The Haemostatic System in Polycystic Ovarian Syndrome (PCOS)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Doctor of Philosophy with Publication Student Declaration

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Genia Burchall                                        27/02/2018
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### Abbreviations

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<thead>
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<tbody>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AES</td>
<td>Androgen Excess Society</td>
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<td>APC</td>
<td>Activated protein C</td>
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<td>ASRM</td>
<td>American Society of Reproductive Medicine</td>
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<td>AT</td>
<td>Antithrombin</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>b.d.</td>
<td>Bi-daily</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CAT</td>
<td>Calibrated Automated Thrombogram</td>
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<tr>
<td>sCD40L</td>
<td>Soluble cluster of designation 40 ligand</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>COC</td>
<td>Combined oral contraceptives</td>
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<td>CV</td>
<td>Coefficient of variation</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CV</td>
<td>Cardiovascular</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone sulfate</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<td>DD</td>
<td>D-dimer</td>
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<td>EE</td>
<td>Ethinyl estradiol</td>
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<td>ESHRE</td>
<td>European Society of Human Reproduction and Embryology</td>
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<tr>
<td>FF</td>
<td>Follicular fluid</td>
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<td>FSH</td>
<td>Follicular-stimulating hormone</td>
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<tr>
<td>FVIIa</td>
<td>Activated factor VII</td>
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<tr>
<td>GC</td>
<td>Granulosa cells</td>
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<td>GFC</td>
<td>Global fibrinolytic capacity</td>
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<td>GnRH</td>
<td>Gonadotropic releasing hormone</td>
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<td>GP</td>
<td>Glycoprotein</td>
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<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>HOMA</td>
<td>Homeostasis model assessment</td>
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<td>Acronym</td>
<td>Term</td>
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<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
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<tr>
<td>IR</td>
<td>Insulin resistance</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<td>MI</td>
<td>Myocardial infarct</td>
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<td>MOU</td>
<td>Memorandum of Understanding</td>
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<td>MPV</td>
<td>Mean platelet volume</td>
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<td>NIH</td>
<td>National Institute of Health</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>OCP/OCPs</td>
<td>Oral contraceptive pill/s</td>
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<td>OCP-S</td>
<td>OCP+spironolactone</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>PA</td>
<td>Plasminogen activators</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
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<td>PCO</td>
<td>Polycystic ovary/ies</td>
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<td>Term</td>
<td>Definition</td>
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<td>PCOS</td>
<td>Polycystic ovarian/ovary syndrome</td>
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<tr>
<td>PF1 &amp; 2</td>
<td>Prothrombin fragments 1 and 2</td>
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<tr>
<td>PROTEIN C/S</td>
<td>Activated protein C and protein S complex</td>
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<td>PRP</td>
<td>Platelet rich plasma</td>
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<td>RCT</td>
<td>Randomised controlled trial</td>
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<td>ROI</td>
<td>Region of interest</td>
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<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
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<tr>
<td>TC</td>
<td>Theca cells</td>
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<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>TG</td>
<td>Thrombin generation</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>TTM</td>
<td>Thrombin-thrombomodulin complex</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>TXM</td>
<td>Thromboxane metabolite</td>
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<tr>
<td>T2D</td>
<td>Type II Diabetes</td>
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<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
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<td>Description</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<td>VTE</td>
<td>Venous thromboembolism</td>
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<tr>
<td>vWf</td>
<td>Von Willebrand factor</td>
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ABSTRACT

Polycystic ovary syndrome (PCOS), diagnosed based on hyperandrogenism, ovulatory dysfunction and polycystic ovaries, is one of the most common disorders of reproductive aged females affecting up to 18% of women in this age group. The aetiology of PCOS is still unknown but includes both genetic and environmental/lifestyle factors contributing to both insulin resistance and hyperandrogenism. Clinically PCOS has reproductive, psychological and metabolic features, the latter predisposing to cardiovascular disease (CVD). Haemostatic abnormalities have an association with and a demonstrated pathophysiological role in CVD in non-PCOS populations, yet have not been adequately explored in PCOS.

Women with PCOS appear to have altered coagulation and fibrinolysis with a prothrombotic state, with epidemiological evidence of increased venous thromboembolism. In an established case-control cohort of lean, overweight and obese women with (n=107) and without (n=67) PCOS, and existing measures of plasminogen activator inhibitor 1 (PAI-1) and asymmetric dimethylarginine (ADMA), other haemostatic markers were measured in plasma samples including prothrombin fragments 1 and 2 (PF1 & 2), plasminogen, tissue plasminogen activator (tPA) and thrombin generation (TG). Higher levels of ADMA (0.70 vs 0.39 μmol/L, p<0.01), PAI-1 (4.80 vs 3.66 U/mL, p<0.01) and plasminogen (118.39 vs 108.46%, p<0.01) were seen in women with PCOS versus controls, which persisted after adjustment for age and body mass index (BMI). PF1 & 2 was marginally lower (180.0 vs 236.0 pmol/L, p=0.05), while tPA and TG were not different between groups, following adjustment for age and BMI. Significant relationships were observed between hormonal and metabolic factors with ADMA and PAI-1. Impaired fibrinolysis was demonstrated in PCOS. In the context of abnormal endothelial
function, known hormonal and metabolic abnormalities, outcomes of this study suggest there is an increased risk of cardiovascular disease and venous thrombosis in women with PCOS.

Women who have PCOS have an increased risk of cardiovascular and venous thromboembolic disease (VTE) related to metabolic and hormonal features, obesity and a hypofibrinolytic state, and current PCOS treatments may possibly exacerbate these risks. The haemostatic impacts of common pharmacological treatments administered to women with the syndrome was investigated, and involved a mechanistic sub-study using biobanked samples from a six month randomised comparative trial of pharmacological treatments. Pro- and anti-thrombotic markers and overall haemostatic activity were measured. Overweight women (mean BMI of 36.5±7.0 kg/m²) of mean age 33.9±6.7 years with PCOS (n=60) were randomised to either: (1) metformin, (2) higher-dose oral contraceptive pill (OCP) or (3) low-dose OCP+spironolactone (OCP+S). The main outcome measures included changes over the six month investigation period in PAI-1, ADMA, PF1 & 2, plasminogen, tPA, thrombin activatable fibrinolysis inhibitor (TAFI) and TG as well as relevant hormonal and metabolic markers. PAI-1 activity fell in all groups (p<0.015), ADMA decreased with higher-dose OCP (p=0.0125), PF1 & 2 increased with metformin and higher-dose OCP (p<0.044), TG increased (p<0.009) and tPA decreased in both OCP groups (p<0.013), plasminogen increased in all (p<0.038) and TAFI increased after higher-dose OCP (p=0.0004). Endothelial function (marker of the primary haemostatic response) improved with higher-dose with some improvement with low-dose OCP+S and metformin. Both OCPs however increased coagulation and induced a hypofibrinolytic state in the higher-dose form, with a subsequent increased risk of thrombosis. Metformin did not show (net) negative effects on overall coagulation. The results suggest an additional dimension of treatment (haemostatic system effects) that favours metformin over that of OCPs in PCOS.
The fibrinolytic system and relevant inhibitors play a number of roles, apart from their function in blood haemostasis and thrombosis, namely in ovulation processes. Plasminogen is converted to plasmin at the time of follicular rupture through a decrease in PAI-1 and an increase in plasminogen activators. Only active plasmin is involved in follicular wall breakdown. PCOS is the leading cause of anovulatory infertility with oligo-/anovulation and ovarian follicle arrest as key characteristics. The presence and distribution of fibrinolytic/proteolytic markers plasminogen, plasminogen/plasmin, tPA and uPA and inhibitor PAI-1 in the ovaries of control and PCOS mice were examined using an existing PCOS mouse model with dihydrotestosterone treated mice that display extensive ovarian, endocrine and metabolic features of humans affected by the syndrome. The expression of PAI-1, tPA, uPA, plasmin/plasminogen and plasminogen on six PCOS and six control ovaries were examined by immunohistochemistry using the appropriate antibodies and quantitative comparisons using digital image analysis were completed. There was a difference in the ovarian distribution of PAI-1 that was localised throughout the PCOS ovary unlike a peripheral distribution seen in control ovaries and plasminogen that was observed in small follicles only in PCOS and not in small follicles of control ovaries. While no differences were noted in the overall expression (mean total percentage and mean colour intensity) of ovarian staining of markers assessed, these findings showed a potential role for the plasminogen system in both the physiological mouse ovary and in the pathological PCOS state. Further studies evaluating these markers at different time-points of ovulation will help to further clarify both physiological and potential pathological roles these markers play in ovulation processes distorted in PCOS.

Collectively these related studies enhance our understanding of the haemostatic and fibrinolytic/proteolytic systems in women with PCOS. As a hypofibrinolytic state was
demonstrated in the first study, this serves as a potential further CVD and VTE risk factor in a group of women already at high-risk. Additionally, use of the commonly prescribed OCPs in the management of women with PCOS has also demonstrated potential further risk factor/s for thrombosis following observations of increased coagulation and a hypofibrinolytic state with use of the higher-dose form. Metformin however did not show any net negative coagulation outcomes and was noted to be the treatment of choice over the use of both OCPs in the management of women with this syndrome. While at a systemic level a hypofibrinolytic state may increase venous thrombotic risks, the third study showed that at a local ovarian level fibrinolytic system changes are also observed in the PCOS ovary in comparison to normal ovaries and may be associated with the aberrant ovulation frequently noted in women with PCOS. Overall the results from these studies will enhance our understanding of PCOS, help to better inform clinical practice as well as assist with development of improved treatment options.
Declaration of Co-Authorship and Co-contribution: Papers incorporated in
Thesis with publications

This declaration was completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Genia Burchall  Signature:  Date: 10/02/2018

Paper Title: Hemostatic Abnormalities and Relationships to Metabolic and Hormonal Status in Polycystic Ovarian Syndrome. \textit{(Note that both Chapters 1 and 2 include the aforementioned published paper)}

In the case of the above publication, the following authors contributed to the work as follows:

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<td>Genia Burchall</td>
<td>70%</td>
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<td>5%</td>
<td>Undertook revision for important content, constructed one of the figures and approved the final version for publication.</td>
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<tr>
<td>Helena J. Teede</td>
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<tr>
<td>Terrence J. Piva</td>
<td>15%</td>
<td>Assisted with reviewing articles, undertook critical revision for important content and approved the final version for publication.</td>
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DECLARATION BY CO-AUTHORS/SENIOR SUPERVISOR

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to

a) granting bodies,

b) the editor or publisher of journals or other publications, and

c) the head of the responsible academic unit; and

5. The original data is stored at the following location(s):

Location(s): School of Health and Biomedical Sciences, RMIT University and will be held for at least five years from the date indicated below.

Date:

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CHAPTER 1: General Overview of PCOS

1.1 Preface

The present chapter provides an overview of the background of the thesis where the pathogenesis of the syndrome, clinical presentations, diagnosis and current treatments including gaps and controversies in our knowledge, with emphasis on links to cardiovascular disease (CVD) and cardiovascular (CV) risk markers frequently present in women with PCOS is discussed. I discuss recently published data that suggests an additional clinical complication of elevated CV risk in PCOS, potentially relating to disturbed haemostasis. In this context, the normal haemostatic system and its functions and the role/s this may play as a potential further CVD risk marker are also discussed. Additionally, as PCOS is a primary cause of anovulatory infertility in reproductive-age women, the role of specific haemostatic markers (fibrinolytic system and relevant inhibitors) in the ovulation process as well as the links these have to fertility are introduced. The critical need for an in-depth review of the literature into the haemostatic system in women with PCOS is ultimately identified.

