated in the rat ovary following the use of specific antibodies to tPA and α-2 antiplasmin. In another study on rat ovaries (Peng et al 1993), using in-situ hybridisation PAI-1 mRNA was mainly found in the cell types located in the outer layer of follicles including the theca interna, theca externa, interstitial cells and ovarian stroma and following HCG stimulation, the dominant pre-ovulatory follicles which protrude onto the surface of the ovary were surrounded by less interstitial and stromal tissue that expressed PAI-1 compared to the smaller non-ovulatory follicles that were in the interior part of the ovary which were surrounded by layers of PAI-1 expressing tissue. These studies suggest that PAI-1 may play a role in peri-follicular proteolysis, protecting relatively immature follicles from premature rupture and any excess PAI-1 might prevent follicular growth and rupture.

Because of the close correlation between insulin resistance and PAI-1, it has been suggested that an imbalance in the intraovarian plasmin pathway (Sampson et al 1996) may partly be responsible for the disordered folliculogenesis found in PCOS. It is thought that this imbalance arises secondary to an insulin driven overproduction in PAI-1 with a reduction in the amount of plasmin available for extracellular proteolysis in the ovary.
that they may contain unwanted antibodies against other constituents present in human
tissue, resulting in a high incidence of non-specific staining of tissue sections. Monoclonal
antibodies are made from hybridomas prepared by the fusion of immune spleen cells and a
myeloma cell line. They are highly specific as the antigen-combining site in all the
molecules are of identical specificity. These antibodies may be labelled with fluorescin or
an enzyme to enable microscopic detection of antigen/antibody hybrids in-situ. The
principle behind immunofluorescent labelling is that a fluorescent marker molecule is
stimulated at one wavelength and emits light at a different wavelength. This fluorescent
signal is viewed against a dark background. The fluorochrome may either be coupled
directly to the primary antibody, or indirectly via the labelling of a secondary reagent.
Direct techniques are rapid but of lower sensitivity and rely on the supply of labelled
primary antibodies, whereas indirect labelling can be used with any primary antibody.

The most common enzyme used with immunoenzymatic labelling is horseradish peroxidase.
It can be detected by reactions, which produce an insoluble product. The commonest
substrate contains diaminobenzidene (DAB) and hydrogen peroxide. Aminoethylcarbazole
(AEC) and hydrogen peroxide may also be used, as DAB may be carcinogenic. Calf
intestinal alkaline phosphatase is an alternative enzyme to peroxidase. Substrates to this
catalytic enzyme include napthol phosphate and hexazotized new fuschin or fast red. The advantage
of calf intestinal alkaline phosphatase over horseradish peroxidase is that endogenous tissue
peroxidase activity is not a problem. Endogenous tissue peroxidase in white cells and red
cells may cause problems if the substrate reacts with both the endogenous enzyme and the
horseradish peroxidase label. This can be prevented by introducing a blocking step during
the procedure. Tissue alkaline phosphatase activity is less of a problem, but its activity can
be blocked by levamisole.
Table 6.1 (Individual patient characteristics. Patients 1-5 had polycystic ovaries and 6-11 were controls).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Weight(kg)</th>
<th>Parity</th>
<th>Hormones</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>51.7</td>
<td>1+0</td>
<td>n/a</td>
<td>Infertility and oligomenorrhoea</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>59.7</td>
<td>0+0</td>
<td>T= 3.8nmol/L.</td>
<td>Amenorrhoea and hirsutism</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>95</td>
<td>0+0</td>
<td>LH/FSH ratio=2</td>
<td>Amenorrhoea</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>57.6</td>
<td>0+1</td>
<td>T= 2.7nmol/L.</td>
<td>Amenorrhoea and infertility</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>60</td>
<td>0+0</td>
<td>n/a</td>
<td>Amenorrhoea</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>69.8</td>
<td>2+0</td>
<td>n/a</td>
<td>Heavy periods</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>66</td>
<td>n/a</td>
<td>n/a</td>
<td>Pelvic mass and oligomenorrhoea</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>91</td>
<td>0+1</td>
<td>n/a</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>88.5</td>
<td>0+3</td>
<td>n/a</td>
<td>Infertility recurrent miscarriage</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>67.7</td>
<td>0+0</td>
<td>n/a</td>
<td>Abdominal pain</td>
</tr>
</tbody>
</table>

T = Testosterone (normal range 0.6-2.2 nmol/L)

n/a = information not available from records

General principles of immunohistochemistry

Immunohistochemistry is the technique by which antigens are detected in-situ in human cell and tissue samples. It involves the incubation of these tissues with a known antibody to the antigen in question. The antibody is linked with a marker molecule, which can be detected microscopically by fluorescence, or the ability of the marker molecule to react with a substrate and yield a visible reaction product.

Samples used for immunohistochemistry may be paraffin embedded formalin fixed tissue, or frozen sections. With the former, tissue morphology is well preserved, but antigenic material tends to be masked or denatured. The preservation of tissue architecture is not as good with frozen sections, but antigenic denaturation is minimal. Antigenic reactivity can be restored by treating sections with proteolytic enzymes (e.g. trypsin or pronase). The antibodies used may be polyclonal or monoclonal. Polyclonal antibodies are raised by immunising animals with the purified antigen. The main drawback of these antibodies is
However the in-situ location of PAI-1 has never been previously described in the human ovary. The aim of this study was to determine the in-situ localisation of PAI-1 in the human ovary, and then compare the amount of PAI-1 protein detected in polycystic ovaries with control ovaries. This was expected to provide a better understanding of the pathophysiology of impaired folliculogenesis and anovulation in PCOS.

METHODS

The study design and case selection are as described in chapter 3. Five women with polycystic ovaries and six with normal ovaries were suitable for inclusion in the study. Three women within the polycystic ovary group had documented biochemical evidence in support of their diagnosis. The hormone results of the remaining two were not available in the notes. Except for one woman with menstrual cycles lasting 60 days, all the women in the control group had regular 28-day cycles. Four of them were in the follicular phase of the menstrual cycle, while the other one was in the luteal phase at the time of their surgery. It was felt that as this study had never been done before, this sample size was all that was required to detect any marked differences in PAI-1 staining, and so they were processed as a pilot. Table 6.1 shows the clinical and biochemical profile of the subjects. The biopsies taken from these women were then processed for the immunohistochemical detection of PAI-1.
that they may contain unwanted antibodies against other constituents present in human tissue, resulting in a high incidence of non-specific staining of tissue sections. Monoclonal antibodies are made from hybridomas prepared by the fusion of immune spleen cells and a myeloma cell line. They are highly specific as the antigen-combining site in all the molecules are of identical specificivity. These antibodies may be labelled with fluorescin or an enzyme to enable microscopic detection of antigen/antibody hybrids in-situ. The principle behind immunofluorescent labelling is that a fluorescent marker molecule is stimulated at one wavelength and emits light at a different wavelength. This fluorescent signal is viewed against a dark background. The fluorochrome may either be coupled directly to the primary antibody, or indirectly via the labelling of a secondary reagent. Direct techniques are rapid but of lower sensitivity and rely on the supply of labelled primary antibodies, whereas indirect labelling can be used with any primary antibody.

The most common enzyme used with immunoenzymatic labelling is horseradish peroxidase. It can be detected by reactions, which produce an insoluble product. The commonest substrate contains diaminobenzidene (DAB) and hydrogen peroxide. Aminoethylcarbazole (AEC) and hydrogen peroxide may also be used, as DAB may be carcinogenic. Calf intestinal alkaline phosphatase is an alternative enzyme to peroxidase. Substrates to this enzyme include napthol phosphate and hexazotized new fuschin or fast red. The advantage of calf intestinal alkaline phosphatase over horseradish peroxidase is that endogenous tissue peroxidase activity is not a problem. Endogenous tissue peroxidase in white cells and red cells may cause problems if the substrate reacts with both the endogenous enzyme and the horseradish peroxidase label. This can be prevented by introducing a blocking step during the procedure. Tissue alkaline phosphatase activity is less of a problem, but its activity can be blocked by levamisole.
Immunoenzymatic techniques may also be direct or indirect. In the direct method, the enzyme is usually coupled to the antibody covalently. However the enzyme may also be bound by antibody which has been raised against the enzyme. These immune complexes of enzyme and antibody can be linked to the primary antibody by a bridging antibody raised in the same animal as the primary antibody. It is important that the anti-enzyme antibody is raised in the same animal in which the primary antibody was raised. Some examples of this are the peroxidase antiperoxdase method and the alkaline phosphatase antialkaline phosphatase method.

Enzymes may also be coupled to the antibody through a reaction between the protein avidin and the vitamin biotin. This is based on the fact that avidin has a very high affinity for biotin and is thought to ensure strong binding of the final enzyme label to the tissue. In this method, a biotinylated link antibody forms a bridge between the unlabelled primary antibody and a pre-formed complex of avidin and biotinylated enzyme complex.

Staining may be specific, non-specific or a false negative. Specific staining reveals the true antigenic content whereas non-specific staining does not. Common causes of non-specific staining include polyclonal antisera and non-specific binding of the antibodies to collagen in the tissue. Polyclonal antisera may contain unwanted antibodies against antigens other than the one of interest, and monoclonal antibodies reduce this problem. A blocking step in which the tissue is first incubated with non-immune serum is occasionally used to prevent non-specific staining. It is worth noting that an antigen may be detected in a cell in which it was not originally synthesised as a result of diffusion and artefactual uptake of extracellular antigens. False negative staining may occur as a result of the
denaturation of antigens during tissue processing, a low level of antigen in the cell, or rapid exportation of an antigen from a cell in which it was originally synthesised.

Controls reactions are usually performed to detect false positive and negative staining. These include omitting the incubation of the tissues with the primary antibody to detect false positives, and staining a different tissue already known to produce the antigen of interest to detect a false negative. Measurement of staining in cells can be done subjectively or objectively. Subjective measurement involves scoring the intensity of staining of histological slides through the light microscope as “+”, “++”, or “+++” over a background negative area. Objective measurement involves the use of image analysers (Bradbury et al 1982).

**Immunohistochemical detection of PAI-1 (See appendix 3 for details of experimental work).** For this study, the steptavidin-biotin method was used (Hsu et al 1981). The tissues had been fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry was initially performed on sections of tissue known to express PAI-1; inflamed appendix (Whawell et al 1993) and placenta (Estelles et al 1994) to determine ideal working dilutions. For the study, four-micron sections were cut. After mounting on 3-aminopropyltriethoxysilane coated glass slides, they were left in a 37°C incubator overnight. The next morning, these slides were transferred to a 60°C incubator for a maximum of 2 hours. The antibody used was a commercially available monoclonal antibody to PAI-1 (murine monoclonal antibody raised against purified active PAI-1 secreted by the human melanoma cell line: American Diagnostica Incorporated, product number 3785) which had been successfully used in formalin-fixed, paraffin-embedded tissue (Jankun et al 1993). The epitope specificity of this antibody was unknown, however there was no cross reactivity with PAI-2 or PAI-3.
The ovarian sections were dewaxed in xylene, rehydrated in alcohol and treated in 3% volume hydrogen peroxide in 100 mls of methanol for ten minutes to block endogenous peroxidase. They were then washed well in running water, and trypsinised at a pH of 7.80 at 37°C for 30 minutes for antigen retrieval. Next, the sections were blocked in 20% normal goat serum for 10 minutes at room temperature to reduce non-specific background staining and then incubated with the primary antibody at a dilution of 1 in 25 and 1 in 50 for one hour at room temperature in a humid chamber. The primary antibody was drained off the slide and the sections were washed with tris (hydroxymethyl) methylamine buffer. Following this, the sections were incubated with the secondary antibody (biotinylated goat antimouse/rabbit IgG (Dako UK)) for 30 minutes, and washed with tris (hydroxymethyl) methylamine buffer again before incubation with steptavidin-biotinylated horseradish peroxidase complex for 30 minutes. After washing the slides, they were incubated with a solution of 3,3'-diaminobenzidine (DAB) for ten minutes and nuclear staining was performed with hematoxylin. The sections were then dehydrated in graded alcohol, cleared in xylene and mounted for viewing. One negative control slide was subjected to all these steps except for the incubation with the primary antibody. Areas containing PAI-1 stained brown.

Slides were evaluated by two independent histologists blind to the diagnosis in each case, the location of PAI-1 was noted and the intensity of staining assessed subjectively.

**Objective quantification of staining.**

Prior to image analysis, hematoxylin was removed from the sections by placing them in a 3.5% solution of hydrochloric acid in methylated spirit for one hour, and histomorphometric analysis was done with a Leica Quantimet 570 image processor.
The system is equipped with a 3CCD Hitachi HV-C20 camera on a Zeiss photomicroscope II. Images were acquired as 512 x 512 pixels, in 256 grey level digital images. Using the principle of Beer's Law, this system processed the amount of light transmitted through the sections and provided a numerical value for each pixel as grey levels, transmittance or optical density. For this study, grey level measurement data was used. High pixel values represented increased light transmittance, reflecting an area of limited staining and low pixel values dense staining. Under low magnification, on a Kyola SDZ trinocular microscope x 45 magnification, the maximum diameter of every intact follicle in each section was measured and the total number of follicles present was noted. The sections were viewed under high magnification 400x (OBJ40x photomag x10), and interactive thresholding performed to eliminate artefacts from the images. An image frame 65 µm horizontally and 12 vertically was defined for quantitation of staining. This provided a horizontal profile of grey levels for 65 µm obtained from 234 pixels within the frame. Horizontal profiles were then taken from four randomly selected points evenly distributed radially around the margin of each follicle. Images were oriented such that this profile started just within the follicle antrum and extended outwards through the granulosa cell layer, into the theca cell layer and stroma.