This chapter has been published in Trends in Cardiovascular Medicine (refer to the end of Chapter 2 for the original paper). Additional information has been added here in this chapter (as well as in Chapter 2) as is relevant to this thesis and to provide more comprehensive and up-to-date information since this original paper was published.
1.2 Introduction and definition of PCOS

Polycystic ovarian syndrome is a condition diagnosed based on anovulation and irregular menstrual cycles, hyperandrogenism and polycystic ultrasound appearance of ovaries (Teede, Misso et al. 2011, Sirmans and Pate 2013). PCOS also presents with hypertension and metabolic abnormalities, including glucose intolerance, insulin resistance (IR), hyperinsulinaemia, and dyslipidaemia, and increased risks for diabetes and CVD (Teede, Misso et al. 2011, Sirmans and Pate 2013). The exact cause/s of PCOS remains to be elucidated. It is a complex disease believed to be similar to diabetes mellitus, rheumatoid arthritis, and CVD; a combination of genetic predisposition and environmental factors contribute to the phenotypic expression of this heterogeneous disorder (Teede, Deeks et al. 2010). Occurrence of PCOS within families and following high prevalence of the syndrome amongst first-degree relatives supports the genetic link to the disorder (Sirmans and Pate 2013). Early research reported on a genetic link in PCOS with genes involved in insulin action (glucose metabolism), weight and energy regulation, gonadotropin secretion and function and androgen secretion and function (Table 1.1). More recent large scale genome wide association studies, reported in a review by Azziz, implicated genes involved in neuroendocrine and ovarian androgen synthesis, in addition to genes involved in modulation of gonadotropin and insulin function in women with the syndrome (Azziz 2016). PCOS is one of the most common disorders of females of reproductive age and is the most common endocrine disorder of females in this age group around the world (March, Moore et al. 2010, Goodman, Cobin et al. 2015), affecting up to 20% of women (March, Moore et al. 2010). It should be noted that the distribution of the disease is not restricted to developed nations nor to Caucasian populations (Azziz 2017). Ethnicity as well as environmental contributions including obesity modify prevalence rates and severity of the condition. In Australian Indigenous women the incidence is as high as 21% (Davis, Knight et al. 2002). Furthermore the diagnostic criteria used also impacts reported prevalence rates.
(Sirmans and Pate 2013). Use of the Rotterdam criteria has noted prevalence rates two to three times greater than use of the National Institute of Health (NIH) criteria (Sirmans and Pate 2013), though the differences noted may be related to the sensitivity of the testing methods used to diagnose the syndrome (Azziz, Carmina et al. 2016). Systematic review evidence shows more a limited impact of diagnostic criteria on prevalence (Original NIH criteria: 6% and Rotterdam criteria: 10%) (Bozdag, Mumusoglu et al. 2016).

Given its prevalence and significant clinical complications, PCOS presents a major burden to its sufferers and the health care system (Teede, Misso et al. 2011). In 2005, based on US data, PCOS was estimated to cost the Australian health care system approximately $400 million per year (Azziz, Marin et al. 2005). Given the underestimated prevalence from this initial evaluation (6%), the actual financial burden is estimated to be significantly higher (Azziz, Marin et al. 2005, March, Moore et al. 2010).
### Table 1.1 Genes considered to play a role in PCOS.

<table>
<thead>
<tr>
<th>Physiological process</th>
<th>Genes that may be affected in PCOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonadotropin secretion</td>
<td>activin receptor 1, activin receptor 2A, activin receptor 2B, follicle-stimulating hormone receptor, follistatin, inhibin subunit A, inhibin subunit βA, inhibin subunit βB, inhibin subunit C, luteinizing hormone β subunit, luteinizing hormone/choriogonadotropin receptor, Müllerian inhibiting substance, sex hormone-binding globulin, mothers against DPP homolog 4</td>
</tr>
<tr>
<td>Androgen production &amp; secretion</td>
<td>androgen receptor, bone morphogenic protein, growth differentiation factor 9, cytochrome P450, family 11, subfamily A polypeptides, cytochrome P450, family 17, subfamily A, polypeptide 1, cytochrome P450, family 19, subfamily A, polypeptide 1, hydroxyl-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 1, hydroxyl-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 2, hydroxysteroid (17-β)-dehydrogenase 1, hydroxysteroid (17β)-dehydrogenase 2, hydroxysteroid (17β)-dehydrogenase 3, steroidogenic acute regulator</td>
</tr>
<tr>
<td>Insulin secretion and obesity</td>
<td>adiponectin, calpain 10, insulin gene variable number of tandem repeats, insulin receptor, insulin receptor substrate 1, insulin receptor substrate 2, insulin-like growth factor 1, insulin-like growth factor 2, insulin-like growth factor 1 receptor, insulin-like growth factor 2 receptor, insulin-like growth factor binding protein 1 plus insulin-like growth factor binding protein 3, peroxisome proliferatoractivated receptor γ, Leydig insulin-like protein, leptin receptor, leptin, melanocortin-4 receptor, tumor necrosis factor, uncoupling protein 2, uncoupling protein 3, propiomelanocortin</td>
</tr>
</tbody>
</table>

Adapted from (Urbanek 2007).
1.3 Pathogenesis

1.3.1 Hyperandrogenism

PCOS has a complex pathogenesis with features that ultimately feedback in a chain reaction-like manner to further aggravate clinical observations. Hyperandrogenism, the predominant feature of the syndrome, is primarily of ovarian origin (~60% of cases) (Azziz 2017) with some contribution from the adrenal gland (~40% of cases) (Luque-Ramirez and Escobar-Morreale 2015, Krishnan and Muthusami 2017). Increased luteinizing hormone (LH) activity from the pituitary gland and increased LH/FSH levels as well as increased LH response to theca cells in polycystic ovaries has been observed in PCOS and is likely involved in the pathogenesis of the hormonal imbalance of the disorder (Krishnan and Muthusami 2017).

1.3.2 Insulin Resistance and Hyperinsulinaemia

IR, which occurs independently of obesity, is observed in 75% of lean and 95% of obese PCOS females (Stepto, Cassar et al. 2013, Teede and Shorakae 2017) and contributes to the pathophysiology of this syndrome (Sirmans and Pate 2013, Azziz 2017). Insulin stimulates secretion of androgens by the ovarian theca cells (Krishnan and Muthusami 2017). In PCOS, these cells appear to be hyper-responsive to insulin in secreting androgens (Azziz 2017). It was previously thought that hyperandrogenism may act on vascular tissues to induce an IR state (Carmassi, De Negri et al. 2005), however more recent literature is focusing on androgen action via the adipocyte tissues as a potential link to IR (Keller, Chazenbalk et al. 2014). IR has also been linked with an abnormal lipid profile in PCOS compared to more insulin-sensitive controls. This is probably due to excessive hypersecretion of apolipoprotein B and very low-density lipoprotein (VLDL) from the liver following insulin stimulation, ultimately resulting in hypertriglyceridaemia (Alexander, Tangchitnob et al. 2009). Low levels of high-density
lipoprotein (HDL) have also been reported in PCOS, as have high levels of low-density lipoproteins (LDL) and triglycerides (Berneis, Rizzo et al. 2007, Wild, Rizzo et al. 2011, Sirmans and Pate 2013). Insulin-sensitizing agents such as metformin and lifestyle interventions that reduce IR have been shown to ameliorate the clinical findings of PCOS (Teede, Misso et al. 2011). Additionally insulin sensitisers can reduce dehydroepiandrosterone sulfate (DHEAS) androgen levels in some but not all women with PCOS (Luque-Ramirez and Escobar-Morreale 2015). In contrast, obesity, which increases IR, exacerbates PCOS (Teede, Deeks et al. 2010, Sirmans and Pate 2013). IR and compensatory hyperinsulinaemia however are insufficient for development of the disorder (Azziz 2017) and the presence of the syndrome in insulin-sensitive PCOS females may indicate further/other causative factor/s or an underlying aetiology in the pathogenesis of the syndrome.

1.3.3 Disturbed Oestrous Cycle and Polycystic Ovaries (PCO)

In healthy women not affected by the syndrome, the menstrual cycle is initiated by the pituitary gland, following stimulation by the hypothalamic gonadotropic releasing hormone (GnRH), secreting LH. This in turn stimulates the ovarian theca cells to increase their production of androgens (testosterone, androstenedione and DHEAS). Pituitary gland secretion of FSH, also following stimulation by GnRH, ensures aromatization of the androgens to oestrogens by the ovarian granulosa cells (Azziz 2017). LH production also induces the maturation and growth of the ovarian follicles (composed of theca and granulosa cells and enveloped in an outer capsule). The follicles, in the early stage of the oestrous cycle, start as small primordial follicles, then grow in size to develop into primary and then secondary follicles and finally form the mature or Graafian follicles. An LH surge mid-cycle assists in the ovulation process by arrest and rupture of the mature ovarian follicles with subsequent release of the oocyte (Figures 1.1 and 1.2).
Figure 1.1 The physiological ovulation process (taken from https://www.ovulationcalculator.com/img/uploads/2016/01/ovulation-hormones.png Last Accessed 21/11/2017).
The normal ovary is a small, walnut sized tissue that is of smooth appearance in girls prior to menstruation. As a woman begins to ovulate, the ovary will display a number of protrusions on the surface that will vary in size depending on the stages of the menstrual cycle (Figure 1.3). These are formed as a result of follicular maturation and once a size of 10-12mm in diameter is reached in the mature follicle, follicular wall rupture takes place, which is then replaced by the corpus luteum (Figure 1.2). Other developing follicles in that cycle then undergo atresia. Progesterone is then produced by the corpus luteum however if pregnancy is not achieved these levels fall, menstruation occurs and a new cycle commences.

In women affected by PCOS the normal menstrual cycle is disturbed. Relatively elevated LH levels induce excessive androgen production by the ovarian theca cells as well as a failed
The ovulation process following a lack of full ovarian follicular maturation and rupture. Arrested follicles in various stages of maturation (atresia), ranging from small or pre-antral follicles to more mature or secondary (antral) follicles are noted in women with PCOS due to excessive LH stimulation (elevated LH level as well as pulse frequency) (Krishnan and Muthusami 2017). The ovary then appears enlarged (up to three times the size of a normal ovary) with multiple (>10–12) underdeveloped follicles (~2–9 mm in diameter) on the surface and a thickened capsule (Figure 1.3). Therefore the inclusion of ‘polycystic’ within the name of the syndrome is an inaccurate representation of its features; these are not cysts but rather underdeveloped follicles.

![Figure 1.3 Polycystic (left) versus normal (right) ovary.](image)

1.3.4 Obesity

Women affected by PCOS are more likely to be overweight, obese and display central obesity than those who are not, and the incidence and severity of this syndrome increases with increased weight (Teede, Misso et al. 2011, Lim, Davies et al. 2012, Behboudi-Gandevani, Ramezani Tehrani et al. 2017). Obesity exacerbates both IR and hyperandrogenism.
Additionally central obesity is a risk factor for development of IR as well as a main component of the metabolic syndrome (Behboudi-Gandevani, Ramezani Tehrani et al. 2017). Although some studies have found abnormal fasting insulin levels and IR only in obese PCOS females (Rajendran, Willoughby et al. 2009), most studies have also observed these abnormal metabolic profiles in lean counterparts (Dereli, Ozgen et al. 2003, Ozgurtas, Oktenli et al. 2008). Two-thirds of studied non-obese PCOS females are reported to have excessive body fat and central visceral adiposity inducing greater IR (Cascella, Palomba et al. 2008). Excess adipose tissue also ensures increased extraglandular aromatization of androgens into oestrogens resulting in a feedback loop on the hypothalamus which then increases LH and subsequent androgen production. It is not clear as yet whether excessive weight gain is an aetiological factor or symptom of the syndrome however it appears to be bidirectional (Teede, Joham et al. 2013).

1.4 Clinical and Diagnostic Features

Clinical manifestations of PCOS are highly variable and may include reproductive, metabolic, as well as psychological features. When fully expressed, features of PCOS may include hirsutism, acne and/or alopecia, menstrual irregularities, sub-fertility and pregnancy complications including miscarriage and gestational diabetes, obesity, increased risks of Type 2 diabetes (4-7 fold) and potentially cardiovascular disease (Rosenfield 2009); increased psychological features including depression and anxiety (Lobo, Granger et al. 1983, Deeks, Gibson-Helm et al. 2010) and increased risks of various cancer types such as endometrial carcinoma. PCOS symptoms however can be restricted to only a few of these features, making both diagnosis and treatment a significant challenge (Teede and Shorakae 2017).
Common reproductive abnormalities include oligo or amenorrhoea and infertility. PCOS women that are successful in conceiving are then faced with increased risks of pregnancy associated complications including pre-eclampsia, gestational diabetes and early delivery (Teede and Shorakae 2017).

While reproductive abnormalities predominate in younger women with the syndrome, metabolic features have also been noted as key characteristic and are independent of weight, though a high body mass index (BMI) will increase these risks (Teede and Shorakae 2017). The incidence of metabolic syndrome is also increased in women with PCOS (Sirmans and Pate 2013). Other metabolic aberrations include glucose intolerance and increased risks of pre-diabetes, gestational diabetes and type 2 diabetes, dyslipidaemia and obstructive sleep apnoea (Teede and Shorakae 2017), many of which predispose to and are well known risk factors for CVD.

Psychological abnormalities include depression, anxiety, eating disorders, psychosexual dysfunction and reduced self-esteem, that can be related to but may also occur independent of obesity and reduce the quality of life of women with the syndrome (Teede and Shorakae 2017).

A summary of the aetiology and clinical features of PCOS is represented in Figure 1.4 (Teede, Misso et al. 2011, Goodman, Cobin et al. 2015, Teede and Shorakae 2017).
The initial diagnostic criteria from 1990 by the NIH incorporated the presence of polycystic ovaries as a main diagnostic component in conjunction with hyperandrogenism, made following clinical (hirsutism or acne) or biochemical (elevated androgen levels, mainly testosterone) evaluation and abnormal ovulation, demonstrated through oligo or anovulation (6-9 or fewer menstrual cycles per year or abnormal bleeding) following exclusion of other causes of hyperandrogenism or menstrual irregularities (such as congenital adrenal hyperplasia, androgen secreting tumours and hyperprolactaemia) (Zawadski and Dunaif 1992). In 2003, the diagnostic criteria was revised by the European Society of Human Reproduction and Embryology (ESHRE) in conjunction with the American Society of Reproductive Medicine.
The Haemostatic System in PCOS

(ASRM) or the so called “Rotterdam criteria” that expanded to include ultrasound features of PCOS (Rotterdam 2004). Diagnosis then required two of three core clinical manifestations: hyperandrogenism, anovulation and PCO on ultrasound, in addition to exclusion of other related disorders (Table 1.2).

Table 1.2 Various diagnostic criteria used for defining PCOS (Zawadski and Dunaif 1992, Rotterdam 2004, Azziz, Carmina et al. 1996).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 1990</td>
<td>To include all of the following and exclude related disorders:</td>
</tr>
<tr>
<td></td>
<td>- Clinical hyperandrogenism and/or hyperandrogenaemia</td>
</tr>
<tr>
<td></td>
<td>- Chronic anovulation</td>
</tr>
<tr>
<td></td>
<td>- Polycystic ovaries</td>
</tr>
<tr>
<td>ESHRE/ASRM (Rotterdam) 2003</td>
<td>To include two of the following, in addition to exclusion of related disorders:</td>
</tr>
<tr>
<td></td>
<td>- Oligo-anovulation</td>
</tr>
<tr>
<td></td>
<td>- Hyperandrogenism and/or hyperandrogenaemia</td>
</tr>
<tr>
<td></td>
<td>- Polycystic ovaries</td>
</tr>
<tr>
<td>AES 2006</td>
<td>To include all of the following:</td>
</tr>
<tr>
<td></td>
<td>- Hyperandrogenism (hirsutism and/or hyperandrogenaemia)</td>
</tr>
<tr>
<td></td>
<td>- Ovarian dysfunction (oligo-anovulation and/or polycystic ovaries)</td>
</tr>
<tr>
<td></td>
<td>- Exclusion of related disorders</td>
</tr>
</tbody>
</table>

Both the original NIH as well as the Rotterdam criteria however received criticism. The diagnosis of hyperandrogenism could be made both clinically as well as biochemically. The clinical manifestation of hyperandrogenism can vary significantly between individuals and therefore some argued that only laboratory evaluation of hyperandrogenism should have been used (Rosenfield 2009, Barbieri and Ehrmann 2017). The introduction of two out of the three clinical symptoms in the Rotterdam criteria made by ESHRE/ASRM also meant that women presenting with polycystic ovaries and abnormal ovulation would be diagnosed as having PCOS, despite no hyperandrogenism. Women could also have PCOS and be asymptomatic with PCO on ultrasound and having biochemical hyperandrogenism, although this scenario was uncommon as women are not presenting with a clinical problem. These were then argued as
inappropriate inclusions and it was contested that hyperandrogenism must be present in order for diagnosis to be made (Rosenfield 2009). One of the recommendations why non-hirsute women with PCO and normal circulating androgens should still be considered as having PCOS is due to the non-accurate commercial kits for determining androgen levels (Rosner 2001, Goodman, Cobin et al. 2015). These diagnostic shortcomings then made way for the Androgen Excess Society (AES) to introduce a further diagnostic criterion in 2006. These suggested that PCOS is a condition presenting with mandatory androgen excess (clinical and/or biochemical) plus one of ovarian dysfunction (oligo-anovulation and/or polycystic ovarian morphology) and exclusion of other androgen excess or ovulatory disorders (Azziz, Carmina et al. 2006). While the name Polycystic Ovarian Syndrome persists, this clinical feature is not mandatory for diagnosis in the AES criteria (Azziz, Carmina et al. 2006). The AES recommendation may also fall short, in that it too accepts the inclusion of clinical not just biochemical diagnosis of the syndrome. Furthermore, due to the high prevalence of the symptoms in PCOS, it is likely that obesity and IR should also be incorporated when diagnosis is made.

At present up to 70% of women with PCOS remain undiagnosed due to the heterogeneity of the syndrome (Teede and Shorakae 2017). In 2012 an Expert Panel from a NIH Evidence Based Methodology Workshop on PCOS, reinforced the use of the wider Rotterdam criteria (NIH Disease Prevention Evidence-based Methodology Workshop on PCOS 2012). This criteria, based on the presence of only two of the following three clinical features: hyperandrogenism (clinical or biochemical), chronic anovulation and polycystic ovaries on ultrasound remains the most widely accepted and used diagnostic criteria by clinicians internationally.
1.5 Treatment

The current management strategies use to ameliorate the symptoms of PCOS are highly variable, in line with the diverse range of clinical features of the syndrome (Table 1.3) (Teede, Misso et al. 2011, Goodman, Cobin et al. 2015). Conventionally most treatments have targeted only the symptoms but not focused on prevention of long-term complications and have not recognised the important role of lifestyle change, metabolic and psychosocial impacts of PCOS. At present women with PCOS display reduced satisfaction with the care and management provided by health professionals and are often diagnosed late (Teede and Shorakae 2017). Current approaches should comprise an initial thorough evaluation of each woman affected by the syndrome as well as incorporate a multidisciplinary approach to treatment to ensure the most appropriate management strategy is tailored. These should also seek to address all features of the syndrome including reproductive but also metabolic and psychological characteristics and focus on prevention of long-term complications including the increased risks of CVD (Teede and Shorakae 2017).

Table 1.3 Management therapies used to treat women with PCOS (Teede, Misso et al. 2011, Teede and Shorakae 2017).

<table>
<thead>
<tr>
<th>Clinical feature/complication</th>
<th>Management strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomenorrhoea/amenorrhoea</td>
<td>Lifestyle change (5%–10% weight loss + structured exercise)</td>
</tr>
<tr>
<td></td>
<td>Oral contraceptive pill (OCP) (low oestrogen doses [eg, 20 μg] may have less impact on IR, though more evidence is needed to confirm this)</td>
</tr>
</tbody>
</table>
| **Hirsutism** | Choice of options depends on patient preferences; impact on wellbeing; and access and affordability:

Self-administered and professional cosmetic therapy are first line (laser recommended)

Eflornithine cream can be added and may induce a more rapid response

If cosmetic therapy is not adequate, pharmacological therapy can be considered

Pharmacological therapy

- Medical therapy if patient is concerned and cosmetic therapy is ineffective/inaccessible/unaffordable

- Primary therapy is the OCP (monitor glucose tolerance in those at risk of diabetes)

- Anti-androgen monotherapy (eg, spironolactone or cyproterone acetate) should not be used without adequate contraception

- Trial therapies for $\geq 6$ months before changing dose or medication

- Combination therapy if $\geq 6$ months of OCP is ineffective, add anti-androgen to OCP (twice daily spironolactone $>50$ mg or cyproterone acetate $25$ mg/day, days 1–10 of OCP) |

| **Infertility** | Lifestyle intervention (to optimise preconception health and fertility and reduce pregnancy and long-term complications) |

Cyclic progestins (eg, 10 mg medroxyprogesterone acetate 10–14 days every 2–3 months)

Metformin (improves ovulation and menstrual cyclicity)
Clinical features of excess androgen levels are primarily approached with cosmetic treatments. They can also be controlled along with menstrual cycle abnormalities using combined oestrogen- and progestin-based oral contraceptive pills (OCP) (Teede and Shorakae 2017). These also act to protect against endometrial hyperplasia that can develop due to chronic anovulation, due to their progestin component (Teede and Shorakae 2017). If hirsutism or alopecia are severe, then an anti-androgen drug such as spironolactone or cyproterone acetate can be introduced (in combination with oral contraceptives) (Teede and Shorakae 2017). The frequently prescribed OCP present with the well-accepted risk of venous thromboembolism. This risk is further amplified because many women with PCOS are overweight (Teede, Misso et al. 2011). OCP also have a negative effect on blood lipids, blood pressure and may increase the risk of insulin resistance (McCartney and Marshall 2016, Teede and Shorakae 2017),

| **Cardiometabolic risk** | Lifestyle change: >5% weight loss in those who are overweight reduces diabetes risk by approximately 50%–60% in high-risk groups
| | Optimise cardiovascular risk factors
| | Consider metformin (reduces the risk of diabetes by ~50% in adherent high-risk groups) |
increasing metabolic and CVD risk factors. Low-dose OCP are currently recommended to reduce some of these risks (Teede and Shorakae 2017). OCP use however is inappropriate for women wishing to conceive and those who have side effects. It is not clear if there are any specific interactions between OCP and PCOS. Metformin has been shown to reduce hyperinsulinaemia (McCartney and Marshall 2016), improve IR and menstrual cycles and appears to improve fertility (Teede, Misso et al. 2011). Metformin has been shown to increase pregnancy rates but not live birth rates (Legro, Barhart et al. 2007, Tang, Lord et al. 2012). Further study is needed to define the role of metformin in PCOS management (Teede, Misso et al. 2011).