Data storage and analysis

The data array from each profile were stored electronically as individual files which were then all imported into a spreadsheet for basic data analysis. The total, mean and minimum grey level pixel value for each horizontal profile was calculated. Comparison of the means of all these values derived from all the horizontal profiles obtained from polycystic ovaries was compared with those obtained from the control group using a Student's t test. This t-test assumed that the profiles were independent but as they came from just 11 patients in total, this was not the case. A more appropriate analysis was felt to be a nested analysis of
variance (ANOVA), which took account of variation within and between patients in both groups. A linear regression analysis was used to evaluate the overall correlation between the mean minimum grey level pixel value derived from the 4 profiles taken from each follicle and the maximum diameter of that follicle.

RESULTS

Immunohistochemical detection of PAI-1 was predominantly found in the granulosa and theca cells in the follicles in both polycystic and normal ovaries with some weak signal in the endothelial cells and serosal surface (figure 6.1). There was good agreement between the histopathologists that overall, there was more intense staining was found in the granulosa cells (figure 6.2), but no obvious difference was identified between the control and study groups on visual inspection.

In both the PCOS and control groups, PAI-1 was detected around the developing follicle at all stages (pre-antral and antral) leading up to ovulation. PAI-1 staining was present in the low columnar follicular cells surrounding the primary (pre-antral) follicle (figure 6.3), the granulosa and theca cell layers of the secondary (pre-antral) follicle (figure 6.4), the theca cell layer of the tertiary (antral) follicle (figure 6.5) and the granulosa and theca cell layers of the atretic follicles (figures 6.1 and 6.2).
Figure 6.1 Follicle from a polycystic ovary: immunostaining of granulosa cell layer (GCL) and theca cell layer (TCL) with anti PAI-1 monoclonal antibody; brown reaction product.

This section was one of those where hematoxylin was removed prior to image analysis.
Figure 6.2 Edge of follicle from a polycystic ovary under high magnification:

immunostaining of granulosa cell layer (GCL) and theca cell layer (TCL) with anti PAI-1 monoclonal antibody; brown reaction product.

*This section was one of those where hematoxylin was removed prior to image analysis.*
Figure 6.3. PAI-1 staining in the single layer of low columnar follicular cells surrounding the primary (pre-antral) follicle; brown reaction product.
Figure 6.4 PAI-1 staining in the granulosa and theca cell layers of the secondary (pre-antral) follicle; brown reaction product.
Figure 6.5 PAI-1 staining in the theca cell layer of the tertiary (antral) follicle; brown reaction product.
There were, in total, 17 intact follicles in the polycystic ovary group and 15 in the control group. There was no significant difference in the mean diameter of the follicles between polycystic and control ovaries (3.02mm (0.4-5.79) vs 3.26mm (0.77-10)). A total of 68 horizontal profiles from the follicles in the polycystic ovary group and 63 in the control group were used for quantification. Using the students t-test, the mean of the minimum grey level pixel values derived from every horizontal profile in the polycystic ovary group was significantly lower than in the control group suggesting more intense PAI-1 staining (57 sd ± 37.6 vs 73.4 sd ± 42.7 grey levels, p= 0.02), but there was no evidence of a difference in the total or mean grey level pixel values (see table 6.2). However, a nested analysis of variance of the minimum grey levels with three factors: the group (Controls or PCOS), the patient (nested within group) and the follicle (nested within each patient) revealed that there was no evidence of a significant difference between controls and PCOS (p = 0.53). The mean minimum grey level was calculated for each subject, giving 11 scores in total, six controls and five PCOS. A student's t-test applied to these values also did not find a difference between groups (p = 0.29).

There was a significant inverse correlation between the mean minimum grey level pixel value derived from the 4 profiles taken around the follicles and their maximum diameter in the polycystic ovary group but not in controls (r = -0.58, p = 0.01 in PCOS and r = -0.34, p = 0.2 in controls).
Table 6.2 (Pixel values from the entire horizontal profile expressed as grey levels).

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 63 profiles)</th>
<th>PCOS (n = 68 profiles)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total pixel values (SD)</td>
<td>34534 (6043)</td>
<td>33245 (6567)</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean average pixel value (SD)</td>
<td>147(25)</td>
<td>142(28)</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean minimum pixel value (SD)</td>
<td>73(42.7)</td>
<td>57(37.6)</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

* P = 0.53 on ANOVA.

DISCUSSION

The finding of PAI-1 in the cells surrounding the oocyte, from the early stages of follicular development (primary follicle), through the secondary follicle, the graafian (tertiary) follicle and the atretic follicle is consistent with a clear role for PAI-1 in human folliculogenesis. However, this study did not demonstrate a significant difference in the intensity of PAI-1 staining between the PCOS and control groups. Thus, the hypothesis, suggesting that raised PAI-1 levels may contribute to impaired folliculogenesis in women with PCOS has not been supported by the findings of this study.

The main drawbacks of this study were the small sample size, and the fact that the ovarian biopsies from the control population had been obtained in both the luteal and proliferative stages of the menstrual cycle, and as such had been subject to different levels of gonadotrophin stimulation. This could potentially have caused an inconsistent expression of PAI-1 in the ovaries in this study group making the quantitative data derived from this group on image analysis less than ideal for analysis. However it was difficult to obtain a reasonable number of biopsies from "normal" ovaries taken from women in a similar age
group as the PCOS group to make exclusion on the basis of cycle stage possible probably because there were very few or no indications to biopsy a normal looking ovary from a young woman in the reproductive age group. It may be argued that to increase the number of control ovarian biopsies used in this study one could have removed the age limit. However PAI-1 expression increases with age and this would have caused a bias, and a significant number of ovarian biopsies from women in the older age group were diseased and unsuitable for the study.

It would have been ideal to obtain biochemical confirmation of PCOS in the PCOS group, but this was not possible in all cases as evidence from the records of the women in this study showed that a biochemical diagnosis of PCOS was not always necessary for a diagnosis of PCOS to be made and wedge resection performed. Efforts were made to contact the women without biochemical evidence of PCOS to come in for blood tests, but this was not achieved because most of the women had either moved out of the area or had changed their names and addresses making it difficult to trace them.

The end product from the immunohistochemical reactions was objectively quantified using microdensitometry. This technique has been used before in immunohistochemistry, during which the staining intensity was shown to be proportionally related to time and antigen concentrations (Van Der Loos et al 1994). However, quantitative data can show poor inter-experimental reproducibility owing to variability in antibody dilution, incubation time, temperature and the state of preservation of antigens after fixation. All the sections used in our study were processed in one batch on the same day, and all the biopsies were taken within the same period to minimise any possible bias from the above factors. However the grey level data may have been subject to inconsistencies in the state of preservation of antigens after fixation as it was not possible to control for this.
The absence of a significant difference in the total grey level scores from the horizontal profiles in the PCOS and control groups did indeed suggest that overall, there was no difference in PAI-1. However because there was no consistency in the thickness of the granulosa and theca cell layers and the distance of the horizontal profile within the follicle antrum it was felt that the minimum grey level score would provide the most objective measure of staining. These minimum grey level data did indeed suggest that overall more PAI-1 was detected around the follicles in polycystic ovaries. This difference was not statistically significant on analysis of variance, which may have been because of the small sample size. The correlation between follicle size and perifollicular minimum grey level scores suggesting that more PAI-1 was detected around the larger follicles compared to the smaller ones especially in women with polycystic ovaries was an unexplained finding as from the studies on PAI-1 mRNA expression in rat ovaries (Peng et al 1993), one would have expected that more PAI-1 would be expressed in the smaller sized follicles.

In summary, this study was the first to demonstrate the in-situ location of PAI-1 in the human ovary by immunohistochemistry, and it showed that PAI-1 was mainly expressed in the granulosa and theca cell layers which was in support of it’s previously suspected role in ovulation. Computer image analysis for quantitation suggested that there was a significant increase in the intensity of PAI-1 staining in polycystic ovaries when all the 131 acquired image profiles were used in the data analysis, but this difference disappeared when the fact that these profiles were obtained from a total of 11 subjects was taken into account in an analysis of variance. The role of PAI-1 in disordered folliculogenesis in women with polycystic ovaries by limiting plasmin production and proteolysis around the developing follicles was not clear from these data, however a larger study may shed more light on this subject. Future experimental work looking at ovarian granulosa cell PAI-1
production in response to different potential stimulants of PAI-1 such as insulin, in polycystic and control ovaries may also be helpful.
CHAPTER SEVEN

PLASMINOGEN ACTIVATOR- INHIBITOR-1 MESSENGER RNA
EXPRESSION IN THE POLYCYSTIC OVARY.

OUTLINE

The methods and results of a study to demonstrate the expression of PAI-1 mRNA by in situ hybridization with synthetic oligonucleotide probes in ovarian biopsies from women with PCOS and normal ovaries are presented. The general principles of in-situ hybridisation, the experimental procedures used in this study and the problems encountered are discussed.

INTRODUCTION

Women with PCOS have poorly developed ovarian follicles, and they suffer from anovulatory infertility of poorly explained aetiology. Systemic levels of PAI-1 are raised in some women with PCOS, and it has been suggested that an insulin driven increase in PAI-1 may be partly responsible for the disordered folliculogenesis in women with polycystic ovary syndrome (Sampson et al 1996, Atiomo et al 1998, and see chapter 5) by inhibiting the conversion of plasminogen to plasmin by tissue plasminogen activator.

In the previous chapter, the tissue distribution of PAI-1 antigen in the human normal and polycystic ovary was studied by immunohistochemistry and found to be localised to the granulosa and theca cell layers in both groups with most PAI-1 found in the granulosa cell layer. The expression of the PAI-1 gene by the different ovarian cell types in the human ovary has never been described although PAI-1 mRNA has been reported in the granulosa cells of women undergoing assisted conception (Piquette et al 1993). In a previous study
on rat ovaries (Peng et al 1993), using in-situ hybridisation PAI-1 mRNA was mainly found in the cell types located in the outer layer of follicles including the theca interna, theca externa, interstitial cells and ovarian stroma which was in contrast to my findings on immunohistochemistry. In addition, following HCG stimulation, the dominant pre-ovulatory follicles which protrude onto the surface of the ovary were surrounded by less interstitial and stromal tissue that expressed PAI-1 compared to the smaller non-ovulatory follicles that were in the interior part of the ovary which were surrounded by layers of PAI-1 expressing tissue. Based on these results, it was interesting to speculate whether an increased expression of the PAI-1 gene contributed to the limited follicle growth seen in women with PCOS.

This study was therefore set up to describe the gene expression of PAI-1 in human normal and polycystic ovaries in situ and determine whether PAI-1 played a role in impaired folliculogenesis in PCOS.

METHODS

The study design and case selection were described in chapter 3. Paraffin embedded histological sections from the same ovarian biopsies used in chapter 6 (immunohistochemical localisation of PAI-1) were used for this study. The biopsies were taken from five women with PCOS and six controls between 1982 and 1985. These numbers were considered sufficient to initially establish the technique of in-situ hybridisation, as this had not been performed in our laboratory at Derriford hospital Plymouth before. The clinical and biochemical profile of these women was described in chapter 6 and table 6.1.
General principles of \textit{in situ} hybridisation

\textit{In situ} hybridisation was first described by research workers in the United Kingdom and United States of America in 1969 (John et al, 1969, Gall and Pardue, 1969). It allows the demonstration of nucleic acid sequences in their cellular environment by the introduction of an exogenous labelled but complementary polynucleotide strand (probe) to a milieu of denatured DNA, which associates (hybridises) with a dissociated polynucleotide strand (target) to form a hybrid duplex in situ. This hybrid is detected by autoradiography if the label is a radioisotope, or by regular light or fluorescence microscopy otherwise.

The principles of the technique involve the pre-treatment of cellular preparations to unmask target nucleic acids, hybridisation of a nucleic acid probe of complementary base sequence to the target, and the detection of the label attached to the probe which allows the demonstration of specific nucleic acid sequences within cells.

The following types of nucleic acid probes may be used in \textit{in situ} hybridisation.

These include, double-stranded DNA probes derived from an isolated and cloned segment of genomic DNA, single-stranded cDNA probes made from mRNA using reverse transcriptase, chemically synthesised oligonucleotides and single stranded RNA probes. The larger fragment DNA probes are more sensitive than synthetic oligonucleotide probes (Berent et al 1985), because the smaller size of oligonucleotide probes relative to conventional cDNA probes covers a smaller target. Cocktails of several oligonucleotide probes are therefore required to compensate for the low specificity. Oligonucleotide probes are however readily available through automated synthesis, and their small size gives them good cellular penetration properties which largely compensates for the smaller target they cover. Complementary single stranded RNA probes (cRNA) have a high binding stability.
Probes used for *in situ* hybridisation may be labelled with isotopic or non-isotopic reporter molecules. Biotin and digoxigenin are examples of non-isotopic reporter molecules, and examples of isotopic reporter molecules include $^3$H, $^{32}$P, $^{35}$S, or $^{125}$I. Biotin labelled probes unfortunately have a disadvantage as biotin has a widespread endogenous tissue distribution (Wood and Warnke 1981) occasionally making it difficult to differentiate between true and false positive results. Digoxigenin (Herrington et al 1989) on the other hand has superior sensitivity to biotin (Morris et al, 1990; Furunta et al, 1990) with very low non-specific background staining. Radioisotopes generally have a short half-life, require long exposure times for detection, and come with the known hazards of radioactivity.

Prior to the procedure, it is important to achieve optimal fixation for good results, as it allows the preservation of cell morphology and nucleic acids, particularly for RNA detection, which is very susceptible to digestion by endogenous ribonucleases. Tissues are therefore best fixed or frozen as soon as possible after surgical excision to prevent this. Many different fixatives can be used, however cross-linking fixatives, such as formalin and paraformaldehyde, give the best retention of nucleic acids, by forming chemical bonds with cellular proteins (Singer et al, 1986).

DNA and RNA can be degraded by nuclease activity. Nucleases present on the skin may contaminate solutions used in hybridisation methods sufficiently to degrade the quality of naked DNA and RNA. For this reason, gloves should be used in handling tissues, and precautions have to be taken to ensure the absence of exogenous nucleases from solutions.
It is important to coat the glass slides used for in-situ hybridisation to ensure section adhesion, and either aminopropyl-triethoxysilane (Van Prooijen-Kneght et al, 1983) or poly-L-lysine (Huang et al, 1983) may be used.

A number of pretreatments are used, both to reduce non-specific background and increase probe penetration, and these depend on the method of fixation. Hydrochloric acid (0.1M) for example reduces non-specific background in formol-sublimate fixed tissue. Acetylation can also used to reduce binding to basic groups within the tissue (Hayashi et al, 1978).

Permeabilisation of the section is necessary for formalin fixed tissue to reduce the effect of cross-linking. This can be achieved by proteolytic digestion with proteinase K (Pringle et al, 1987) or pronase (Brigati et al, 1983). It is important to control this step carefully as underdigestion will result in insufficient exposure of the nucleic acid, and over digestion can sufficiently weaken the protein structure surrounding the sequences as to bring about its loss into solutions.

A prehybridisation solution applied prior to addition of the probe contains high concentrations of single stranded sheared DNA and anionic macromolecules, which block sites of potential non-specific probe interaction.

The aims of hybridisation are to ensure maximal reaction of the probe with target sequence with minimal non-specific interaction with nucleic acids and other cellular components. A number of variables in the conditions used for hybridisation affect this aim and these include; Temperature and ionic strength, accelerators (dextran sulphate), buffers, probe concentration, and blocking agents.
(i) Temperature and ionic strength - the optimum temperature for efficient hybridisation of homologous nucleotide sequences is 20-30°C below the melt temperature (Tm) (denaturation temperature) of the specific hybrids. The Tm is the temperature at which 50% of the duplexes are dissociated and is proportional to the guanine-cytosine (G-C) base pair content. Where there is a high percentage of these, the third hydrogen bond associated with the base pair results in a higher melt temperature than in sequences in which adenosine and thymidine (A-T) pairing predominates. The inclusion of formamide, a helix destabiliser, into the hybridisation mixture reduces the Tm by 0.7°C for each 1% formamide.

(ii) Accelerators - inclusion of dextran sulphate increases effective probe concentration and hybridisation rates by volume exclusion. Dextran sulphate has the added advantage of reducing non-specific binding of probe to positively charged molecules in the tissue.

(iii) Buffers - Tris/phosphate/sodium citrate based buffers are used to maintain neutral pH.

(iv) Probe concentration - low probe concentrations are important for the reduction of non-specific binding.

(v) Blocking agents - a number of other macromolecules can be included in the hybridisation mixture to reduce background, including salmon sperm DNA, transfer RNA and Denhart's solution.

Post-hybridisation washes, are used to wash off mismatched and non-specifically bound probe. Usually these washes include the sequential immersion of the specimens in salt solutions alone or with formamide. The temperature, formamide and ionic concentration of this solution can be varied to improve the specificity of the final detected signal.
The choice of a detection system is determined by the probe label used, and this may be direct (e.g. with fluorescin labelled probes), or indirect by the sequential application of histochemical reagents (e.g. with biotin).

In order to confirm the specificity of signal a number of controls are usually employed. A positive control and a no hybrid control are usually included. A positive control probe usually demonstrates RNA/DNA preservation in the tissue, and this will exclude one cause of false negative results.

For negative controls, omission of probe from the hybridisation mixture, allows the exclusion of non-specific reactions in the detection systems. However this does not provide information on background staining engendered by the probe, and negative control probes (designed to unrelated RNA species) will provide this additional information.

It is important to prevent contamination from exogenous ribonucleases during all steps of the procedure until hybridisation by treating all glassware and solutions with diethyl pyrocarbonate (DEPC).

Localisation of PAI-1 mRNA by in situ hybridisation.

i) PAI-1 Probes

Prior to the study, I tried to obtain a cDNA probe to PAI-1 which had been used in the UK for a previous study on the gene expression of PAI-1 in transplant kidneys (Wang et al 1994), but it was not possible to track down the author who had specifically obtained the probe. A 40-oligomer probe for the detection of PAI-1 was commercially available (Cambridge bioscience UK). This probe was supposed to have been tested on paraffin embedded tissue, but the
company could not provide any publications in support of this claim. Therefore for practical reasons, it was decided to use a cocktail of synthetic oligonucleotide probes. It was anticipated in addition to being easy to obtain, that these probes would provide the added potential benefits of a high efficiency of hybridisation, and increased sensitivity compared with the single commercial oligonucleotide probe.

Oligonucleotides of 30 bases in length were synthesised. Shorter sequences would have formed less stable hybrids, and with longer ones there were potential problems with homology. Probes of this length also allow more flexible limits of stringency to be used and still remain specific as well as being long enough to diminish the chance of cross-hybridisation with similar sequences in the human genome. Base sequences complementary to 5 regions of the known nucleotide sequence of the DNA coding for human PAI-1 (Ny et al 1986) were selected so that they did not occur too closely to each other (greater than 20 bases apart) to avoid any possibility of interference of probe hybridisation once they have been labelled by the addition of a tail. The base sequences (see figure 7.1) selected also had approximately a 50% G-C content to increase the stability of DNA/RNA hybrids.

### Table 7.1

Oligonucleotide sequences selected for synthesis of PAI-1 probes.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>Nucleotide Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAT,AGC,TGC,TTG,AAT,CTGCTG,CTG,GGT,TTC</td>
<td>198-169</td>
</tr>
<tr>
<td>2</td>
<td>TCC,ATG,GCC,CCA,TGA,GCT,CCT,TGT,ACA,GAT</td>
<td>271-242</td>
</tr>
<tr>
<td>3</td>
<td>CTT,GAC,CGT,GCT,CCG,GAA,CAG,CCT,GAA,GA A</td>
<td>378-349</td>
</tr>
<tr>
<td>4</td>
<td>CTT,CAC,CCA,GTC,ATT,GAT,GAT,GAA,TCT,GGC</td>
<td>435-406</td>
</tr>
<tr>
<td>5</td>
<td>CTT,CCA,CTG,GCC,GTG,GAA,GTA,GAG,GGC,ATT</td>
<td>540-511</td>
</tr>
</tbody>
</table>
To ensure the specificity of these sequences for PAI-1, the following human nucleic acid databases GenBank, EMBL and HGMP-RC cDNA sequences, on the UK Human Genome Resource Centre internet web site were searched using a search engine (BLAST) found on the web site. The results confirmed a perfect match with PAI-1 and no other known DNA sequences.

The first three (three and not all five because of financial considerations) oligonucleotide sequences were then chosen for synthesis. These PAI-1 probes were synthesised using phosphoramadite chemistry (Beaucage and Caruthers, 1981) on a DNA synthesiser at the department of Pathology, University of Bristol.

The oligonucleotide probes were labelled (see appendix 4) at the 3′ hydroxy end with digoxigenin using the homopolymer tailing method with terminal deoxynucleotide tranferase (Boerhinger Mannheim). Digoxigenin was chosen as the label because the laboratory used for this experiment did not carry a licence for radioactive experiments, and because of the previous experience of the supervisor of this project.

**ii) Control probes**

In order to demonstrate RNA preservation in the tissues, oligonucleotide probes complementary to mitochondrial ribosomal RNA normally expressed in most tissues were used as positive controls. Mitochondrial RNA is highly expressed within all tissues and there was also previous experience within the Department with this probe (Hilton et al 1994).

For negative controls, the probe was omitted from the hybridisation mixture.
iii) In situ hybridisation

The details of the method including the preparation of the solutions and labelling of the probes with digoxygenin are given in appendix 4.

Preliminary experiments performed on paraffin embedded sections of inflamed appendix in 1997 using the mitochondrial ribosomal RNA probe alone established that the technique of hybridisation worked and determined the optimal dilutions for some of the reagents. Following this, the experiment was performed on the study material using the cocktail of oligonucleotide probes for PAI-1 and mitochondrial RNA probes. For negative controls, no probe was included in the hybridisation mixture and inflamed appendix which had previously been known to express PAI-1 (Whawell et al 1993) were used as positive controls.

In situ hybridisation was carried out as follows;

Four micron sections mounted on aminopropyl silane coated slides were dewaxed in xylene and graded alcohols, then washed in hot SSC (sodium chloride and trisodium citrate dihydrate solution) to denature any RNA secondary structure. They were then treated with proteinase K (at two concentrations 2 and 5 µg/ml to expose the target sequences). These two dilutions of proteinase K were used in the preliminary experiments to determine an optimal dilution for the test material. Sections were then briefly refixed in 0.4% paraformaldehyde for 20 minutes at 4°C. Next the slides were then incubated with the prehybridisation solution for 30 minutes at 37°C and with the hybridisation solution overnight at 37°C. The hybridisation solution consisted of 50 µl of buffer containing 0.1ng/µl probe, 30% formamide, 600mM NaCl, 0.1 M phosphate buffer, 10% dextran
sulphate and 150μg/ml sheared SSDNA (Salmon sperm DNA). Post-hybridisation washes were performed with 2 x SSC/30% formamide at 37°C to wash off any unhybridised or mismatched probe. Sections were blocked for 10 minutes in a solution of 3% bovine serum albumin, triton X and tris-buffered saline to prevent non-specific antibody binding. Following this they were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase at 1:600 and 1:300 (Boehringer-Manheim) for 30 minutes. Two dilutions of alkaline phosphatase were used in the preliminary experiments to determine an optimal dilution for the test material. NBT/BCIP (Nitrobluetetrazolium, and bromochloroindolylphosphate) were applied as substrates for the colour reaction and left in the dark overnight. The next morning, the slides were rinsed, counter-stained with hematoxylin and mounted in Apathy's medium.

RESULTS
There was clear expression of mitochondrial ribosomal RNA in the cytoplasm of cells from the inflamed appendix used in the preliminary experiment (Figure 7.1). No detectable staining for PAI-1 mRNA was found in the cytoplasm of any of the ovaries, and only a weak expression of mitochondrial ribosomal RNA was detected (Figures 7.2 & 7.3). Some staining for PAI-1 mRNA was noted in the nucleus of a few granulosa cells, however this was most likely non-specific staining, as mRNA is usually found in the cytoplasm. There was also no detectable staining for PAI-1 mRNA in the sections of inflamed appendix, which had served as a known positive control tissue. No staining for PAI-1 or mitochondrial RNA was found in the negative control tissue.
Figure 7.1  Clear expression of mitochondrial ribosomal RNA in mucosa of inflamed appendix used as positive control for in situ hybridisation.
Figure 7.2  Absent signal for PAI-1 mRNA in cytoplasm of cells in polycystic ovaries, but note granulosa cell nuclear staining (dark reaction product).
DISCUSSION

The results from this study showed that PAI-1 mRNA was not detected in either polycystic or normal ovaries, and that it was also not detected in inflamed appendix which had served as the positive control tissue for PAI-1 (Whawell et al 1993).

The fact that mitochondrial RNA was detected in the ovaries and inflamed appendix suggests that there was no problem with the technique of in situ hybridisation itself, however the negative results for PAI-1 may have occurred for any or a combination of the following factors. Firstly the cocktail of PAI-1 oligonucleotide probes used for this study may not have been sensitive enough for the detection of PAI-1 mRNA and secondly overall tissue RNA preservation may have been poor in the sections from ovaries and
inflamed appendix samples used for this study because of the effects of endogenous ribonucleases (RNAse) on PAI-1 mRNA.

It is unlikely that a reduced sensitivity of our probes was the single contributory factor to the negative results, as with previous research using northern blot, a single 17 base oligonucleotide probe containing one internal mismatch provided enough signal to be distinguishable from background signal in a study comparing long DNA fragments with oligonucleotides (Berent et al 1985). However it is important to note that these material would not have had the potential problem of endogenous RNAse present in our study material. It is therefore likely that our results arose because of a combination of poor tissue RNA preservation and low probe sensitivity.

Degradation of mRNA by intrinsic RNAse can be limited by rapid fixation of tissue specimens, and this is shown by the decreasing yields of RNA obtained from freshly fixed biopsies, laparotomy and post mortem gut specimens (Yap & McGee 1992). It is therefore important that tissue to be used for in situ hybridisation is fixed as soon as possible after surgical biopsies, for optimal RNA preservation. The ovarian biopsies used for this study were surgically excised 12-15 years prior to the study, and it is not possible to comment on the speed of their fixation. It may be argued that the successful detection of mitochondrial RNA in the ovarian samples suggests that poor RNA preservation in isolation may not have been the problem, however the weak and patchy demonstration of mitochondrial RNA indicates that poor RNA preservation was a major factor as staining for mitochondrial RNA in these ovarian biopsies was weaker than that found in the inflamed appendix used as a positive control (see figures 7.3 and 7.1).
The non-specific nuclear signal possibly occurred because nucleotides in the digoxigenin tail were hybridising in a non-specific manner to nucleotides in the nucleus, many of, which were undergoing apoptosis leading to fragmentation and destruction of DNA (Hilton et al 1997).

The following options were considered in an attempt to improve the detection of PAI-1; obtaining a cDNA probe, repeating the study with sections from fresh fixed ovarian biopsies, increasing the number of oligonucleotide probes in the cocktail, and performing in-situ gene amplification (in situ polymerase chain reaction).