For women wishing to conceive, lifestyle intervention including regular exercise and caloric restrictions is the first line of treatment (Teede and Shorakae 2017). Ovulation induction can be achieved through weight loss (Barbieri and Ehrmann 2016) in women who are overweight. The data relating to the use of insulin sensitizing agents such as metformin is inconclusive. Metformin can assist in the treatment of metabolic abnormalities as well as menstrual irregularities but has limited or no effect in the treatment of infertility (Legro, Arslanian et al. 2013). Others have noted that overall metformin has a variable effect on ovulation function (McCartney and Marshall 2016). A consensus group has advised the use of metformin be restricted only to PCOS women with glucose intolerance (Barbieri and Ehrmann 2016). Clomiphene, a drug that stimulates the pituitary gland to increase its secretion of FSH, is currently first-line treatment for women with PCOS that require ovulation induction (Legro, Arslanian et al. 2013). Gonadotropins, aromatase inhibitors and laparoscopic ovarian drilling are further possible treatment options in suitable cases for PCOS women requiring assisted fertility (Teede and Shorakae 2017).
Recently guidelines and recommendations for early and regular screening of women with PCOS for detection of metabolic features and complications have been put forth (Teede and Shorakae 2017). Screening for glucose intolerance, type 2 diabetes, gestational diabetes (during pregnancy), dyslipidaemia, hypertension and obstructive sleep apnoea may help to reduce the risks of CVD development these factors attract. It is also recommended to screen women with PCOS for other risk factors of CVD including BMI, fatty liver and smoking status.

It is recommended that women with PCOS be screened for psychological features including anxiety and depression which constitute the most common mood disorders. Treatment options are varied however and it is important to consider management of weight gain and treatment of hirsutism to help improve the quality of life of women affected by this syndrome (Teede and Shorakae 2017).

In summary, despite significant developments in the treatment of women with PCOS over the last few years, areas of controversy still remain and further research is still needed.

1.6 The haemostatic system under a physiological state

The structure and function of the haemostatic system and its components under physiological state have been described here in order to provide the necessary background information and for improved understanding of the system’s role and links to CVD and VTE as well as to provide for clearer comprehension of the methodology/ies used in several of the studies completed.

The haemostatic system functions to keep blood in a fluid state within the vasculature and ensure adequate supply of oxygen and nutrients to tissues. It also assists to prevent blood loss
when blood vessel injury occurs through the formation of a stable blood clot or thrombus at the site of damage. It is composed of four major systems or responses, including the primary and secondary haemostatic systems, the fibrinolytic system and coagulation inhibitors as well as inhibitors of fibrinolysis, as discussed below.

1.6.1 The primary haemostatic system

The primary haemostatic system/response predominantly includes the vasculature and platelets. The endothelial cell lining, as the inner most layer of blood vessels, plays a critical role in blood haemostasis, which under a normal physiological state and when intact, releases nitric oxide (NO), that inhibits vascular smooth muscle contraction and growth as well as preventing platelet aggregation, allowing adequate flow of blood through the vessel lumen to reach the tissues (Turgeon 2017). Prostacyclin, also secreted by intact endothelial cells, acts to inhibit platelet adhesion and aggregation (Turgeon 2017). The basement membrane acts to support the endothelial cell layer and is known as the sub-endothelium. This is composed of collagen and is highly thrombogenic, ensuring platelet activation and initiation of thrombosis when exposed. The endothelium and sub-endothelium are surrounded by a layer of smooth muscle and elastic fibres (tunica media) and finally the outermost layer of blood vessels (tunica externa) is composed of fibrous connective tissue (Turgeon 2017).

Following blood vessel injury vasoconstriction ensures to reduce loss of blood from the vasculature (Hoffbrand, Moss et al. 2006). Damage to endothelial cells exposes the thrombogenic sub-endothelium and activation of platelets ensures. Platelets then adhere to the collagen in the subendothelial connective tissue (either directly or through the ‘bridge’ molecule von Willebrand factor), change shape, from a disc to a sphere with pseudopods to increase their surface area, and release their contents (Turgeon 2017). Platelets are composed of
a plasma membrane surrounded by a glycocalyx containing various glycoproteins (GP) including GPIb and GPIIb/IIIa that assist in platelet adhesion (to damaged blood vessel walls) and aggregation (platelet adhesion to each other) processes (Turgeon 2017). Platelets are composed of three major storage components related to haemostasis: alpha granules, dense granules (bodies) and lysosomes. Alpha granules include fibrinogen, factor V and factor VIII as factors part of the coagulation cascade (described later) have a predominant pro-coagulant effect (Turgeon 2017). Dense granules include ADP and ATP that assist in platelet activation and aggregation, calcium which is required at various stage in the coagulation cascade and serotonin which can assists in vasoconstriction (Turgeon 2017). Lysosomes contain several hydrolytic enzymes. Following platelet adhesion to the damaged blood vessel wall and release of platelet contents, a chain reaction of events ensures whereby each platelet, upon adhesion to the blood vessel wall or other platelets, releases its granules and causes further platelet recruitment, adhesion and aggregation, ultimately resulting in the formation of a primary platelet thrombus (Hoffbrand, Moss et al. 2006, Turgeon 2017). This is then further stabilised through the activation of the coagulation cascade, also known as the secondary haemostatic system/response.

1.6.2 The coagulation system

In vitro the coagulation cascade exerts its effects through the extrinsic pathway (activated by tissue factors including thromboplastin released from damaged tissue cells) and the intrinsic pathway (activated by exposed collagen or platelet phospholipid) (Figure 1.5) with further potential interactions observed in current in vivo coagulation cascade concepts (Figure 1.6), which also recognise the importance of cellular (surfaces) interactions with coagulation proteins. The coagulation cascade factors (denoted mainly by Roman numerals) are composed predominantly of serine proteases and their cofactors, which, through proteolytic activation, act
to transform downstream enzymes from their inactive to active forms (denoted by inclusion of subscript ‘a’ next to each Roman numeral number) and ultimately result in the conversion of soluble fibrinogen into insoluble fibrin by the action of thrombin (Turgeon 2012). Thrombin is also able to cause further coagulation cascade amplification by activation of coagulation cascade factors V, VIII and XI. Thrombin is also a powerful platelet activator and able to stimulate further platelet activation and aggregation steps (Turgeon 2017). Conversely thrombin can also act as a coagulation inhibitor following attachment to antithrombin (AT) that ultimately inhibits its action, stimulates endothelial cell release of plasminogen activators as well as attaching to thrombomodulin which then results in the inactivation of factors Va and VIIIa (Turgeon 2017). Thrombin activation of coagulation factor XIII results in formation of cross-linked fibrin polymers. Ultimately in the coagulation cascade, cross-linked fibrin acts to enmesh the initial primary platelet thrombus into a more stable form and further reduce the loss of blood (Figure 1.5) (Hoffbrand, Moss et al. 2006, Turgeon 2012, Turgeon 2017).
Figure 1.5 The ‘classical’ *in vitro* coagulation cascade. Coagulation is activated through both the intrinsic pathway which involves contact activation factors prekallikrein (PK), high molecular weight kininogen (HMWK) and factors XII and XI while the extrinsic pathway follows from release of tissue thromboplastin (T. Thromboplastin) from damaged tissues to ultimately converge at a common pathway (following activation of factor X to Xa) and convert insoluble fibrinogen into fibrin (cross-linked) to enmesh the initial platelet thrombus formed into a much more stable form. PK, prekallikrein; HMWK, high molecular weight kininogen; Kall., Kallikrein; T. Thromboplastin, tissue thromboplastin; PF3, platelet factor 3 (adapted from Turgeon 2017).
Figure 1.6 The current ‘in vivo’ (cell-based) coagulation is thought to occur in three stages: initiation, amplification and propagation. In the initiation stage a tissue factor (TF) bearing cell such as a monocyte presents TF to factor VII which then can activate factors X and IX. Activated factor X (Xa) is then able to activate thrombin (or factor IIa) from prothrombin (factor II). Thrombin is then able to activate platelets and co-factors V and VIII. The amplification stage may then commence whereby the platelet surface acts to provide the phospholipid necessary for activated factors VIIIa and IXa and allows for further thrombin formation to ultimately generate large amounts of thrombin. Finally in the propagation and final stage of the cell based coagulation concept a large volume of thrombin has been generated on the platelet surface to ultimately allow for the formation of a large fibrin thrombus. TF, tissue factor; PLT, platelet/s (adapted from Turgeon 2017).
1.6.3 The fibrinolytic system

The thrombus is ultimately digested through another system known as fibrinolysis as collagen is deposited at the site of injury to seal the damaged area. The fibrinolytic system also acts to break down thrombi that have formed inappropriately due to abnormal activation of blood coagulation within the blood vasculature and ensures that blood is maintained within its fluid form. Tissue plasminogen activator (tPA), one of the principal fibrinolytic system components, acts to convert fibrinolytic pro-enzyme plasminogen into active plasmin. Plasmin is able to digest fibrinogen, fibrin as well as hydrolise coagulation factors V, VIII and XII (Turgeon 2017). Plasmin is further able to activate the complement cascade (Turgeon 2017). Other activators of plasminogen include the ‘kinases’ urokinase plasminogen activators (uPA), streptokinase, staphylokinase but also thrombin (Turgeon 2017). Ultimately plasmin acts to break down the thrombus formed releasing fibrin and fibrinogen degradation products (Hoffbrand, Moss et al. 2006). These degradation products of split fibrin can exert an antithrombin effect as well as inhibit formation of fibrin polymers and platelet aggregation (Turgeon 2017).

1.6.4 Inhibitors of coagulation

Inhibitors of the coagulation cascade including AT, protein C and cofactor protein S as well as tissue factor pathway inhibitor (TFPI) act to control coagulation and prevent aberrant growth of the thrombus beyond needs to prevent loss of blood. Continued release of NO and prostacyclin by intact endothelial cells surrounding the mechanically damaged tissue also prevents growth of the thrombus beyond requirements (Turgeon 2017). Antithrombin (formerly known as Antithrombin III), is a glycoprotein that inactivates procoagulant factors thrombin and activated factor X (Harper 2017). As a serine protease inhibitor and as indicated earlier AT acts to inhibit
plasmin, however it does this to a lesser extent than its negative effects on the procoagulant system, resulting in a net anticoagulant effect. This is demonstrated following observations of increased risk of both arterial as well as venous thrombosis in patients deficient for AT (Harper 2017). Protein C, once converted to functional form, activated protein C (APC) following attachment to and activation by endothelial-cell associated cofactor thrombomodulin, plays a critical role in the anticoagulant pathway. APC, in the presence of cofactor Protein S, is able to dramatically reduce the conversion of prothrombin to thrombin by proteolytic cleavage of factors Va and VIIIa. APC is also able to break-down inhibitors of tPA and therefore promote fibrinolysis (Turgeon 2017).

1.6.5 Inhibitors of fibrinolysis

Inhibitors of the fibrinolytic system including plasminogen activator inhibitor-1 (PAI-1), α2 antiplasmin, α2 macroglobulin and thrombin activatable fibrinolysis inhibitor (TAFI), act to control fibrinolysis (Hoffbrand, Moss et al. 2006). PAI-1 is a primary inhibitor of serine proteases and fibrinolytic activators tPA and uPA and is secreted predominantly by endothelial cells but also adipose tissue and liver hepatocytes. PAI-1 is able to down regulate fibrinolysis by preventing cleavage of pro-enzyme plasminogen by serine proteases to active protease plasmin (Yasar Yildiz, Kuru et al. 2014). TAFI, secreted mainly by the liver, cleaves lysine residues from partially degraded fibrin preventing binding and conversion of plasminogen into active plasmin (Karakurt, Gumus et al. 2008). α2 antiplasmin inhibits active plasmin by binding to and forming complexes with the latter inhibiting its function. Plasmin in fluid phase (plasma) is rapidly converted to plasmin-α2antiplasmin or neutralized by α2 macroglobulin (Syrovets and Simmet 2004, Syrovets, Lunov et al. 2012). α2 antiplasmin is present in the plasma at high enough concentrations to immediately neutralize up to 50% of available plasmin (Syrovets and Simmet 2004, Syrovets, Lunov et al. 2012). The remainder of the 50% is neutralized by α2
macroglobulin. Plasmin cannot normally be found in blood as it is inactivated by excess presence of inhibitors (Turgeon 2017).

Overall the haemostatic system is a highly interwoven and complex system (Figure 1.7). Although markers of haemostasis have been attributed predominantly pro-coagulant or anticoagulant roles, a number of factors can, either directly or indirectly, exert both roles and while initially having one predominant action, will have downstream effects within the haemostatic system (and other systems) that activate markers to counteract initial effects or further propagate these actions. It is therefore critical that any assessment on the haemostatic system include a thorough evaluation of all relevant components within this system but also incorporate global markers of assessment.
Figure 1.7 The systems that ensure balanced Haemostasis: primary haemostasis including vascular endothelium and supporting tissues as well as platelets (not shown); secondary
haemostasis that includes the coagulation cascade factors; inhibitors of coagulation including antithrombin (AT), protein C (and active form activated protein C, APC) and co-factor protein S, tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM); the fibrinolytic system including plasminogen and active plasmin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and lastly inhibitors of fibrinolysis including plasminogen activator inhibitor 1 (PAI-1), α2 antiplasmin and α2 macroglobulin. HMW, High molecular weight; XL-FDP, cross-linked fibrin degradation products (adapted from http://diapharma.com/coagulation/ Last accessed 19/12/2017; Note that for clarity and ease of understanding not all haemostatic factors have been included in this figure).

1.7 PCOS, CVD and Haemostasis

PCOS is underpinned by genetic IR, environmental IR (obesity related), as well as metabolic abnormalities in the majority of cases (Teede, Misso et al. 2011, Sirmans and Pate 2013). Women with PCOS also have evidence of subclinical CVD (Teede, Misso et al. 2011, Zhao, Zhu et al. 2016) and appear to have an increased risk of CV events and death (Teede, Misso et al. 2011, Hart and Doherty 2015, Azziz, Carmina et al. 2016) especially when combined with other risk factors, such as obesity and hypertension (Wild, Carmina et al. 2010), although overall evidence is still unclear (Goodman, Cobin et al. 2015, Azziz, Carmina et al. 2016). The incidence of metabolic syndrome is increased in both lean and obese females with PCOS (Alexander, Tangchitnob et al. 2009, Moran, Misso et al. 2010, Sirmans and Pate 2013). Between 30-40% of women with PCOS have impaired glucose tolerance and diabetes, a major CV risk factor, is increased fourfold in this patient group (Moran, Brinkworth et al. 2006, Sirmans and Pate 2013). Other features associated with metabolic syndrome may occur in
PCOS, including a proinflammatory (Shorakae, Teede et al. 2015) and prothrombotic state, both of which have well-accepted links to CVD (Alexander, Tangchitnob et al. 2009).

The underlying mechanism/s of increased cardiometabolic risk in PCOS is/are unclear. Although IR is clearly a candidate, studies have shown that both obese and lean women with PCOS, both hyperinsulinaemic and those with ambient insulin levels, have increased CVD risk (Macut, Micic et al. 2001, Health 2017). Hyperandrogenaemia (with IR) is also linked with increased metabolic and CV morbidity in PCOS (Charitidou, Farmakiotis et al. 2008) and high androgens and low SHBG levels are associated with increased CVD risk factors in both pre- and post-menopausal PCOS females (Health 2011). An elevated testosterone level has also been shown to be an independent risk factor for myocardial infarct (MI) and coronary atherosclerosis (Kebapcilar, Taner et al. 2009). Others have reported contrary findings indicating either a neutral or a beneficial effect of androgens on the vasculature (Worboys, Kotsopoulos et al. 2001, Heutling, Schulz et al. 2008). There have also been reports of increased inflammatory markers in PCOS, including C-reactive protein (Boulman, Levy et al. 2004, Health 2017), and of endothelial dysfunction, arterial stiffness, and early atherosclerosis as surrogate indicators of CV damage (Moran, Hutchison et al. 2009, Health 2011, Teede, Misso et al. 2011). A large number of PCOS females are obese or have excess weight that can both directly and indirectly increase CV risk, with increased IR, hyperandrogenaemia, dyslipidaemia, and potential activation of the haemostatic system (Teede, Misso et al. 2011, Azziz 2017).

Further research is needed, but OCP may also place women with PCOS at an increased risk of developing CV problems through increased risk of arterial thrombotic events, arterial dysfunction (Meyer, McGrath et al. 2007), altered glucose metabolism and IR (Meyer, McGrath et al. 2007, Charitidou, Farmakiotis et al. 2008), which may increase the risk of type 2
diabetes. Women with PCOS taking OCP are also at increased risks of VTE (Bird, Hartzema et al. 2013), risks that are well accepted for those in the general population taking the hormonal therapy.

Potentially disturbed haemostasis may also underpin cardiometabolic abnormalities in PCOS. Although no epidemiological studies to date demonstrate a clinically evident increased venous thrombotic risk, epidemiological studies in PCOS are limited. It was found that 29% of PCOS females have a positive family history of venous thrombosis compared to 8% of controls (Atiomo, Fox et al. 2000). Shaw et al observed that the incidence of CVD was elevated in PCOS females compared to controls (Shaw, Bairey Merz et al. 2008). Although still contentious, recent large scale studies have shown that women with PCOS have increased rates of both CVD and VTE in comparison to controls (Bird, Hartzema et al. 2013, Hart and Doherty 2015). Arterial thrombi are the usual cause of strokes and MI, and disturbed haemostasis may contribute to CVD risk in PCOS. The haemostatic system is also integrally linked to the endothelium and vessel wall. Demonstrated endothelial dysfunction and vessel wall functional and structural abnormalities seen in PCOS may also be linked pathophysiologically to disturbed haemostasis in PCOS (Moran, Hutchison et al. 2009). Ultimately, a greater understanding of the potential pathophysiological role that haemostatic factors play in the cardiometabolic features may make way for novel therapeutic measures in PCOS. This is particularly important because women are becoming increasingly obese, living longer, and experiencing higher rates of diabetes and because CVD is the leading cause of death in women in Australia (Oral, Mermi et al. 2009, Health 2011).
1.8 Ovulation, fertility and pregnancy outcomes in PCOS and links to fibrinolytic system markers

An excess androgen level in addition to a lack of ovulation has seen PCOS develop into a primary cause of infertility, with ~75% of women who have anovulation also being affected by PCOS (Homburg 2003, 2009, Azziz, Carmina et al. 2016). Women with PCOS are 15 times more likely to be infertile than those without, and this is independent of obesity (Azziz, Carmina et al. 2016). Studies have also shown that if women with the syndrome are successful in conceiving, they are then faced with increased risks of pregnancy associated complications (Glueck, Awadalla et al. 2000, Glueck, Wang et al. 2004, Azziz, Carmina et al. 2016) as well as increased risk factors to infants born to affected women including higher risk of being large for gestational age and having a low Apgar score (that assesses the condition of the newborn by measuring factors such as breathing effort, heart rate, muscle tone, reflexes and skin colour) (Azziz, Carmina et al. 2016).

The fibrinolytic system has been shown to have a number of roles other than its involvement in the breakdown of thrombi at a system level; this has been shown to have a critical role in ovulation processes. In animal models PAI-1 and plasminogen are key components of ovulation regulation (Politis, Srikandakumar et al. 1990). Degradation of the follicular layers is necessary for oocyte escape. Rupture of the ovarian follicles is associated with a net increase in plasmin activity, achieved following an increase in tPA and/or uPA which convert plasminogen to active plasmin, and a reduction in PAI-1 (Politis, Srikandakumar et al. 1990). Plasminogen plays a central role in ovarian follicle extracellular matrix degradation as well as regulation of proteins involved in follicular rupture (Cao, Sahmi et al. 2004). The importance of these markers in ovulation processes have also been identified in human studies with tPA found in female ovarian granulosa cells and within the follicular fluid and PAI-1 also in theca cells.
The Haemostatic System in PCOS

(Beers 1975, Piquette, Crabtree et al. 1993, Atiomo, Hilton et al. 2000). Few studies have evaluated these fibrinolytic/proteolytic system markers and inhibitors within the PCOS ovaries in either humans or in animal models though plasminogen was found to be degraded within the follicular fluid of the ovary in women with PCOS (Ambekar, Kelkar et al. 2015) and no significant difference in PAI-1 antigen levels in the ovaries of PCOS women versus controls have been observed (Atiomo, Hilton et al. 2000). In contrast PAI-1 was found to be prevalent in the PCOS ovary but lacking or scant in normal human ovaries (Devin, Johnson et al. 2007). Abnormal levels/function of fibrinolytic parameters and inhibitors may, at a systemic level contribute to aberrant haemostasis and thrombosis and be linked to CVD and VTE, however at a local ovarian level this/these haemostatic distortion/s may contribute to aberrant ovulation and have the potential to explain, at least in part, some of the reasons behind the frequent fertility problems noted in women with PCOS.

Women with PCOS also have elevated risks of pregnancy associated complications (Glueck, Awadalla et al. 2000, Glueck, Wang et al. 2004, Azziz, Carmina et al. 2016). Women with the syndrome have a markedly increased rate of miscarriage with an estimated 44% of pregnancies resulting in foetal loss (Glueck, Wang et al. 1999). Early pregnancy loss occurs in around 30-50% of PCOS women versus only 10-15% of those in the general population (Kamalanathan, Sahoo et al. 2013). Pregnancy associated complications including increased rates of abortion may potentially be related to an aberrant haemostatic and fibrinolytic system, following increased risks of thrombi formation and placental insufficiency. It is well accepted that pregnancy, for those in the general population, is a known hypercoagulable state, likely to ensure cessation of bleeding following delivery. Increase in clotting factors occurs during pregnancy as well as a fall in fibrinolysis (Shan, Wang et al. 2013). PAI-1 activity has been demonstrated as an independent risk factor for increased miscarriage rates (Glueck, Wang et al. 1999). Studies which evaluated the use of metformin and effects on birth outcomes in pregnant
women with PCOS noted correlations between PAI-1 and miscarriage rates with improvement in live births following a reduction in PAI-1 however the increased miscarriage rates persisted in women where PAI-1 activity increased or remained unchanged during treatment (Palomba, Orio et al. 2005, Glueck, Sieve et al. 2006, Kamalanathan, Sahoo et al. 2013). Furthermore, a recent study that looked into the haemostatic and fibrinolytic potential of pregnant women with PCOS during the first trimester of pregnancy noted that pregnant females with the syndrome are more prone to a prothrombotic state (Shan, Wang et al. 2013).