All previous studies of PAI-1 mRNA detection on human granulosa cells had used a cDNA probe during northern blot (Piquette et al 1994, Piquette et al 1993, Reinthaller et al 1990, Jones et al 1988). A cDNA probe for PAI-1 may therefore have improved the sensitivity from our sections, however there was no guarantee that it would have been easy to obtain one, or that it would have worked if the samples had been fixed suboptimally leading to poor RNA preservation. Obtaining freshly fixed ovarian biopsies from women with PCOS was considered, however we anticipated ethical constraints as ovarian biopsies were no longer performed in our hospital as part of the treatment for infertility in PCOS because of the risk of peri-ovarian adhesions and subsequent infertility. In situ gene amplification, a recent technique, which is based on the in situ annealing and polymerisation of oligonucleotides to complementary nucleotides located at each side of the target DNA or RNA (Walker et al 1995), was considered. In this technique, the product obtained is then visualised directly by the incorporation of labelled nucleotides, or indirectly by following the amplification by insitu hybridisation with labelled probes. All of these options including the synthesis of additional oligonucleotide probes would have incurred additional financial and human resources, which were already in short supply at
this stage of my research projects. It was therefore felt that as the results of the large clinical study on PAI-1 in women in polycystic (chapter 5), and the study on immunohistochemical localisation of PAI-1 in polycystic ovaries (Chapter 6) did not show a significant difference in systemic or local PAI-1 compared with controls, the study was best discontinued.

In summary this study to evaluate the expression of PAI-1 mRNA in ovarian biopsies from women with and without PCOS using a cocktail of synthetic oligonucleotide PAI-1 probes did not find any detectable PAI-1 mRNA, although signals for the positive control probe (mitochondrial ribosomal RNA) were detected. The possible explanation of these findings were a combination of the effect of endogenous ribonucleases and an insufficiently sensitive oligonucleotide probes. The options considered in order to improve mRNA detection included obtaining a cDNA probe, increasing the number of oligonucleotides in the cocktail, performing in-situ gene amplification, and repeating the study with fresh ovarian biopsies and frozen sections. However these were not done because of limited financial and human resources.
CHAPTER EIGHT

ACTIVATED PROTEIN-C (APC) RESISTANCE AND POLYCYSTIC OVARY
SYNDROME

OUTLINE
This chapter presents the results of a study to determine whether there was any relationship between the increase in the prevalence of a positive family history of thrombosis in the PCOS group (see chapter 5) and an increased prevalence of activated protein C resistance.

INTRODUCTION
In the normal physiology of haemostasis (figure 8.1), clotting factors, natural anticoagulants, fibrinolysis, platelets and endothelial cells are the main factors, which determine the risk of venous thrombosis. The production and structure of these components may be affected by genetic or acquired risk factors, which may increase the risk of venous thrombosis. Some of the acquired risk factors for venous thrombosis include venous stasis from prolonged bed rest, surgery, obesity, pregnancy, smoking, increased age and damage to the blood vessel wall. The heritable defects which are at present accepted as proven to be associated with familial venous thromboembolism are deficiency of antithrombin III, protein C or protein S and factor V leiden mutation (activated protein C resistance) (Walker 1997). Antithrombin is a naturally circulating anticoagulant, which inhibits activated factors XII, XI, IX and X. The rate of interaction between antithrombin III and activated clotting factors is increased in the presence of heparin. Protein C is a vitamin-K dependent plasma protein, and it is a key player in physiological anticoagulation. After its activation on the surface of endothelial cells by a
complex of thrombin with thrombomodulin, activated protein C inhibits coagulation by selectively degrading coagulation factors Va and VIIIa. Protein-S, another vitamin K -dependent plasma protein is thought to function as a cofactor to APC.

Figure 8.1 The coagulation cascade

Of the heritable coagulation defects, the commonest appears to be that associated with APC resistance, which has been shown to be present in 30 to 50% of cases with venous thrombosis (Griffin et al 1993, Koster et al 1993, Svensson & Dahlback 1994) and 45% of relatives of people with venous thrombosis (Svensson & Dahlback 1994). Antithrombin III deficiency appears to be present in 1 in 200 to 400 individuals (Tait et al
1994) and protein C deficiency in 1 in 300 to 500 (Tait et al 1995). So far no large study has reported the prevalence of protein S deficiency (Walker 1997).

The role of APC resistance in familial thrombophilia was first identified in 1993 by Dahlback et al (1993) during the investigation of 51 year old man with a history of multiple thrombotic events. They found that the addition of activated protein C to his plasma did not result in a normal anticoagulant response as measured by prolongation of an activated partial thromboplastin time (APTT). Four of the proband's relatives also had histories of thrombosis, and they and several other family members also responded poorly to APC in the APTT. After ruling out several other theoretical possibilities that could have provoked the poor anticoagulant response to APC, the authors postulated that the findings were due to a deficiency of a hitherto unknown co-factor to APC.

Subsequently, DNA studies have shown that APC resistance is caused by a point mutation in the factor V gene (named Factor V Leiden or factor V:R506Q) which renders the activated factor V molecule resistant to APC inactivation (Bertina et al 1994, Voorberg et al 1994, Zoller et al 1994a, Zoller et al 1994b). The mutation replaces arginine (R) with glutamine (Q) at position 506 of the factor V molecule. A remarkably high prevalence of APC resistance and the factor V:R506Q mutation have been found in healthy controls in several European countries, most notably Greece (14%), France (10%), United kingdom (9%), Germany (8%), Sweden (7%) and the Netherlands (5%) (Koster et al 1993, Svensson et al 1994, Beauchamp et al 1994, Soubrier et al 1995, Rees et al 1995). So far the mutation has not been identified in Italian, Japanese, Chinese, African or Arab populations which could account for the relatively low incidence of venous thrombosis in these races.
Recurrent pregnancy loss has been associated with deficiencies of antithrombin III, protein C and protein S (Zanardi et al 1995), and APC resistance has been shown in 20% women with a history of second trimester miscarriage and 5% in the first trimester but the exact role of these thrombophilic defects in recurrent pregnancy loss is debatable. APC resistance has also been linked with an increased risk of pre-eclampsia (Davidson et al 1995). Polycystic ovaries are the commonest finding in women with recurrent pregnancy loss (Clifford et al 1996) and women with PCOS have an increased risk of developing pre-eclampsia (Gjonnaess et al 1989, Urman et al 1997). In the study carried out in chapter 5 (PAI-1 and metabolic features of PCOS), although none of the women themselves had suffered from a thromboembolic event, 29% of women with PCOS gave a positive family history of thrombosis compared to 8% in the control group. A family history of strokes and myocardial infarction was also elicited separately and this family history of thrombosis was assumed to refer to venous thromboembolism. This suggests that women with PCOS may have an increased prevalence of inherited thrombophilic defects, which in addition to placing them at increased risk of thrombosis could also possibly explain the link between PCOS and recurrent pregnancy loss. Prior to this study there had been no previous studies in the literature on the prevalence of these thrombophilic defects in women with PCOS, and this study was therefore performed to determine the prevalence of APC-resistance in women with PCOS compared with controls. The prevalence of deficiencies of protein C, protein S and anti-thrombin were also determined in the women with a positive family history of thrombosis.

SUBJECTS AND METHODS

The overall recruitment of subjects and their general characteristics are described in chapter 3 (study group II). Table 8.1 shows the characteristics of the women who gave a positive family history of thrombosis and their affected relatives. Approval was sought
from the local research ethics committee, and stored frozen platelet free plasma from
patients who took part in the study in chapter 5 were thawed within one year of collection
for the assays on APC-resistance, protein S, protein C and anti-thrombin III.

Assays

**APC resistance:** In general, the standard screening test for the APC resistance phenotype
measures two APTT's, one performed in the presence of APC which results in a
prolonged APTT, and the other in its absence. The relationship between the two APTTs is
usually expressed as an APC-ratio. APC resistance is indicated when the ratio is below or
equal to a cut off value defined as the lower limit of a normal range (calculated as the
mean APC-ratio from 50 to 100 healthy controls minus two standard deviations). Typical
APC ratios obtained on most instruments are ≤ 1.5 for homozygotes, ≤ 2.3 for
heterozygotes and ≤ 2.5 for individuals with a normal factor V genotype. With a
diagnosis based on an APC-ratio <2.23 (mean-2SD) the standard APC resistance test has
been shown to have a sensitivity of 76% and specificity of 98% for the factor V:506Q
mutation in a group of patients with venous thrombosis (Ailluad et al 1995). However by
using a modified APC resistance test, in which the patient sample is diluted in factor V-
deficient plasma the sensitivity and specificity for the factor V:R506Q mutation is
increased to almost 100% (Dahlback 1995).

For this study, APTT ratios for APC resistance were calculated using a commercially
available kit (Coatest APC resistance, chromogenix Sweden). With this kit, the
measurement principle of APTT is that plasma is incubated with a standard APTT reagent,
and coagulation is triggered by the addition of CaCl 2. All the controls and test cases were
diluted 1:5 in factor V deficient plasma (Immuno LTD, Kent). The precision for APC
ratios using the chromogenix kit showed a between series coefficient of variation of 1.4%
for ratios of 2.0 and 3 to 5% for ratios of 3.6 to 4.1. The normal range of APC ratios established from the local Plymouth population is 1.85 to 2.80 (mean ± 2SDs), and so far the sensitivity and specificity for the detection of the factor V:R506Q mutation using this range has been 100% (Condon J, personal communication).

Anti-thrombin III, Protein C and Protein S activity were determined using automated coagulation analysers based on chromogenic reactions (Instrumentation Laboratory-Milano, Italy).

The principle of the antithrombin III assay is that antithrombin III has a powerful and immediate antithrombin action in the presence of heparin. The assay involves the incubation of the test sample with an excess of thrombin in the presence of heparin, and the detection of the residual thrombin on a synthetic chromogenic substrate. The paranitroaniline release, monitored at 405nm, is inversely proportional to the antithrombin III level. The normal range is 80%-120% and the sensitivity is 15%.

The protein C assay is based on the initial activation of protein C in a test sample with protein C activator (derived from the venom of the copperhead snake). The amount of activated protein C is determined by measuring the amidolytic activity on a chromogenic substrate. The normal range is 70%-140% and the sensitivity is 15%.

The assay for Protein S is based on the principle that the functional activity of protein S, cofactor of activated protein C, is proportional to the prolongation of the PT of a protein S deficient plasma to which diluted sample has been added. The normal range is 60-140% and the sensitivity is 10%.
All assays had been successfully subjected to the UK national external quality assessment scheme for blood coagulation based in Sheffield.

**Statistical Analysis**

Results were stored on a spreadsheet (microsoft excel) for analysis, and are presented as proportions of the overall population with results outside the normal reference range. Comparisons between groups were performed using the chi-squared test. A p value of < 0.05 was accepted as statistically significant.

**RESULTS**

Of the 41 women with PCOS and 25 controls, 12 women in the PCOS group and 2 in the control group gave a positive family history of thrombosis as detailed in table 8.1. In all of these women with a family history of thrombosis, the APC ratios, protein C, protein S and antithrombin III levels were normal (table 8.2).

There was no significant difference in the overall proportion of women with low APC ratios in women with PCOS compared with controls (three women in the PCOS group (7%) vs one woman in the control group (4%), p=0.5), and the prevalence of APC resistance in the entire study population was 6.5%.
Table 8.1 General Characteristics Of The Women With a Positive Family History Of Thrombosis.

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<tr>
<th>Study Number</th>
<th>Diagnosis</th>
<th>Age (M)</th>
<th>Height (M)</th>
<th>Weight (kg)</th>
<th>BMI</th>
<th>Affected Relative</th>
<th>Age (yrs) of relative on diagnosis</th>
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<td>103</td>
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Table 8.2  APC ratios, protein S, protein C and antithrombin III levels in the Women With a Positive Family History Of Thrombosis.

<table>
<thead>
<tr>
<th>Study Number</th>
<th>Diagnosis</th>
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<th>Antithrombin III (%)</th>
<th>Protein C (%)</th>
<th>Protein S (%)</th>
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<td>86</td>
<td>110</td>
</tr>
<tr>
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<td>PCOS</td>
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<td>92</td>
<td>102</td>
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</tr>
<tr>
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<td>PCOS</td>
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<td>101</td>
<td>100</td>
</tr>
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<tr>
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<td>130</td>
<td>120</td>
</tr>
<tr>
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<td>Control</td>
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<td>66</td>
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<td>71</td>
<td>PCOS</td>
<td>2.4</td>
<td>95</td>
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<td>107</td>
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DISCUSSION

The results showed that women with PCOS did not have an increased prevalence of APC-resistance, compared to controls, that the prevalence of APC resistance in both groups were within the normal range expected for the general population in the UK (Rees et al 1995) and that none of the women with a positive family history of thrombosis were positive on the thrombophilia screen for antithrombin III, protein C and protein S. Although these results suggest APC-resistance most probably does not play a role in the PCOS, it is not possible to conclude that the same applies to antithrombin III, protein C and protein S deficiency because they were only tested in the 14 women with a family history of thrombosis and not the entire population.

On closer inspection of the characteristics of the 14 women with a family history of thrombosis in only six cases (five PCOS and one control) did the thrombotic event occur before the age of 50 years. As inherited thrombophilic defects are relatively rare but more common in the younger age group, one could argue that this study was not justified based
on the fact that it was unlikely that any thrombophilic defect was going to be found in this small "at risk" population of six, and that a larger study may have been appropriate with a prior sample size estimation. However this study could be justified by the putative links between these thrombophilic defects and recurrent pregnancy loss and pre-eclampsia. These are unexplained phenomena, which occur in women with PCOS, and the results of this study could serve as a pilot study on which to base any calculation for a sample size. The results from this study did not suggest that an increased prevalence of APC-resistance in PCOS could be found in larger studies but that further studies on other inherited thrombophilic defects might be necessary.

APC-resistance is ideally detected by DNA studies to determine whether patients are heterozygotes or homozygotes for the factor V leiden mutation. However in our laboratory, the sensitivity and specificity so far for the detection of heterozygotes for the Factor V Leiden mutation has been 100% when factor V deficient plasma is used. This is consistent with previous studies (Dahlback 1995) and supported by the fact that genetic tests confirmed that all of the women with low APC-ratios in this study were heterozygotes for the factor V Leiden mutation on follow in the haematology outpatients clinic (Prentice AG, personal communication).