1.9 Summary and research gaps

PCOS is a highly prevalent disorder with reproductive, psychological and metabolic features with increased CVD risk factors. Aetiology/ies is/are still unknown and women affected by the syndrome can present with a wide-range of clinical features making both diagnosis and treatment a significant challenge. Haemostatic abnormalities have an association with and a demonstrated pathophysiological role in CVD in non-PCOS populations, yet appear unexplored in PCOS. The haemostatic system is highly intricate and a comprehensive and systematic assessment is needed to ensure accurate evaluation. Additionally, the OCP, which presents with well-accepted risks of venous thromboembolism, is frequently prescribed to this group of women. The additional proteolytic role of the fibrinolytic system markers and inhibitors also needs to be investigated further within the ovary of women with PCOS to explore aetiology/ies of PCOS and assist with future treatment options including for the frequent infertility noted in women with the syndrome.

Key research gaps remain in this area and include the need for:
1. An in-depth review of the literature to identify the state of the haemostatic system in women with PCOS and evaluate potential relationships to the aberrant metabolic and hormonal features frequently present;

2. A comprehensive and systematic analysis of the overall haemostatic system and its components in women with PCOS and review of links to metabolic and hormonal parameters to identify potential underlying causes or improved treatments strategies;

3. An in-depth review of the literature to identify the state of the haemostatic system in women with PCOS following commonly and currently prescribed therapies such a high- and low-dose OCP and metformin as well as review of potential effects/relationships to metabolic and hormonal markers;

4. Clear demonstrable evidence of the haemostatic effects in women with PCOS following administration of commonly prescribed therapies such a high- and low-dose OCP and metformin and review of potential effects/relationships to metabolic and hormonal markers;

5. A better understanding of the role fibrinolytic/proteolytic markers and inhibitors have in the PCO and in PCOS.

1.10 Aims of the Thesis

Given the gaps in our knowledge, the body of work presented in this thesis aims to:

a) Review the literature to evaluate for a relationship between PCOS and disturbed haemostasis, including assessment of any associations between haemostatic markers and metabolic and hormonal parameters in women with the syndrome.(Chapter 2)
b) Comprehensively and systematically assess the haemostatic system in an established case-controlled cohort of lean and overweight women with and without PCOS. (Chapter 3)

c) Assess the haemostatic system in a cross-sectional study of overweight women with PCOS following commonly prescribed therapies such as higher- and low-dose OCP and metformin and identify potential effects/relationships to metabolic and hormonal markers. (Chapter 4)

d) Evaluate and compare the expression and potential roles of fibrinolytic/proteolytic markers: plasminogen, plasmin, tPA, uPA and inhibitor PAI-1 in the PCOS ovary and in normal ovaries using ovarian tissue from a PCOS mouse model and normal control mice, respectively. (Chapter 5)
CHAPTER 2: Haemostatic Abnormalities and Relationships to Metabolic and Hormonal Status in Polycystic Ovarian Syndrome

2.1 Introduction

PCOS is diagnosed based on hyperandrogenism (clinical and/or biochemical), ovulatory dysfunction and polycystic ovaries on ultrasound. Aetiological factor(s) include both genetic and environmental/lifestyle factors contributing to both insulin resistance and hyperandrogenism. It is one of the most common disorders of females of reproductive age, yet as a syndrome, it is difficult to diagnose and treat (Teede and Shorakae 2017). Clinical findings include reproductive, psychological and significant metabolic features. The metabolic abnormalities predispose patients to type II diabetes and CVD. Haemostatic abnormalities are closely related to metabolic abnormalities and to CVD in non-PCOS populations.

A comprehensive review of the literature of the haemostatic system in PCOS must/should systematically evaluate all appropriate markers of its major components/systems including those of the primary haemostatic system/response, secondary haemostasis or the coagulation cascade and inhibitors of coagulation, fibrinolytic system components and relevant inhibitors of fibrinolysis as well as assess global markers of haemostasis and fibrinolysis, as a number of the haemostatic components have both pro-thrombotic and anti-thrombotic outcomes (see Section 1.6). This must also review and identify if studies evaluated for markers which interfere with haemostatic function including BMI, age, relevant medications and smoking status; if studies were adequately powered and if sufficient evidence is available to draw any definitive conclusions.
In this context and to evaluate the primary haemostatic system, studies assessing both quantitative and qualitative markers of platelets in women with PCOS were evaluated. This included evaluation of platelet numbers but also platelet structure and function through a review of the literature of mean platelet volume (MPV); platelet activation and aberrant thrombotic stimuli were also investigated by examining the literature for soluble CD40 ligand (sCD40L) and urinary thromboxane metabolite (TXM) in women with the syndrome, as in vivo markers of platelet activation (FitzGerald, Pedersen et al. 1983, Oktem, Ozcimen et al. 2009). Further assessment of primary haemostasis in women with PCOS was completed through investigation of endothelial function by examining its synthesized products including von Willebrand factor (vWf) and PAI-1 as well as asymmetric dimethyl-arginine (ADMA), a major nitric oxide inhibitor (De Gennaro Colonna, Bianchi et al. 2009).

Evaluation of secondary haemostasis in the literature was completed through review of all individual clotting factors from the coagulation cascade (coagulation factors I through to XIII) in women with the syndrome but also by looking into screening tests that measure components of this cascade. Prothrombin Time (PT) measures the levels and function of coagulation factors I (fibrinogen), II (prothrombin), V, VII and X part of the extrinsic and common pathways in the ‘classical’ in vitro coagulation cascade (Figure 1.5) through observation of the time taken to form a fibrin thrombus following exposure to a tissue factor-rich substance such as thromboplastin (in addition to phospholipids and calcium) (Turgeon 2017). Activated Partial Thromboplastin Time (APTT) measures the intrinsic and common pathways (Figure 1.5), and determines the levels and function of all clotting factors of the cascade with the exception of factors VII and XIII (Turgeon 2017). APTT reagents usually contain activating agents including phospholipids and negatively charged particles such as kaolin, silica, ellagic acid or celite to improve responsiveness and reproducibility of the test (Fritsma, Dembitzer et al. 2012). Thrombin time (TT) assesses formation of polymerized fibrin by thrombin and will
identify the presence of inhibitors of thrombin or substances that affect fibrin formation (Turgeon 2017).

A comprehensive review of the literature for all major inhibitors of haemostasis including AT, TFPI, Protein C and its cofactor Protein S as well as APC in women with the syndrome was performed. Review of principal fibrinolytic system markers plasminogen (and active plasmin) as well as activators of plasminogen including tPA and uPA and inhibitors of fibrinolysis PAI-1, α2 antiplasmin, TAFI and α2 macroglobulin was also carried out for women with PCOS.

Global markers of coagulation and fibrinolysis were explored including the Global Fibrinolytic Capacity (GFC), a test which evaluates the amount of fibrin degradation products (FDP) generated when a freeze-dried fibrin clot is stopped by the addition of aprotinin (an antifibrinolytic molecule) (Yildiz, Haznedaroglu et al. 2002). The level of PF1 & 2, generated following conversion of prothrombin to thrombin, were evaluated in the literature in women with PCOS to identify both the effectiveness of the coagulation cascade but also the ability of some coagulation factors inhibitors to prevent conversion of prothrombin to its active form. PF1 & 2 is also a marker of subclinical in vivo coagulation-mediated thrombosis. The Thrombin Generation (TG) test, which looks at the haemostatic system overall, evaluating both the pro-coagulation effects of the coagulation cascade, but also inhibitory substances that prevent the generation of thrombin, mainly TFPI, AT and Protein C was also investigated (Chaireti 2013). DDimer (DD) levels were explored in the literature for women with the syndrome to identify formation of cross-linked fibrin following coagulation cascade activation and simultaneous breakdown by fibrinolysis.
Even minor alterations in the levels and/or function of any of the components of the haemostatic system can have serious consequences on a patient’s ability to prevent loss of blood following vascular harm or to ensure adequate flow of blood following aberrant procoagulant stimulus/i within the vasculature. Assessing the haemostatic system in females affected by PCOS is critical due to their already increased risk of CVD and VTE to ensure appropriate preventative measures are implemented against CVD and venous thrombosis development and their complications in the younger population group as well as appropriate management strategies are introduced for those already in a high-risk range.

2.2 PCOS and the Primary Haemostatic System

Assessment of primary haemostasis (in females with PCOS) can be undertaken by evaluating its principal components, namely platelets and the vascular endothelium (Figure 2.1).

2.2.1 Platelet volume, number and function

Platelets with a high volume have been shown to have a higher thrombotic potential (Gursoy, Ertugrul et al. 2006); these aggregate more rapidly, have elevated levels of Thromboxane A2 (a platelet aggregation agonist) as well as have increased levels of some procoagulant surface proteins (Kebapcilar, Taner et al. 2009). Enhanced platelet aggregability may also increase the risk of thrombosis and of CVD. Increased mean platelet volume (MPV) is considered an independent risk factor for CVD (Gursoy, Ertugrul et al. 2006). It is also well accepted that increased platelet numbers are linked to increased CVD risks.
Figure 2.1 Relationships and interactions between endothelial function, platelet activation and aggregation, inflammation, coagulation and fibrinolysis in PCOS. Studies have shown abnormal platelet function, inhibition of endothelial function and inhibition of fibrinolysis in PCOS resulting in a net prothrombotic phenotype. ADP, adenosine diphosphate; ADMA, asymmetric dimethylarginine; CD40L, CD40 ligand; FVIIa, activated factor VII; GPIIb/IIIa, platelet glycoprotein IIb/IIIa; PAI-1, plasminogen activator inhibitor 1; PAR, protease activated receptor; PROTEIN C/S, activated protein C and protein S complex; tPA, tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor; TTM, thrombin-thrombomodulin complex; TxA₂, thromboxane A₂; vWF, Von Willebrand factor.
Earlier studies demonstrated elevated platelet volume in PCOS versus controls (Gursoy, Ertugrul et al. 2006, Kebapcilar, Taner et al. 2009). More recent studies however have revealed equivocal results with some noting no significant difference in MPV between lean women with PCOS and controls (Dogan, Arduc et al. 2014, Silfeler, Kurt et al. 2014); or increased MPV in only obese PCOS versus controls (Cakiroglu, Vural et al. 2016) while increased MPV has also been observed in lean counterparts (Yilmaz, Duran et al. 2016). Silfeler et al (Silfeler, Kurt et al. 2014) suggest that obesity drives the increased MPV in PCOS though Kucur et al (Kucur, Gozukara et al. 2015) have noted increased MPV in PCOS versus controls independent of BMI. A positive correlation between MPV and IR has been observed (Kebapcilar, Taner et al. 2009, Kucur, Gozukara et al. 2015) but not always (Gursoy, Ertugrul et al. 2006). Differences in relationships between MPV and testosterone levels have been observed (Gursoy, Ertugrul et al. 2006, Kebapcilar, Taner et al. 2009), while in one study a negative correlation between androgens and MPV was noted (Dogan, Arduc et al. 2014).

Platelet numbers were shown to be higher in PCOS in one study (Luque-Ramirez, Mendieta-Azcona et al. 2009) and similar to controls in most (Dereli, Ozgen et al. 2003, Gursoy, Ertugrul et al. 2006, Kebapcilar, Taner et al. 2009, Cakiroglu, Vural et al. 2016, Yilmaz, Duran et al. 2016). However, no correlations between platelet numbers and free testosterone levels have been observed (Kebapcilar, Taner et al. 2009).

Earlier studies have noted platelet dysfunction in women with PCOS compared to controls with one study showing increased platelet aggregation following agonist stimulation (ADP, collagen, epinephrine) (Dereli, Ozgen et al. 2003) and the other impairment of platelet responsiveness to the anti-aggregation effects of NO, independent of obesity (Rajendran, Willoughby et al. 2009). A recent small study performed with 21 PCOS and 19 BMI- and age-matched controls showed no significant difference in platelet function between the two.
groups following stimulation with ADP or inhibition by prostacyclin (Kahal, Aburima et al. 2013).

Investigation of platelet activation and exocytosis and abnormal thrombotic stimulus (on platelets) can be performed using soluble CD40 ligand (sCD40L), which is a thromboinflammatory marker. With 95% of its circulating levels originating from activated platelets (Oktem, Ozcimen et al. 2009), elevated sCD40L levels represent an indirect marker of \textit{in vivo} platelet activation. Elevated sCD40L levels may represent ongoing subclinical platelet-mediated thrombosis and are highly correlated with cardiovascular outcome (Lutgens, van Suylen et al. 2003). Identification of an abnormal procoagulant stimulus on a normal primary haemostatic system can also be undertaken by evaluation of thromboxane metabolite (TXM). Thromboxane A2/B2 metabolite, a major prostanoid synthesized by platelets, can be measured through a sensitive, specific and non-invasive method known as urinary thromboxane metabolite (Eldor, Lellouche et al. 1991). Enhanced urinary TXM represents a time-integrated index of ongoing platelet activation \textit{in vivo} (FitzGerald, Pedersen et al. 1983). This method would therefore demonstrate superiority to platelet aggregation studies which assess platelet functionally \textit{in vitro} and only for the small period of time during which the blood sample was collected.

In general sCD40L levels in PCOS have been shown to be elevated compared to controls (Zwirska-Korczala, Sadowski et al. 2008, Oktem, Ozcimen et al. 2009, Kebapcilar, Kebapcilar et al. 2011, El-Mesallamy, Abd El-Razek et al. 2013) though in a recent study reduced sCD40L levels in PCOS were observed in comparison to controls, irrespective of body weight (Sarray and Almawi 2016). Oktem et al found that sCD40L levels were affected independently of the metabolic (fasting insulin and HOMA-IR) or hormonal (total and free testosterone) profile in PCOS (Oktem, Ozcimen et al. 2009). In another study no correlation between sCD40L and
hyperandrogenaemia or IR was observed (Kebapcilar, Bilgir et al. 2010). A more recent study by El-Mesallamy et al. found an association between sCD40L and IR indices (El-Mesallamy, Abd El-Razek et al. 2013). There have not been any publications identified to date assessing the levels of urinary TXM in women with PCOS.

Although there is disagreement in the literature, available data indicate a potential role for abnormal platelet activation/function, suggesting disturbed primary haemostasis in PCOS, although the mechanism/s by which these effects are exerted are unclear. The cause of the platelet abnormalities or their aberrant activation is uncertain. Further research is needed to determine the relationships of MPV, sCD40L, urinary TXM and possibly platelet numbers to metabolic and hormonal profiles in PCOS. Insulin is believed to affect platelet function, and variation in insulin sensitivity can affect platelet responses to various agonists (Dereli, Ozgen et al. 2003). Platelet dysfunction in PCOS, following impairment of responsiveness to NO, was also shown to be independent of IR (Rajendran, Willoughby et al. 2009). Further research is necessary to both demonstrate the presence of abnormal platelet function/activation in PCOS compared to matched controls and ascertain whether this is linked to IR. Assessment of platelet activation following OCP administration in PCOS may also identify thrombotic and CV risk factors, which are important considering the frequency of OCP use in PCOS.

2.2.2 Endothelial function

A dysfunctional endothelium has been linked with an increased risk of CV events following its key contribution to atherosclerosis (Moran, Hutchison et al. 2009). Its function can be assessed indirectly through measurement of its synthesized products, namely vWF and PAI-1 as well as through assessment of ADMA, a major NO inhibitor. These products, along with an abnormal endothelium, have all been independently linked to type 2 diabetes, further increasing the
potential for CV injury. ADMA is also an independent marker for CV morbidity and mortality (Heutling, Schulz et al. 2008).

Most studies have revealed that vWF levels in PCOS are similar to those of controls (Dahlgren, Janson et al. 1994, Kelly, Lyall et al. 2002, Kuscu and Var 2009, Moran, Hutchison et al. 2009, Manneras-Holm, Baghaei et al. 2011) though others have shown increased levels of the endothelial dysfunction marker in females with the syndrome versus age- and BMI-matched controls (Foltyn, Strzelczyk et al. 2011, Koiou, Tziomalos et al. 2012). One study showed a positive link between insulin and vWF antigen (Dahlgren, Janson et al. 1994) and others between the latter and triglycerides (Dahlgren, Janson et al. 1994, Foltyn, Strzelczyk et al. 2011), suggesting a potential link between vWF and the metabolic system. A larger sample size and age-matched controls in an earlier study by Kelly et al would have assisted in clarifying these findings (Kelly, Lyall et al. 2002).

(Demirel, Bideci et al. 2007, Charitidou, Farmakiotis et al. 2008, Pamuk, Torun et al. 2010, Turkcuoglu, Engin-Ustun et al. 2011). It has been suggested that body weight may affect ADMA levels (Kielstein, Donnerstag et al. 2006, Meinitzer, Puchinger et al. 2007), but not all studies have used BMI-matched controls (Demirel, Bideci et al. 2007, Rajendran, Willoughby et al. 2009).

studies suggest a link (Carmassi, De Negri et al. 2005, Aziz, Sidelmann et al. 2015) while others did not (Sampson, Kong et al. 1996, Orio, Palomba et al. 2004).

Conclusions from many of the studies that assess PAI-1 (and ADMA) in PCOS are limited by suboptimal experimental design and between-study heterogeneity (Toulis, Goulis et al. 2011). Both age and weight are known to affect PAI-1 (Vague, Juhan-Vague et al. 1986, Heinrich, Dirkeskersting et al. 1992, De Pergola, De Mitrio et al. 1997). Some of the earlier studies were neither age- nor weight-matched (Glueck, Wang et al. 2004, Carmassi, De Negri et al. 2005) or only matched for age (Glueck, Morrison et al. 2008). In some there was a significant difference in age (Yarali, Yildirir et al. 2001) or in weight (Atiomo, Bates et al. 1998) between PCOS and controls. Some would have been supported by larger sample sizes (Atiomo, Bates et al. 1998, Slopien, Lewandowski et al. 2006, Oral, Mermi et al. 2009). Some selected participants other than healthy controls (Dahlgren, Janson et al. 1994) and others indicated elevated PAI-1 levels in PCOS versus controls, but did not reach statistical significance (p>0.05) (Macut, Micic et al. 2001). One study did not mention whether the PCOS females were on medications known to interfere with PAI-1 (Lin and Yongmei 2008).

Although discrepancies are noted, correlation studies reveal a link between PAI-1 and the aberrant metabolic (and potentially hormonal) profile in PCOS. If this is indeed the case, it is also important to determine whether IR and consequent hyperinsulinaemia are a result of abnormal endothelial function limiting insulin action. Alternatively, the disturbed metabolic system in PCOS may have caused endothelium dysfunction. Insulin induces vasodilation (Carmassi, De Negri et al. 2005), and high levels may contribute to hyperstimulation of the endothelium, potentially resulting in dysfunction. Studies have shown that PAI-1 declines following metformin administration or weight loss, suggesting IR as a contributor (Ibanez, Aulesa et al. 2002, Sills, Drews et al. 2003). However, intervention studies using OCP reduced
ADMA levels in PCOS despite increases in IR (Charitidou, Farmakiotis et al. 2008, Heutling, Schulz et al. 2008). Insulin can also directly affect PAI-1 levels by up regulating its transcription (Sills, Drews et al. 2003).

While earlier research is generally of suboptimal quality and studies show significant between-study heterogeneity with conflicting results including in some studies completed more recently, the overwhelming majority of studies reveal an aberrant endothelium in PCOS, as reflected by elevated ADAM levels and PAI-1 levels/activity, that is related to the aberrant metabolic and potentially hormonal profiles. Further research with robust studies assessing endothelial function and its potential links to the metabolic and hormonal profiles in PCOS will help to further clarify or confirm these outcomes and may yield valuable aetiological information, potentially providing future therapeutic targets.

2.3 PCOS and Secondary Haemostasis

Assessment of secondary haemostasis in PCOS can be carried out by examining significant coagulation factors and pathways within the coagulation cascade (Figure 2.1).

2.3.1 Coagulation Factor FVII and Fibrinogen

There is evidence that elevated plasma levels of coagulation factor VII (fVII) are independently linked to risks for coronary heart disease (Kelly, Lyall et al. 2002), cardiovascular death (Dahlgren, Janson et al. 1994) and atherosclerosis due to its affinity to lipoproteins (Dahlgren, Janson et al. 1994). Elevated fibrinogen concentrations have been linked with coronary artery disease (Dahlgren, Janson et al. 1994) and MI (Yildiz, Haznedaroglu et al. 2002, Karakurt, Gumus et al. 2008) and they are a known risk factor for atherosclerosis (Nasiek, Kos-Kudla et
Fibrinogen may promote thrombosis because it affects platelet aggregability and increases plasma viscosity (Nasiek, Kos-Kudla et al. 2007).

Studies on fVII in PCOS have yielded conflicting results. One study reported a significantly higher mean concentration of fVII antigen (Dahlgren, Janson et al. 1994), whereas others observed no significant differences compared to controls (Kelly, Lyall et al. 2002, Yildiz, Haznedaroğlu et al. 2002). Positive correlations to insulin in PCOS and triglycerides in both PCOS and controls with fVII antigen were noted (Dahlgren, Janson et al. 1994). A recent study evaluating tissue factor, the receptor for coagulation fVII, revealed elevated levels in lean women with PCOS compared to lean controls, that positively correlated to androgens and inversely to insulin sensitivity (Gonzalez, Kirwan et al. 2013).

Fibrinogen levels in PCOS were observed to be similar (Kelly, Lyall et al. 2002, Yildiz, Haznedaroğlu et al. 2002, Andiran, Yordam et al. 2005, Slopien, Lewandowski et al. 2006, Nasiek, Kos-Kudla et al. 2007, Karakurt, Gümüş et al. 2008, Kebapcilar, Taner et al. 2009, Pamuk, Torun et al. 2010, Glintborg, Sidelmann et al. 2015), lower than (Dahlgren, Janson et al. 1994) or higher than (Atiomo, Bates et al. 1998, Erdogan, Karadeniz et al. 2008, Heutling, Schulz et al. 2008, Bagir, Bakiner et al. 2016) those of controls. No correlation between fibrinogen and free testosterone levels in PCOS was observed (Kebapcilar, Taner et al. 2009), whereas a direct association between fibrinogen and insulin levels was noted (Dahlgren, Janson et al. 1994).