Although these inherited thrombophilic defects did not appear to be related to the finding of an increased family history of thrombosis in PCOS, it is important to remember that women with PCOS have other features which could place them at increased risk of thromboembolic disease such as insulin resistance and obesity. As there appears to be a genetic component in the PCOS making insulin resistance and obesity more likely in their relatives, it is possible that these factors may have contributed to the increased family history of thrombosis observed in the PCOS group. Unfortunately this hypothesis could
not be tested because there were no details on the insulin states and BMIs of these relatives. However the finding of a correlation between a positive family history of thrombosis and PAI-1 in the multiple regression analysis in chapter 5 does suggest that insulin resistance and elevated PAI-1 may explain the increased family history of thrombosis observed in the PCOS group. On the other hand the study in chapter 5 also suggests that, although women with PCOS were insulin resistant compared to weight matched controls, their PAI-1 levels were not correspondingly elevated.

In summary, the prevalence of APC resistance was determined in women with PCOS and controls using an APTT based assay. The prevalence of deficiencies of antithrombin III, protein C and protein S in a subgroup of women with a positive family history of thrombosis were also determined. The results showed that the prevalence of APC resistance was similar in both the control and study groups and similar to the prevalence in the general population. No deficiency of antithrombin III, protein C or protein S was detected in the subgroup of women with a positive family history of thrombosis. Further studies are suggested to determine the prevalence of venous thrombosis in women with PCOS.
CHAPTER NINE

CONCLUSIONS

This study was designed to determine whether elevated PAI-1 levels were part of the metabolic aspects of PCOS and to investigate the expression of PAI-1 protein and mRNA in the polycystic ovary compared with normal ovaries. It was thought that the results might help discover whether PAI-1 and the PAS contribute to the pathophysiology of anovulation and pregnancy failure in women with PCOS and whether these women were at increased risk of thromboembolic disease from a prothrombotic PAS. Although some of the stated aims were achieved, questions have been raised.

The clinical pilot study of systemic plasminogen activation in chapter 4 showed that 11 oligomenorrheic women with PCOS had a prolonged ECLT and PAI-1 compared to 12 controls with regular menstrual cycles, which suggested that elevated PAI-1 was a feature of the PCOS and that in addition to placing women with PCOS at increased risk of thromboembolic disease, it was consistent with the hypothesis that PAI-1 played a role in the pathophysiology of anovulation and miscarriage in PCOS.

Unfortunately, women in the pilot group of PCOS were of a significantly greater BMI compared with the controls, which may have explained the elevated PAI-1 in PCOS as obesity correlates positively with PAI-1 levels.

This pilot study enabled us to calculate the required sample size for the larger clinical study (chapter 5), determine the overall feasibility of a large clinical study on the PAS in the PCOS in the setting of a district general hospital where these studies were carried out, and highlight any potential difficulties which were to be avoided in the larger study.
The large clinical cross-sectional study of PAI-1 and the metabolic aspects of the PCOS in chapter 5 showed that systemic PAI-1 activity or any other measured component of the PAS were not significantly different in 41 oligomenorrhoeic women with PCOS compared with 25 regularly menstruating controls of a similar BMI. It also showed that the only significantly different metabolic features of the PCOS group were insulin resistance, hyperinsulinaemia and lower HDL cholesterol ratios. The study did not confirm that hypertriglyceridaemia, hypertension and raised PAI-1 described in syndrome X were present. These findings were not consistent with the hypothesis that elevated PAI-1 levels put women with PCOS at increased risk of thromboembolic disease or that it contributed to their anovulatory infertility and miscarriage.

Another interesting but unexplained finding from this study was the approximately three-fold (but not significant) increase in the presence of a positive family history of thrombosis in women with PCOS.

The results from the clinical studies in this thesis must however be interpreted with caution for the following reasons. Firstly they were primarily designed to detect statistical differences in the systemic levels of metabolic and haemostatic variables, which may not be the same, as differences that, are biologically important. Secondly these systemic PAI-1 measurements were one aspect of a dynamic plasminogen activator system which could have been influenced by the many variables which affect the PAS. The observed variability of the mean and median PAI-1 values observed in these studies may therefore have been larger than one would have observed if all these variables were perfectly controlled, increasing the potential of a type II statistical error. Although very good
standardisation was obtained by the measures taken in the studies, perfect standardisation was impractical to achieve.

Experimental work at the level of the end organ often provides more insight into the exact role of a putative factor in a biological process of interest involving that organ, and the studies on the ovarian expression of PAI-1 protein and mRNA were designed for this purpose. The results of the study on the immunohistochemical detection of PAI-1 in polycystic ovaries demonstrated that PAI-1 was mainly expressed in the peri-follicular region in human ovaries supporting the hypothesis of a role in ovulation. The failure to demonstrate a statistically significant difference in PAI-1 staining on quantification by computer image analysis cast doubts on any role in anovulatory infertility in PCOS. The previous argument about the possibility of a false negative result from insufficient standardisation of all the possible variables which may affect a dynamic PAS also applies to any conclusions drawn from the quantification by image analysis.

Nevertheless this was the first study to describe the in-situ localisation of PAI-1 protein in the human ovary. It was disappointing that the study on PAI-1 mRNA expression in stored biopsies of polycystic and control ovaries failed to detect PAI-1mRNA, although signals for the positive control probe were detected. Although it is possible that the negative results occurred either because endogenous ribonucleases had degraded PAI-1 mRNA in the study material or the cocktail of oligonucleotide probes used for PAI-1 the study were not adequately sensitive, this study was very useful in highlighting the occasional difficulties encountered in laboratory research.

The study on APC-resistance in the PCOS is an example of how new questions can be raised in research as it was prompted by the unexpected finding of an increased prevalence
of a positive family history of thrombosis in women with PCOS. The results suggested that women with PCOS were not at increased risk from thrombosis because of APC resistance. The prevalence of APC resistance was similar in both the PCOS and control groups and not significantly different from the prevalence in the general population. No deficiency of antithrombin III, protein C and protein S was detected in the subgroup of women with a positive family history of thrombosis. However the number of women in this subgroup was too small to draw any meaningful conclusions on whether these factors played a role in the PCOS given their relatively low frequency in the general population.

This study did not support the hypothesis that elevated PAI-1 was a feature of the PCOS, but the in-situ expression of PAI-1 protein was shown for the first time to be localised mainly to the granulosa and theca cell layers of both polycystic and normal ovaries supporting a previously suspected role in human ovulation. Women with PCOS were not found to have an increased thrombotic risk from APC resistance. It was not possible to speculate confidently on a clear role for PAI-1 in contributing to the poor reproductive performance observed in the PCOS or in causing an increased thrombotic risk. It however raised questions about the exact pathophysiology of the metabolic consequences of insulin resistance in PCOS and a possible link between PCOS and venous thrombosis.
Further Research Questions Generated By This Work

- Are the metabolic consequences of insulin resistance in women with PCOS different from those in other forms of insulin resistance?

- Are women with PCOS at increased risk of venous thrombosis?

- Is there an increased prevalence of a positive family history of thrombosis in women with PCOS?

- Is the production of PAI-1 by granulosa cells from women with PCOS in response to insulin, gonadotrophins or steroids different from controls?
APPENDICES

Contents

Pro formas for study group 1 163
PAI-1 assay 166
Individual data for study group 1 168
Pro formas for study group 2 172
Individual data for study group 2 176
Pro formas for study group 3 180
Protocol for Immunohistochemistry 181
Reagents & Protocols Used For In-Situ Hybridisation 187
APPENDIX 1 - PROFORMAS FOR STUDY GROUP 1, PAI-1 ASSAY,

INDIVIDUAL DATA FOR STUDY GROUP 1
### PCOS PROFORMA FOR STUDY GROUP 1

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| | 0 |

#### Hirsute: A-F:

| | 0 |

#### Total Hirsute Score:

| | 0 |

#### FAI:

| | |

#### Testosterone:

| | |

#### SHBG:

| | |

#### LH (1st day in cycle):

| | |

#### FSH (1st day in cycle):

| | |

#### Estradiol:

| | DHEAS |

#### Progesterone:

| | TSH |

#### Weight (kg):

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PAI-1 assay

Materials

The reagent PAI kit came with instructions for the preparation of Tris.HAc Buffer stock solution, Acetate buffer, t-PA, PAI depleted plasma (human), S-2403, plasminogen, and stimulator. They were prepared as instructed in the manufacturers data sheet.

Standards

Standards were prepared as follows.

- To make a standard equivalent to 0 AU/ml of PAI-1, 25 μL of 40 IU/ml tPA was added to one test tube. To make 40 AU/ml, 25 μL of Tris buffer working solution was added to another test tube.

- 25 μL of PAI depleted plasma was then added to each of the two test tubes.

- Next, 4.00 ml of sterile water as added to each of the two test tubes.

- The test tubes were mixed well.

- and kept at 2 to 8°C.

Different standards concentrations were obtained by mixing these two standards according to the following:

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<th>PAI level in plasma (AU/ml)</th>
<th>40 AU/ml PAI</th>
<th>0 AU/ml PAI</th>
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A fresh set of standards was used for every test run.

The samples were prepared as follows

- 25 μL of 40 IU/ml, t-PA (20-24°C) was added to the test tube
- 25μL of the sample was added

- The tubes were mixed and incubated for exactly 10 minutes at 20-24°C

- 4.00ml of sterile water were added

- Tubes were again mixed well and kept at 2 to 8°C

We proceeded with the assay immediately after preparation of standards and samples. A uniform time span was kept for both samples and standards from preparation of dilution's to start of assay, avoiding differences exceeding 10 minutes.

Assay

1. Assay mixture

Just before the assay, cold (2 to 8°C) solutions of 1 volume of plasminogen, 1 volume of S-2403 and 3 volumes of Tris working solutions were mixed.

2. The following solutions were added in a test tube; 200μL incubated and diluted test plasma or standard (2 to 8°C), 200μL of Plasminogen/S-2403/Tris (2 to 8°C), 100μL of Stimulator, working solution (20 to 24°C). They were then mixed and incubated at 37°C for 50 minutes. At the end of this period, 20% Acetic acid was added and the solution mixed.

The contents were transferred to a microtitre plate, and the absorbence read against distilled water at 405nm. The absorbence (A) of the standards was plotted against their concentration of PAI on a linear graph paper. The PAI concentration for the corresponding A of the unknown sample was read from the standard curve.
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169
| SHBG(nmol/L) | Testosterone(nmol/L) | DHEAS(umol/L) | Estradiol(pmol/L) | LH(UL) | FSH(UL) | LH/FSH | Plas-1(%) | Plas-2(%) | A2 aP-1 (%) | A2 aP-2 (%) | PAL-1/ent | PAL-1/two |
|-------------|---------------------|---------------|------------------|-------|--------|--------|----------|----------|------------|-------------|----------|-----------|----------|
| 37          | 1.3                 | 10            | 285              | 36.4  | 23.1   | 1.576759| 84       | 100      | 87         | 94          | 69       | 69        | 1.5      |
| 35.9        | 0.9                 | 7.1           | 20               | 3.7   | 7      | 0.528571| 91       | 115      | 94         | 84          | 103      | 7         | 12       |
| 48.8        | 1.1                 | 5.6           | 134              | 5.1   | 3.8    | 1.207692| 100      | 127      | 88         | 121         | 4        | 8         |
| 61.4        | 1.3                 | 6.2           | 137              | 7.7   | 8.1    | 0.950817| 104      | 139      | 108        | 128         | 27       | 27        |
| 62.9        | 1.2                 | 6.1           | 49               | 9.1   | 7      | 0.422857| 107      | 156      | 102        | 125         | 6.5      | 7         |
| 31.8        | 0.8                 | 6.2           | 23               | 5.2   | 7      | 0.742854| 91       | 111      | 94         | 112         | 7        | 7         |
| 33.2        | 1.2                 | 8.8           | 246              | 3.2   | 3      | 1.066657| 97       | 128      | 101        | 123         | 4        | 6         |
| 27.1        | 0.3                 | 4.1           | 150              | 6.4   | 4      | 1.986917| 91       | 94       | 96         | 98          | 22       | 20.5      |
| 55.2        | 0.8                 | 7.4           | 54               | 2.3   | 3      | 0.766657| 89       | 98       | 84         | 100         | 7        | 8         |
| 49.5        | 0.3                 | 6.5           | 353              | 1.8   | 3      | 0.533333| 91       | 104      | 108        | 116         | 10       | 9         |
| 132.8       | 3.4                 | 14.8          | 145              | 4.4   | 2.9    | 1.571241| 114      | 115      | 115        | 118         | 20.5     | 24        |
| 51.3        | 1.7                 | 7.3           | 40               | 2.8   | 5.1    | 0.549022| 91       | 105      | 104        | 108         | 14       | 14.5      |
| 31.8        | 1.1                 | 5.6           | 49               | 1.1   | 3.4    | 0.323529| 104      | 114      | 100        | 102         | 15       | 16        |
| 28.3        | 2.6                 | 7.5           | 456              | 5.9   | 1.8    | 3.277778| 82       | 102      | 98         | 113         | 28       | 29        |
| 31.1        | 2                   | 10.5          | 76               | 3.4   | 4.8    | 0.273541| 80       | 96       | 102        | 110         | 30.5     | 32.5      |
| 19.5        | 0.8                 | 5.7           | 82               | 2.5   | 2      | 1.25    | 75       | 87       | 108        | 120         | 11       | 8.5       |
| 7.6         | 2.5                 | 10            | 87               | 9.2   | 5.2    | 1.789231| 131      | 165      | 90         | 111         | 37       | 37.5      |
| 75.8        | 3.5                 | 13.8          | 14               | 7.7   | 5.2    | 1.346154| 82       | 109      | 102        | 104         | 7        | 4         |
| 28.4        | 2.8                 | 11.4          | 118              | 7.1   | 5      | 1.42    | 116      | 151      | 112        | 130         | 18       | 19        |
| 38.2        | 4.7                 | 17.4          | 463              | 4.3   | 4      | 1.075   | 57       | 71       | 102        | 115         | 16.5     | 15.5      |
| 35.4        | 2.2                 | 5.3           | 47               | 11.1  | 5      | 2.22    | 89       | 132      | 92         | 117         | 2.5      | 5         |
| 25.8        | 3.4                 | 18.6          | 52               | 13.3  | 4      | 3.373   | 122      | 118      | 113        | 111         | 13       | 14        |
| 23.4        | 3.5                 | 15.3          | 97               | 9.6   | 5      | 1.92    | 95       | 101      | 110        | 105         | 38.3     | 37.5      |
APPENDIX 2 - PROFORMAS AND INDIVIDUAL PATIENT DATA FOR STUDY

GROUP 2
The Polycystic Ovary Syndrome Study Proforma

STUDY CODE

INFORMATION SHEET?

CONSENT?

NAME

DATE OF BIRTH

BIRTH WEIGHT

DATE AND TIME

-------------------

MEDICATION

ASPIRIN YES/NO

HEPARIN YES/NO

SEX STEROIDS YES/NO

FERTILITY DRUGS YES/NO

OTHER

-------------------

PARITY

DATE OF LAST PREGNANCY

NUMBER OF LIVING CHILDREN

NUMBER OF MISCARRIAGES

PREGNANCY OUTCOMES

<table>
<thead>
<tr>
<th>YEAR</th>
<th>GESTATION DELIVERED</th>
<th>OUTCOME</th>
<th>OTHER (if miscarried? pregnancy test? d&amp;c)</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
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PERIODS

MENARCHE

LAST MENSTRUAL PERIOD

AVERAGE LENGTH OF CYCLES

NUMBER OF PERIODS IN LAST YEAR

SHORTEST CYCLE IN LAST YEAR

LONGEST CYCLE IN LAST YEAR

VARIABILITY

DURATION OF ABNORMAL PERIODS

SMOKING YES/NO (If yes, how many a day?)

ALCOHOL YES/NO (If yes how many units a week?)
### FERRIMAN-GALLWEY SCORE

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<tr>
<td>C</td>
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</tr>
<tr>
<td>D</td>
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<td>E</td>
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<tr>
<td>F</td>
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</tr>
<tr>
<td>G</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
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<tr>
<td>I</td>
<td>TOTAL</td>
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### MEDICAL HISTORY

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<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>INFERTILITY</td>
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<tr>
<td>PCOS</td>
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### FAMILY HISTORY

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<tr>
<td>OTHER:</td>
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### Measurements

- **HEIGHT (cm)**
- **WEIGHT (kg)**
- **BMI**
- **WAIST CIRCUMFERENCE (cm)**
- **HIP CIRCUMFERENCE (cm)**
- **WAIST/HIP RATIO**
- **% BODY FAT**
- **BLOOD PRESSURE**: 
  - **MAC (cm)**
  - **CUFF SIZE**: Normal / Large
  - **POSITION**: Sitting/Standing/Supine
  - **KOROTKOFF PHASE**: **...**
## ULTRASOUND FINDINGS

### ABDOMINAL / TRANSVAGINAL

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<th>Left Ovary</th>
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<tr>
<td>Number Of Follicles</td>
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<td>Diameter of smallest Follicle (mm)</td>
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<td>Diameter of largest Follicle (mm)</td>
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<td>Distribution of Follicles</td>
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<td><em>x</em> <em>x</em> <em>x</em></td>
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<tr>
<td>Maximum Endometrial Thickness (mm)</td>
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### OTHER COMMENTS

........................................................................................................
........................................................................................................
........................................................................................................

### BIOCHEMISTRY

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### CONCLUSION

SUBJECT / CONTROL / EXCLUDED (give reason)

Follow Up .................................................................

Other Comments ...........................................................

### TRANSPORT

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174
The Polycystic Ovary Syndrome Study Proforma (Visit 2)

STUDY CODE

YES NO

INFORMATION SHEET?

CONSENT?

NAME

DATE OF BIRTH

LMP

DATE AND TIME

Last Meal

Last Cigarette

15 Minute Rest Period

Time Of Pre Occlusion Sampling

Time Of Post Occlusion Sampling

ASSAYS

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APPENDIX 3 - PROFORMA FOR STUDY GROUP 3, PROTOCOLS USED FOR IMMUNOHISTOCHEMISTRY
**PROFORMA FOR STUDY GROUP 3**

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<td>Op findings:</td>
<td>Hormones:</td>
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PROTOCOLS USED IN IMMUNOHISTOCHEMISTRY

I initially tried to detect PAI-1 using a polyclonal antibody, but unfortunately there was a problem with significant background staining in all the slides and it was therefore felt that it was best to switch primary antibodies to a monoclonal antibody to PAI-1 to resolve this problem which was successful.

REAGENTS

Solution for blocking endogenous peroxidase
9mls of 100-volume hydrogen peroxide in 300mls of methanol.

Trypsin Preparation (pH 7.80)
0.1g of calcium chloride was added to 0.1 g of trypsin and dissolved in 100mls of distilled water for 30 minutes. The trypsin temperature was checked 0.01% Sodium hydroxide added in drops to bring the pH of the trypsin up to 7.80. The trypsin was then transferred to a Grant water bath and allowed to warm up at 37 degrees centigrade for 30 minutes.

Primary antibody
Sheep anti-PAI-1 polyclonal antibody, - chemicon international inc AB778. Dilution’s made up as follows; 1: 100 = 10 μL of antibody + 1000 μL of Tris, 1: 1000 = 1 μL of antibody + 1000 μL of Tris & 1: 10000 = 0.5 1 μL of antibody + 5000 μL of Tris. The minimum volume of solution required for the 6 slides on which each antibody was to be tested was 900 μL.

Secondary Antibody
Rabbit anti-sheep IgG (H&L) affinity purified antibody (chemicon international inc AP147). Dilution’s made up as follows; 1: 50 = 20 μL of antibody and 1000 μL of Tris.
\[ 1: 100 = 10 \mu\text{L} \text{ of antibody and } 1000 \mu\text{L} \text{ of Tris. } 1: 200 = 5 \mu\text{L} \text{ of antibody and } 1000 \mu\text{L} \text{ of Tris.} \]

**Tertiary Antibody**

Biotinylated goat anti mouse / rabbit immunoglobulin (DAKO K0492) Prepared as follow; 10 \( \mu\text{L} / \text{mL} \) required. Total number of slides in the run was 20, which meant that 3000 \( \mu\text{L} \) of volume was required. To make up this dilution, 30 \( \mu\text{L} \) of the tertiary antibody was added to 3 mls of Tris.

**Streptavidin-biotinylated horseradish peroxidase complex (Dako K0492)**

30 \( \mu\text{L} \) of biotinylated peroxidase, 30 \( \mu\text{L} \) of steptavidin and 3000 \( \mu\text{L} \) of Tris

**DAB Solution**

Sigma tablet set - 1 tablet of DAB added to 1 tablet of urea and made up to 15 mls with distilled water. Allowed to dissolve and filtered into a small 50ml beaker.

**Bleach Solution Prepared For Disposal Of DAB Contaminated Equipment**

10 tablets of actichlor 0.5gms and tap water.

**IMMUNOHISTOCHEMISTRY USING A POLyclONAL ANTIBODY TO PAI-1**

The steptavidin biotin method was used. Inflamed appendix was used as the positive control tissue (Whawell et al 1993). The primary antibody used was sheep anti-PAI-1 polyclonal antibody, - chemicon international inc AB778. The secondary (link) antibody used was rabbit anti-sheep IgG (H&L) affinity purified antibody (chemicon international inc AP147), and the third antibody was biotinylated goat anti-mouse/ rabbit
immunoglobulin (Dako Denmark, K0492). A checker board titration was used. The dilutions of the primary antibody tested were 1:100, 1:1000 and 1: 10,000. The dilution's of the secondary antibody were 1:50, 1:100 and 1:200. One negative control slide was included. Slides were stained in pairs to determine whether pre-treatment with 10 minutes of trypsinisation or no pre-treatment was required for antigen retrieval. 19 slides in total were available for this run.

1. 4 μm sections were cut, mounted on silane-coated slides and left in a 37°C incubator overnight.
2. The slides were transferred to a 60°C incubator for a maximum of 2 hours the next morning.
3. The slides were dewaxed as follows. 2 minutes in Xylene, 2 minutes in Xylene, 2 minutes in Alcohol and 2 minutes in alcohol.
4. Endogenous peroxidase was blocked with 9mls of 100-volume hydrogen peroxide in 300mls of methanol for 10 minutes.
5. Slides were washed in running water for 5 minutes.
6. Slides requiring no pre-treatment were left in a Tris bath, while the slides for trypsinisation were transferred into a grant water bath set at 37 degrees centigrade.
7. Trypsin was prepared and buffered to a pH of 7.80.
8. Tris in slides for pre-treatment replaced with trypsin for 10 minutes.
9. After trypsinisation both set of slides (trypsinised and no pre-treatment) were drained onto a blotter, washed well in water marked with a Papain pen, and placed in Tris buffer for 2 minutes.
10. The slides were placed in a humid incubation chamber and incubated with the primary antibody (three drops of the primary antibody were added to each slide) for 30 minutes taking care to put plain Tris on the negative control.
13. After incubation, slides were drained and placed in a Tris bath. The bath was emptied and replaced with fresh Tris for 5 minutes.

14. Slides incubated in secondary antibody for 30 minutes.

15. Same as step 13

16. Slides incubated with the tertiary antibody for 30 minutes.

17. Same as step 13

18. Slides incubated with Streptavidin-biotinylated horseradish peroxidase complex for 30 minutes.

19. Same as for step 13, and placed over metal racks over sink.

20. Incubated for 10 minutes in 2-3 drops of DAB.

21. Slides drained off into bleach solution, placed in slide tray and washed well with water.

22. Stained with DAB enhancer for 2 minutes.

23. Washed well in water

24. Stained with hematoxylin for 2 minutes.

25. Hematoxylin washed off well in water.

26. Dipped in acid alcohol 10 times to remove hematoxylin.

27. Washed well in water.

28. Dehydrated in Alcohol, Alcohol, Alcohol, Xylene, Xylene, and Xylene for 1 minute each.

29. Slides Mounted and viewed.

Outcome There was a positive stain for PA1-1 at all dilution’s of the primary antibody in the endothelial cells, serosal cells and inflammatory cells in the sections pre-treated with trypsin and minimal staining in those that were not pre-treated. However there was also a significant amount of background staining in the sections.
To resolve this problem, the experiment was repeated replacing the tertiary antibody with biotinylated swine anti-rabbit immunoglobulin (Dako E0353) at a dilution of 1:200 made up as 15μL of antibody in 3000 μL of Tris. Placental tissue in addition to inflamed appendix was used as positive controls, and all the sections were trypsinised. The dilutions used for the primary and secondary antibodies were as in the first experiment.

There was still a problem with non-specific background staining after this experiment and the negative control slide had stained positive invalidating the whole batch.

To resolve these problems, I repeated the experiment using weaker dilution's of the primary antibody (1:1000, 1:10000, and 1:50000), and secondary antibody (1:200, 1:400 and 1:800). In addition, prior to incubating the sections with the primary antibody, they were incubated with 20% normal rabbit serum for ten minutes, and the primary antibody was diluted in 4% normal rabbit serum.

Unfortunately, there was still a problem with significant background staining in all the slides. It was therefore felt that it was best to switch primary antibodies to a monoclonal antibody to PAI-1 to resolve this problem.
REAGENTS USED FOR IMMUNOHISTOCHEMISTRY USING A MONOCLONAL ANTIBODY TO PAI-1

The following solutions were made up as described for use with the polyclonal antibody; Blocking solution for endogenous peroxidase, Trypsin, Biotinylated goat anti mouse/rabbit IgG, Streptavidin-biotinylated horseradish peroxidase complex, DAB Solution and Bleach Solution for Disposal Of DAB Contaminated Equipment. The volumes made up depended on the number of slides being tested.

Primary antibody Murine monoclonal antibody (American Diagnostica Inc product number 3785). Dilution’s made up as follows for testing on appendix (positive control) 1: 25 = 12 μL of antibody + 300μL of 5% normal goat serum in Tris and tween (NGS), 1:50 = 150 μL of 1: 25 antibody + 150μL of 5% NGS, 1: 100 = 150μL of 1:50 antibody + 150 μL of 5% NGS, 1: 200 = 150μL of 1:100 antibody + 150 μL of 5% NGS, 1: 400 = 150μL of 1:200 antibody + 150 μL of 5% NGS, 1: 800 = 150μL of 1:400 antibody + 150 μL of 5% NGS. The minimum volume of solution required for each slide on which each concentration of antibody was tested was 150 μL. For the ovarian sections, the dilution’s were made up as follows; 1:25 = 80μL of primary antibody and 2000μL of 5% NGS, and 1:50 = 40μL of primary antibody and 2000μL of 5% NGS.

PROCEDURE
Same as used with the polyclonal antibody to PAI-1, except that the primary antibody was incubated for 1 hour, no secondary (link) antibody was required, and the slides were incubated with 20% NGS before incubation with the primary antibody to reduce non-specific binding.
APPENDIX 4 - REAGENTS & PROTOCOLS USED FOR IN-SITU HYBRIDISATION
REAGENTS FOR IN-SITU HYBRIDISATION

**Blocking solution to block non-specific binding prior to adding antibody during ISH.**

Dissolve 3g of bovine serum albumin in 100ml of TBS and add 100 µl of Triton X. Filter through Whatman filter paper and store at 4°C until use.

**BCIP solution for detection of alkaline phosphatase**

1. Weigh 12.5 mg of 5-bromo-4-chloro-3-indolyl phosphate into a bijoux.
2. Add 500 µl of dimethyl formamide in a fume cupboard.
3. Dissolve and store in the dark at 4°C.

**Buffer 3 for the detection of alkaline phosphatase.**

Add: 10 ml 1M Tris Cl, pH 9.5, 2 ml 5M NaCl and 5 ml 1M MgCl2 to 83 ml of sterile ultrapure water.

**Dextran sulphate (50%)**

1. Dissolve 10 g dextran sulphate (Sigma D6001) in 17 ml of DEPC treated water, at 60°C, using RNase free glassware.
2. Store at 4°C.

**DEPC water**

1. Fill empty clean 2 litre bottle with ultrapure water.
2. Add 2 ml of diethyl pyrocarbonate (Sigma D.5758) in a fume cupboard.
3. Mix vigorously until DEPC globules disappear.
4. Leave to stand for at least 1 hour.
5. Autoclave.
6. Store at room temperature.

**Levamisole (1 M)**

1. Dissolve 120 mg levamisole (Sigma, L9756) with 500µL sterile ultrapure water in a bijoux.
2. Aliquot and store in 20 µL aliquots at -20°C.

**MgCl2 (1 M)**

1. Dissolve 20.33 g of MgCl2 (Sigma) into final volume of 100 ml of sterile ultrapure water.
2. Autoclave and store at room temperature.

NaCl (5 M)

1. Add 29.2 g (5 M) NaCl (Sigma) to 100 ml (final volume) DEPC treated water.
2. Autoclave and store at room temperature.

NBT solution for detection of alkaline phosphatase

1. Weigh 18.5 mg of nitroblue tetrazolium into a bijoux bottle.
2. Add 350 μl of dimethyl formamide in a fume cupboard and 150 μl of sterile water.
3. Dissolve and store in the dark at 4°C.

Phosphate buffered saline (PBS)

1. 5 tablets of PBS (Sigma) dissolved in 1 Litre of ultrapure water. 1 tablet in 200mls of water gives 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137 M sodium chloride.
2. Make up to 1 litre with further water.
3. Autoclave and store at room temperature.

PE, modified for ISH (10 X)

1. Add 6.058 g Tris (hydroxymethyl) methylamine and 1.862 g EDTA to 70 ml DEPC treated water in fume cupboard.
2. Adjust pH to 7.5 with concentrated HCl.
3. Add:
   1 g tetra sodium pyrophosphate (Sigma)
   2 g poly vinyl pyrrolidone (Sigma)
   2 g Ficoll (Sigma)
4. Dissolved.
5. Add further DEPC treated water to make final volume 100 ml.
6. Store at room temperature.

Proteinase K (1mg/ml)

1. Weigh out 5 mg proteinase k (Boehringer/P4914) into an RNase free bijoux bottle.
2. Add 5 ml ultrapure water
3. Split into 150 μl aliquots in sterile 0.5 ml eppendorfs.
4. Store -20°C.
**Salmon sperm DNA (10 mg/ml)**

1. Using forceps add 0.1 g deoxyribonucleic acid from salmon testes to a 50 ml beaker (RNase free).
2. Add DEPC treated water to the 20 ml line.
3. Add small flea and seal with nescofilm. Dissolve overnight on the spinmix at 4°C.
4. Passed through a fine bore needle several times to break up.
5. Dispense into 1 ml aliquots in eppendorfs.
6. Pierce lids with sterile needle and boil for 10 minutes to denature.
7. Cool and store at -20°C.

**SDS (10%)**

1. Add 10 g sodium dodecyl sulphate (launyl sulphate) to a 250ml jar (RNase free) in the fume cupboard.
2. Add DEPC treated water to 100 ml level.
3. Cover with nescofilm over the top and dissolve using a spinmix.
4. Store at room temperature.

**SSC (20X)**

1. Dissolve 87.7 g NaCl (analar) and 44.1 g trisodium citrate dihydrate (analar) in 400mls DEPC treated water.
2. Adjust pH to 7 with a small amount of 0.1 M HCl.
3. Add further DEPC treated water to make up to 500mls.
4. Filter, autoclave and store at room temperature.

**Tris buffered saline (TBS)**

1. Dissolve 6.06 g Tris (hydroxymethyl) methylamine (BDH) and 8.76 g NaCl in 500 ml sterile ultrapure water in the fume cupboard.
2. Adjust the pH to 7.65 with concentrated HCl.
3. Make up to a final volume of 1000 ml with sterile ultrapure water.

**Tris-HCl (1 M) (DEPC)**

1. Dissolve 12.1 g of Tris (hydroxymethyl) methylamine in 50 ml of DEPC treated water.
2. Adjust pH to 7.65 with concentrated HCl.
3. Volume made up to 100mls with DEPC treated water.
4. Autoclaved and stored at room temperature.
**In situ hybridisation using digoxigenin labelled synthetic oligonucleotides**

1. Dewax sections (4µm on silane coated slides) with xylene/graded alcohols.
2. Rinse (DEPC treated water).
3. 2x SSC, 70°C for 10 minutes.
4. Rinse (DEPC treated water).
5. Proteinase K (2-5 µg/ml in 0.05M Tris) for 1 hr at 37°C.
6. Rinse (DEPC treated water).
7. 0.4% paraformaldehyde in 1 X PBS for 20 minutes at 4°C.
8. Rinse (DEPC treated water).
9. Prehybridisation solution, 150 µl/section, 30mins at 37°C (30% formamide, 600mM NaCl, 1 X PE, 10% dextran sulphate, 150µl/ml SSDNA).

   e.g. for 4ml: 1060µl water
   
   480µl 5M NaCl
   400µl 10 X PE
   800µl 50% dextran sulphate
   60µl 10mg/ml SSDNA (boil 5 minutes, chill on ice for 5 mins)
   1200µl 100% formamide in a fume hood

10. Hybridisation solution (1ml of prehybridisation solution + 100ng of probe), 60µl /section, overnight with coverslip at 37°C (add probe to the prehybridisation solution to make it at 0.1 ng/µl).

N.B. all glassware and solutions up to this stage are treated with diethyl pyrocarbonate (sigma)-add 1ml to 1 litre of solution, leave overnight, then autoclave).

11.2 X SSC/30% formamide 37°C, 3 X 5 minutes washes.
12. Rinse (ultrapure water) x 4
13. Block at Room Temperature for 10 minutes (100ml TBS, 3g BSA (Bovine Serum Albumin), 100µl triton X).
14. Antidigoxigenin alkaline phosphatase (Boehringer Mannheim) at 1:600/1:300 in blocking solution, 30 minutes on rocker.
15. Rinse TBS x 2
16. Rinse Ultra pure water.
17. Buffer 3, 5 minutes (10ml 1M Tris Cl pH 9.5, 5ml 1M MgCl₂, 2ml 5M NaCl, 83ml UP water).
18. Substrate (5ml buffer 3, 5 µl 1M levamisole, 22 µl NBT, 16.5 µl BCIP), 200 µl/section, coverslip and leave in dark for 1-16 hrs.
19. Rinse and counterstain with hematoxylin.
20. Mount in Apathy's.
3’ end labelling of Mitochondrial ribosomal RNA oligonucleotide probes with Digoxygenin

Add the following in an eppendorf tube:
1. Sterile ultrapure water - 63 μl
2. Oligonucleotide (40ng/μl) - 2 μl
3. CoCl₂ - 10 μl
4. 5 x reaction buffer - 20 μl
5. 1mM digoxigenin-11-dUTP - 2.0 μl
6. 1mM dATP (spacer) - 3.3 μl
7. Terminal Tranferase - 4 μl
Mix and incubate at 37°C for 1 hour then separate.

3’ end labelling of PAI-1 mRNA oligonucleotide probes with Digoxygenin

Add the following in an eppendorf tube:
1. Sterile ultrapure water - 63 μl
2. Oligonucleotide (1000ng/μl) - 2 μl
3. CoCl₂ - 6 μl
4. 5 x reaction buffer - 20 μl
5. 1mM digoxigenin-11-dUTP - 2.0 μl
6. 1mM dATP (spacer) - 3.3 μl
7. Terminal Transferase - 4 μl
Mix and incubate at 37°C for 1 hour then separate.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP</td>
<td>17 Hydroxyprogesterone</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>A.2.aP</td>
<td>Alpha-two antiplasmin</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>AEC</td>
<td>Aminoethylcarbazole</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>Au</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>C</td>
<td>Cytidine</td>
</tr>
<tr>
<td>CAH</td>
<td>Congenital Adrenal Hyperplasia</td>
</tr>
<tr>
<td>CCD</td>
<td>Cathode Couple Device</td>
</tr>
<tr>
<td>CVA</td>
<td>Cerebrovascular accident</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>Cm</td>
<td>Centimetres</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylycarbolineate</td>
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<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone Sulphate</td>
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<td>Diabe</td>
<td>Diabetes</td>
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<tr>
<td>Distr</td>
<td>Distribution</td>
</tr>
<tr>
<td>DLF</td>
<td>Diameter of largest follicle</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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</tbody>
</table>
DSF Diameter of smallest follicle
DVT Deep vein thrombosis
Dx Disease
ECLT Euglobulin Clot Lysis Time
EDTA Ethylenediamine tetra-acetic acid
ELISA Enzyme Linked Immunoassay
EMBL European Molecular Biology Laboratory
ET Endometrial thickness
FAI Free Androgen Index
FG Ferriman Gallwey
FH Family history
FSH Follicle Stimulating Hormone
G Guanine
GD Gestational Diabetes
GnRH Gonadotrophin Releasing Hormone
HDL High Density Lipoprotein
HGMP-RC Human Genome Mapping Project - Resource Centre
HOMA Homeostasis Model Assessment
IDDM Insulin dependent diabetes mellitus
IGF Insulin Like Growth Factor
IgG Immunoglobulin
ISH Insitu-Hybridisation
IU International Units
Kg Kilograms
L Litre
LDL Low Density Lipoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LH</td>
<td>Lutenising Hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>Lutenising Hormone Releasing Hormone</td>
</tr>
<tr>
<td>mAB</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Mid arm circumference</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MPB</td>
<td>Male Premature Balding</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribose nucleic Acid</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometres</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p</td>
<td>Pico</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PAS</td>
<td>Plasminogen Activator System</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCO</td>
<td>Polycystic Ovary</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphate ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>Plas</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>PMH</td>
<td>Past medical history</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>r</td>
<td>Regression coefficient</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>R/L ov NO</td>
<td>Number of follicles in right or left ovary</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulin</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard citrate saline</td>
</tr>
<tr>
<td>SSDNA</td>
<td>Salmon sperm deoxyribose nucleic acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded deoxyribose nucleic acid</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Thro</td>
<td>Thrombosis</td>
</tr>
<tr>
<td>Tm</td>
<td>Melt temperature</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UP</td>
<td>Ultrapure</td>
</tr>
<tr>
<td>USS</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
</tr>
<tr>
<td>VO</td>
<td>Venous Occlusion</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
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<td>β</td>
<td>Beta</td>
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<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>σ</td>
<td>Gamma</td>
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214


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PUBLICATIONS FROM THE WORK DONE IN THIS THESIS.
The plasminogen activator system in women with polycystic ovary syndrome


Department of Obstetrics and Gynaecology and Department of Haematology, Derriford Hospital, Plymouth, United Kingdom

Objective: To evaluate the plasminogen activator system in women with polycystic ovary syndrome (PCOS).

Design: Case-control study.

Setting: A district general hospital in the United Kingdom.

Patients: Eleven women with PCOS and 12 controls.

Intervention(s): Venepunctures for assays of the plasminogen activator system.

Main Outcome Measures: Euglobulin clot lysis times, plasminogen activator inhibitor I (PAI-1) activity, fibrinogen, plasminogen, and alpha-2 antiplasmin concentrations in plasma.

Results: Women with PCOS had a significantly longer euglobulin clot lysis time (mean ± SD, 189 ± 164 minutes vs. 120 ± 110 minutes), a higher PAI-1 activity (mean ± SD, 19.7 ± 12 arbitrary units [AU] per mL vs. 10.9 ± 7.9 AU/mL), and a higher fibrinogen level (mean ± SD, 4.02 ± 0.64 g/L vs. 3.13 ± 0.6 g/L) compared to controls.

Conclusions: Women with the PCOS may have an imbalance in the plasminogen activator system that is tilted toward a reduced production of the proteolytic enzyme plasmin. Systemically, this may increase their risk of cardiovascular disease, but at cellular level in the ovaries, it may result in impaired follicular rupture and anovulation. (Fertil Steril 1998;69: 136–41. ©1998 by American Society for Reproductive Medicine.)

Key Words: Polycystic ovary syndrome, plasminogen activator inhibitor-1, plasmin, ovulation

Polycystic ovaries are the most common findings in women with anovulatory infertility (1), oligomenorrhea (2), and recurrent miscarriage (3). The origin of anovulatory infertility in these women is unknown. Until recently, attention has been focused on endocrine abnormalities and LH hypersecretion (4), abnormal follicle stimulating hormone dynamics (5), androgen effects on the follicle (6), and disturbances in intraovarian growth factors (5) have been put forward as possible mechanisms of anovulatory infertility. However, there may be a relation between abnormalities of the endocrine and coagulation systems involved in the etiology. Insulin resistance is also found in these women independent of body mass index (7), and in diabetes, insulin resistance correlates positively with plasma levels of plasminogen activator inhibitor-1 (PAI-1) (8, 9). Plasminogen activation produces plasmin, a proteolytic enzyme (10) that is important in clot lysis, ovulation (11–13), and implantation (14). Plasminogen activator inhibitor-1 is the main regulator of the plasminogen activator system. Components of the plasminogen activator system have been found in the follicular fluid (15) and granulosa cells of human ovaries (11), and plasmin has been shown to weaken the follicular wall in vitro (12). It also activates the conversion of procollagenase to collagenase (13), which is required for the breakdown of type IV collagen present in the basement membrane around the graafian follicles at ovulation (13).

It is interesting to speculate whether in the polycystic ovary syndrome, an insulin-driven overproduction of PAI-1 leads to distortions in the intraovarian plasminogen-plasmin pathway and anovulation. Before initiating this study, we identified only two studies on the plasminogen activator system in women.
with the polycystic ovary syndrome (PCOS). The first study (16) showed that PAI-1 levels were about the same in 28 women with PCOS and 56 age- and weight-matched controls. However, in the second study (17), there were high PAI-1 levels in nine obese patients with PCOS, and these levels were reduced by dietary intervention in the six patients who completed the study. This reduction in PAI-1 level was associated with the return of regular menstruation and successful pregnancy. Therefore, this study evaluates further the plasminogen activator system in women with PCOS.

MATERIALS AND METHODS

The study was carried out in 1996 at Derriford Hospital (Plymouth, United Kingdom). Institutional review board approval for the study was obtained from the local research ethics committee. The hospital records of patients suspected to have PCOS were reviewed by one of the authors (W. U. A.) and recruited into the study group if they had an ultrasound (US) report suggestive of polycystic ovaries and a history of oligomenorrhea or amenorrhea with or without a history of hirsutism. The US criteria used were the presence of 10 or more 2- to 3-mm follicles distributed around the periphery or all over the ovary associated with the presence of a bright ovarian stroma (18). Women were recruited as controls if they had regular menstrual cycles and US scans that were negative for polycystic ovaries. Women with any of the following were excluded from the study: current pregnancy, delivery or miscarriage occurring within the preceding 3 months, recent surgery (within 3 months), recent myocardial infarction, use of aspirin or heparin within 15 days of the test, sex steroid therapy, and a history of hematologic disease, malignancy, or liver disease.

**TABLE 1**

Clinical variables for patients with polycystic ovary syndrome versus controls.

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Polycystic ovary syndrome (n = 11)</th>
<th>Controls (n = 12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD (y)</td>
<td>27 ± 1.6</td>
<td>31.8 ± 7.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Mean BMI ± SD (kg/m²)</td>
<td>31 ± 3.3</td>
<td>25.9 ± 2.8</td>
<td>0.003</td>
</tr>
<tr>
<td>Median hirsutism score (range)</td>
<td>3 (1-12)</td>
<td>1 (0-6)</td>
<td>0.003</td>
</tr>
<tr>
<td>Median cycle length in weeks (range)</td>
<td>20.70 (17.3-104)</td>
<td>24 (3.7-5.0)</td>
<td>0.00007*</td>
</tr>
<tr>
<td>No. with history of subfertility (%)</td>
<td>7 (64)</td>
<td>4 (33)</td>
<td>0.22</td>
</tr>
<tr>
<td>No. of nulliparas (%)</td>
<td>7 (64)</td>
<td>7 (58)</td>
<td>1.0</td>
</tr>
<tr>
<td>No. with previous miscarriage (%)</td>
<td>2 (18)</td>
<td>3 (25)</td>
<td>1.0</td>
</tr>
<tr>
<td>No. of smokers (%)</td>
<td>4 (36)</td>
<td>7 (58)</td>
<td>1.0</td>
</tr>
<tr>
<td>No. of alcohol units per week (range)</td>
<td>2 (0-31)</td>
<td>3 (0-41)</td>
<td>0.49*</td>
</tr>
<tr>
<td>Mean systolic blood pressure ± SD (mm Hg)</td>
<td>123 ± 14</td>
<td>114 ± 15</td>
<td>0.14</td>
</tr>
<tr>
<td>Mean diastolic blood pressure ± SD (mm Hg)</td>
<td>82.7 ± 8</td>
<td>72.7 ± 9.9</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Note: BMI = body mass index.

* Determined by the Mann-Whitney U test.

Before our study, the only case-control study of the plasminogen activator system in women with PCOS had shown no difference in PAI-1 levels between women with PCOS and controls (16). Therefore, it was difficult to determine what difference in PAI-1 levels was likely to be associated with a clinical finding of oligomenorrhea and to then calculate a sample size. We arbitrarily agreed to test 10-15 women with PCOS and a similar number of controls as a pilot. Eleven women with PCOS were recruited from subfertility and gynecology clinics, and 12 controls of a similar age were recruited from among the female staff members at the hospital and the infertility clinic. Tables 1 and 2 show the clinical and hormonal profiles in subjects and controls; these profiles provide biochemical confirmation that the recruited subjects have PCOS.

All the tests were carried out between 8 A.M. and 10 A.M. because of the diurnal variation in PAI-1 (19), and participants were asked to fast overnight and refrain from smoking beginning at midnight.

On arrival at the hospital, the subjects’ weights and heights were recorded. Participants were then asked to sit upright or in a supine position for 15 minutes, during which clinical details (age and menstrual, obstetric, medical, family, and social histories) were obtained and they were given a Feinman-Gallwey chart (20) to score their perceived hirsutism.

A blood sample (18-20 mL) was then taken from an antecubital vein on the left forearm with minimal or no venous occlusion into four to five citrate tubes for assays of the plasminogen activator system (prolonged venous occlusion can stimulate the production of tissue plasminogen activator and plasminogen activator inhibitor from the endothelial cells, hence the need for minimal or no venous occlusion for standardization). Five milliliters of blood were
collected into a separate tube for hormonal assays. The blood pressure was then checked on the right arm, and the sphygmonanometer cuff was inflated midway between systolic and diastolic pressures for 10 minutes. After this test, another 18-20 mL of blood were collected from below the cuff or posterior occlusion samples.

The samples for the plasminogen activator system assays were immediately placed on ice and centrifuged within 30 minutes of collection at 2,000 × g for 15 minutes at +4°C. Hormonal assays were performed in the hospital laboratory.

Assays
The assays performed to evaluate the plasminogen activator system were euglobulin clot lysis times, fibrinogen, plasminogen, alpha-2 antiplasmin concentrations, and PAI-1 activity. The hormonal assays performed were testosterone, sex hormone-binding globulin, luteinizing hormone, follicle-stimulating hormone, E2, and DHEAS. Plasma was frozen at -70°C and tested within 3 months for PAI-1 activity, alpha-2 antiplasmin, and plasminogen concentrations. All other coagulation tests were done immediately on fresh plasma.

Euglobulin clot lysis time was determined as previously described (21). In this assay plasma is diluted and acidified, forming a precipitate (euglobulin) that contains plasminogen activator, plasminogen, and fibrin. The precipitate is dissolved, the fibrinogen clotted with thrombin, and the time for clot lysis measured. The normal time range for this assay is 90-240 minutes.

Plasminogen activator inhibitor 1 activity was tested using a commercially available chromogenic assay (Chromogenix, Malmö, Sweden). In this assay a fixed amount of tissue plasminogen activator is added to undiluted plasma, where part of it forms an inactive complex with PAI-1. In the presence of a stimulator, the residual tissue plasminogen activator activates plasminogen to plasmin. The plasmin formed is inversely proportional to the PAI-1 activity in the sample. The amount of plasmin is measured from its amidolytic activity on a chromogenic substrate. The normal range is less than 15 arbitrary units per mL (AU/mL).

Plasminogen and alpha-2 antiplasmin levels were also tested using chromogenic assays (Instrumentation Laboratory, Milan, Italy). In the plasminogen assay an excess of streptokinase is added to diluted plasma. The plasminogen present will complex with streptokinase, forming a complex with plasmin-like activity. This acts on a chromogenic substrate, releasing paranitroaniline in amounts directly proportional to the plasminogen level. The normal range for this assay is 80%-120%.

In the alpha-2 antiplasmin assay, diluted plasma samples are incubated with an excess of plasminogen. Alpha-2 antiplasmin has a powerful and rapid plasmin-inhibitory potential, and the residual plasmin is measured by its activity on a chromogenic substrate. The amount of paranitroaniline released is inversely proportional to the alpha-2 antiplasmin level. The normal range of this assay is 80%-120%. Fibrinogen concentration was tested using an automated coagulation laboratory analyzer (Instrumentation Laboratory). This is a nephelometric analysis measuring the intensity of light scattered by a plasma sample before, during, and after clot formation. The light scatter reached is proportional to the fibrin concentration, and therefore, the total clottable fibrinogen. The normal range is 2-6 g/L.

Follicle-stimulating hormone and LH concentrations were tested using the Amerlite-enhanced luminescence method (Johnson and Johnson, New Brunswick, NJ). Serum DHEAS, serum testosterone, E2, and sex hormone-binding globulin levels were tested using RIA kits (Johnson and Johnson; Medegenix, Chevrous, Belgium; Sann Diagnostics; Salluggia, Vercelli, Italy; and Farmos Diagnostics, Espoo, Finland, respectively). Free androgen index was calculated
using the following formula: (testosterone/sex hormone-binding globulin levels × 100). The interassay and intrassay coefficient of variation for all these assays were <9%. The sensitivities for testosterone, DHEAS, sex hormone-binding globulin, E₂, LH, and follicle-stimulating hormone were 0.05 mg/L, 14.7 mg/100 mL, 2.30 ng/L, 0.5 = 0.55 pg/mL, 0.2 IU/L, and 0.5 IU/L, respectively.

Statistical Analysis
Means and standard deviations were calculated for the clinical and biochemical variables and for the assays of the plasminogen activator system (Tables 1 to 3). The analysis used to compare the two groups depended on the distribution of the variables. For those variables where normal probability plots indicated a normal distribution, a Student's t-test was used to compare the means. Two other variables, E₂ and LH (IU/L), were "normalized" by the removal of an outlier. Where appropriate, r-tests took account of any differences in standard deviations between the two groups. For variables that were clearly not normally distributed according to a normal probability plot (hirsutism score, cycle length, alcohol intake, E₂, follicle-stimulating hormone levels, LH, basal euglobulin clot lysis time, postvenous occlusion euglobulin clot lysis time, and postvenous occlusion PAI-1 activity), a Mann-Whitney U test was appropriate for comparing the two groups.

Some of the measured variables had a limited range of values, and for these neither a t-test nor a Mann-Whitney test was appropriate. In these cases, Fisher's exact test was used to compare the proportions of "high" values for the two groups. A P value of <0.05 was defined as statistically significant.

RESULTS
Table 3 shows the results of assays of the plasminogen activator system. The mean basal PAI-1 activity and the mean basal fibrinogen concentrations were significantly higher in women with PCOS than in controls (P = 0.049 and 0.004, respectively). The mean prevenous occlusion euglobulin clot lysis time was longer in women with PCOS (P = 0.016).

Forty-five percent of the women with PCOS had a blunt response to the venous occlusion test as compared to 9% in the control group (P = 0.06). A blunt response to the venous occlusion test was defined as <28% reduction in the euglobulin clot lysis time. This was based on data from a previous study (22), in which a control population of 90 apparently healthy women was used to define a normal response to the venous occlusion test. A reduction in euglobulin clot lysis time of <28% was below the first percentile.

The mean PAI-1 levels were higher in the four women with PCOS who had a normal body mass index (BMI <27) compared with the eight controls who had a normal body mass index, although this difference did not reach statistical significance (mean ± SD = 18.6 ± 16 vs. 12.5 ± 9 AU/mL, P = 0.4).

DISCUSSION
Our results show that women with PCOS have a prolonged euglobulin clot lysis time and high PAI-1 activity. These findings may be explained by the greater body mass index in our PCOS group, as it influences PAI-1 levels (23). However, an article published after the completion of our study (24) showed that PAI-1 levels were elevated in 24 nonobese women with PCOS and extreme menstrual disturbance compared with 10 matched controls with regular menstrual cycles. We found a similar trend in the nonobese women in our study, but because of the small numbers, the difference was not statistically significant.
Currently, we are addressing this issue in an ongoing larger study.

The significance of our findings is unknown at the present time, but it is interesting to speculate whether a disturbed intraovarian plasminogen–plasmin pathway plays a significant role in anovulatory infertility in women with PCOS. An increased systemic level of PAI-1 does not necessarily imply high local ovarian levels and we know of no correlation between follicular fluid and plasma PAI-1 levels. We are now evaluating expression of PAI-1 mRNA and protein in ovarian biopsies from women with PCOS.

Another speculative implication of our findings is that women with PCOS may be at increased risk of thromboembolic disease. This is because elevated PAI-1 levels impair fibrinolysis by limiting plasmin formation, and impaired fibrinolysis is a significant risk factor in the development of fatal ischemic heart disease in women (25). Although there is no direct evidence of an increased risk of thromboembolic disease in these women, indirect evidence suggests that this may be the case, as women with clinical signs of androgen excess often have abnormal coronary angiograms (26). More studies should be performed to determine whether women with PCOS are indeed at increased risk of ischemic heart disease.

High PAI-1 levels are one mechanism that may explain the previously unexplained link between the finding of polycystic ovaries and recurrent miscarriage, but this is entirely speculative. The finding of high PAI-1 levels in many women with recurrent miscarriage (22, 27) and the important role played by plasmin in animal implantation (14) justify further studies to evaluate the link between abnormalities of the plasminogen activator system and the outcome of pregnancies in women with polycystic ovaries.

To date, purely endocrine explanations of the pathophysiology behind this syndrome remains controversial and unproved. For example, hypersecretion of LH has been suggested as a cause (4), but a recent attempt at lowering LH levels in women with recurrent miscarriage did not improve the pregnancy rates (28). Our study suggests an alternative explanation, with the balance in the plasminogen activator system in women with PCOS probably tilted toward a reduced production of the proteolytic enzyme plasmin. Systemically it may increase their risk of cardiovascular disease, but at the cellular level, it may result in impaired follicular rupture, anovulation, failed implantation, and miscarriage. More research is required to test these two hypotheses, which may lead to trials of interventions that encourage plasm formation to improve the reproductive outcome in these women.

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27. Patrasso CM, Smiti MT, Raffatti A, Vier M, Di Leonardo L, Caz-

“So do not worry, saying, “What shall we eat?” or “What shall we wear?” For the pagans run after all these things, and your heavenly Father knows that you need them. But seek first his kingdom and his righteousness, and all these things will be given to you as well. Therefore do not worry about tomorrow, for tomorrow will worry about itself. Each day has enough trouble of its own.”

- Matthew 6:31-34.
I would like to dedicate this work to AC, Lizzy & "Moskito".
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