Earlier studies evaluating fibrinogen in PCOS would also have benefited from larger sample sizes and/or weight- or age-matched controls (Atiomo, Bates et al. 1998, Kelly, Lyall et al. 2002, Andiran, Yordam et al. 2005, Slopien, Lewandowski et al. 2006, Karakurt, Gümüş et al. 2008). Smoking and OCP are known to increase fibrinogen levels (Dahlgren, Janson et al. 1994).
Some studies investigated only OCP effects (Yildiz, Haznedaroglu et al. 2002, Słopień, Lewandowski et al. 2006, Karakurt, Gumus et al. 2008) or did not clarify confounders such as smoking status (Erdogan, Karadeniz et al. 2008).

### 2.3.2 Coagulation Screening Tests

Assessing PT, APTT and TT in PCOS can assist in evaluating the functionality of clotting pathways in the coagulation cascade where abnormal results may be associated with a procoagulant state. PT assesses the extrinsic (and common) coagulation pathway, APTT measures factors of the intrinsic (and common) pathway of the coagulation cascade and TT evaluates for fibrin formation and inhibitors of thrombin.

Studies undertaken to date that looked at PT and APTT in females with PCOS found no significant difference in the times measured when compared to controls (Yildiz, Haznedaroglu et al. 2002, Andiran, Yordam et al. 2005, Karakurt, Gumus et al. 2008, Kebapcilar, Taner et al. 2009, Guldas, Altinkaya et al. 2015). Two of these studies (Yildiz, Haznedaroglu et al. 2002, Andiran, Yordam et al. 2005) also evaluated the TT in this population group and observed no statistically significant differences when compared to controls. Outcomes obtained may have been influenced by limitations of some of the studies including a low sample size (Karakurt, Gumus et al. 2008) and significant differences in age and weight between PCOS and controls (Andiran, Yordam et al. 2005). Kebapcilar et al looked at correlation figures between coagulation parameters and hormonal markers and did not find an association between PT and APTT with testosterone (Kebapcilar, Taner et al. 2009). No correlation studies with metabolic parameters in PCOS were completed for PT, APTT or TT.
2.3.3 Other Coagulation Factors

Only a few studies have evaluated other individual clotting factors within the coagulation cascade. Many of these also revealed contradictory findings with one noting elevated factor VIII levels in PCOS when compared to controls (Glueck, Wang et al. 2004) and the other observed comparable levels between the two groups (Manneras-Holm, Baghaei et al. 2011). Yildiz et al. also assessed factors II (thrombin), V and X and found no significant difference between women with the syndrome and controls (Yildiz, Haznedaroglu et al. 2002).

Further studies assessing coagulation factor fVII and fibrinogen, and potentially other coagulation cascade factors and pathways and continuing to use adequately powered and robust study protocols and correlating to metabolic and/or hormonal parameters, are necessary to address the inconsistency of results of studies published to date. Inclusion of markers of subclinical in vivo coagulation-mediated thrombosis, such as PF1 & 2 (Ota, Wada et al. 2008), would clarify the role of coagulation in PCOS-associated cardiovascular risk. Apart from my paper (Burchall, Piva et al. 2016), I am unaware of any published studies investigating PF1 & 2 in PCOS.

2.4 PCOS, Inhibitors of Coagulation, Fibrinolytic System and Global Haemostatic & Fibrinolytic Markers

The fibrinolytic system and inhibitors of coagulation act to control excessive or inappropriate thrombotic effects. These systems may be important in PCOS for several reasons. Suppression of the fibrinolytic system has been associated with endothelial dysfunction and with the development of CVD (Yildiz, Haznedaroglu et al. 2002). If a procoagulant state is
demonstrated in PCOS within the primary and/or secondary haemostatic systems, it is important to identify whether this/these is/are adequately counteracted by fibrinolytic activation and inhibitors of coagulation. This can be accomplished by examining the literature for individual factors within these systems in women affected by the syndrome but importantly following a review of global markers of haemostasis and fibrinolysis (Figure 2.1).

2.4.1 Coagulation Inhibitors

Individuals heterozygous for protein C, protein S and AT deficiencies are known to have increased risks of thrombosis (Tsanadis, Vartholomatos et al. 2002). AT levels/activity in PCOS are either similar to (Yildiz, Haznedaroglu et al. 2002, Andiran, Yordam et al. 2005) or higher than (Oral, Mermi et al. 2009) those of women who are not affected by this syndrome. Protein C, protein S, and activated protein C percentages have been shown to be comparable to those of controls (Oral, Mermi et al. 2009). Relationships between these haemostatic parameters and metabolic or hormonal markers have not been explored.

Gaps in the literature include the need for larger sample sizes assessing these haemostatic markers in PCOS and correlations between these and hormonal or metabolic PCOS features. Thrombomodulin, which stimulates Protein C activation, has been found to be elevated in women with PCOS versus matched controls (Oral, Mermi et al. 2009).

2.4.2 Thrombin Generation (TG)

Increased thrombin generation is linked to increased risks for both arterial and venous thrombosis (Aziz, Sidelmann et al. 2015). Few studies have been completed to date, apart from my paper (Burchall, Piva et al. 2016), that have evaluated TG or thrombin potential in females with PCOS. de Mendonca-Louzeiro et al have shown a shorter thrombin generation lag-time (time of the lag-phase that follows after addition of activating agent and generation of
thrombin) in females with PCOS compared to age- and BMI-matched controls, that can lead to a hypercoagulable state (de Mendonca-Louzeiro, Annichino-Bizzacchi et al. 2013). Others have noted longer TG lag-time however increased endogenous thrombin potential (noting the total amount of thrombin formed) and peak generation of thrombin in PCOS compared to controls, independent of other risk factors of CVD (Glintborg, Sidelmann et al. 2015). TG measures were related to CRP, fibrinogen, trunk-fat and SHBG (Glintborg, Sidelmann et al. 2015).

Further research is needed to evaluate the recently developed TG test that is currently only used in research settings (Pabinger and Ay 2009, de Mendonca-Louzeiro, Annichino-Bizzacchi et al. 2013). However results so far indicate potential increased generation of thrombin that can have detrimental effects on the haemostatic system including increased fibrin formation as well as increased platelet activation, as thrombin is a well-known and powerful platelet agonist (McNicol and Robson 1997). Thrombin has also been involved in the development of atherosclerosis, endothelial dysfunction and increased TG measures are noted in patients with increased risks for VTE (Borissoff, Spronk et al. 2009, Pabinger and Ay 2009, Glintborg, Sidelmann et al. 2015).

2.4.3 Tissue Plasminogen Activator

Elevated tPA levels in PCOS have been shown to correlate with coronary artery disease and independently with chronic heart disease (Kelly, Lyall et al. 2002). In general, tPA levels are elevated in PCOS (Kelly, Lyall et al. 2002, Sills, Drews et al. 2003, Lin and Yongmei 2008) or only in obese PCOS (Lindholm, Bixo et al. 2010) although others have shown no significant differences compared to controls (Carmassi, De Negri et al. 2005, Manneras-Holm, Baghaei et al. 2011), despite significant differences in IR between groups in one study (Carmassi, De
Negri et al. 2005). tPA levels have also been shown to be higher in PCOS compared to controls even after adjustment for insulin sensitivity (Kelly, Lyall et al. 2002). These levels have also been correlated with fasting insulin (Lin and Yongmei 2008) and an inverse correlation between the insulin sensitivity index and tPA has been suggested (Kelly, Lyall et al. 2002). No correlation between tPA and total testosterone has been observed (Kelly, Lyall et al. 2002, Lin and Yongmei 2008). Greater inclusion of both age- and BMI-matched controls in some studies (Kelly, Lyall et al. 2002, Carmassi, De Negri et al. 2005) as well as information on relevant medication usage (Lin and Yongmei 2008) would assist interpretation.

While tPA levels appear elevated in PCOS, research has shown that these predominantly reflect tPA-PAI-1 complexes, formed due to elevated PAI-1 levels (Carmassi, De Negri et al. 2005, Lin and Yongmei 2008). It is unclear whether elevated tPA levels are linked with elevated insulin levels. Although a link may be plausible, especially following observations of a reduction in tPA antigen levels in PCOS following metformin treatment (Kelly, Lyall et al. 2002), the data are conflicting. Further research is thus necessary, including studies comparing tPA levels in BMI- (and age-) matched insulin-sensitive and IR PCOS women both pre- and post-pharmacological insulin and androgen modulation.

2.4.4 Plasminogen

Although a significant member of the fibrinolytic system, only two studies have been published assessing plasminogen in PCOS (apart from my paper Burchall et al. 2016), one showing similar (Yildiz, Haznedaroğlu et al. 2002) while the other lower activity compared to controls (Slopien, Lewandowski et al. 2006) with no correlation to metabolic markers completed. Additional research is needed to assess plasminogen in PCOS and should incorporate correlation analyses to identify any links with the hormonal and metabolic systems.
2.4.5 Global Fibrinolytic Capacity

GFC is a superior assay for identifying net fibrinolytic potential rather than examining individual fibrinolytic components (Yildiz, Haznedaroglu et al. 2002). A reduced GFC is associated with a number of CV risk factors in healthy individuals (Yildiz, Haznedaroglu et al. 2002).

GFC has been shown to be significantly lower in PCOS versus controls (Yildiz, Haznedaroglu et al. 2002). It has also been shown to be significantly lower in young girls with PCOS compared to young girls controls (Andiran, Yordam et al. 2005). Limitations of this study however included a small sample size and a lack of age- and BMI-matched controls. A positive correlation between GFC level and median fasting glucose:insulin ratio has been observed, with a negative correlation between GFC and both free testosterone and fasting insulin levels (Andiran, Yordam et al. 2005). In contrast, no correlation between GFC and IR parameters (fasting, insulin, HOMA-IR, and fasting glucose:insulin levels) has also been observed, and a negative correlation with testosterone and free testosterone levels (Yildiz, Haznedaroglu et al. 2002).

Although it is an effective and sensitive method for assessing fibrinolytic activity in large study populations (Yildiz, Haznedaroglu et al. 2002), further research is needed on the accuracy and clinical relevance of the GFC. More research is also needed to assess GFC in PCOS and relationships to hormonal and metabolic markers, given the small sample sizes and inconsistent findings to date.
2.4.6 D-Dimers (DD)

DD levels, as a marker of fibrin formation and degradation, have been shown to be elevated in PCOS by some (Kebapcilar, Taner et al. 2009, Oral, Mermi et al. 2009) though unchanged in other studies (Yıldız, Haznedaroğlu et al. 2002, Andiran, Yordam et al. 2005, Karakurt, Gumus et al. 2008, Manneras-Holm, Baghaei et al. 2011, de Mendonça-Louzeiro, Annichino-Bizzacchi et al. 2013) when compared to controls. A correlation between DD levels and free testosterone levels in PCOS have been observed, whereas relationships with IR have not been explained (Kebapcilar, Taner et al. 2009). Further studies to confirm the levels of DD in women with PCOS in comparison to controls and the clinical significance of these are warranted (Wannamethee, Lowe et al. 2005, Oral, Mermi et al. 2009). Where elevated DD levels occur in PCOS, these are likely to represent increased subclinical fibrin generation, rather than elevated fibrin degradation, because the global fibrinolytic capacity is reduced.

All in all inhibitors of coagulation, fibrinolytic system markers and global indicators of haemostasis and fibrinolysis appear poorly explored with significant further robust research studies required to accurately evaluate these markers and correlate to the aberrant metabolic and hormonal profiles in women with PCOS.

### 2.5 PCOS and Inhibitors of Fibrinolysis

Important inhibitors of fibrinolysis include PAI-1, α2 antiplasmin, α2 macroglobulin and TAFI. PAI-1 (discussed previously in section 2.2.2) and TAFI may be relevant in PCOS given their
The Haemostatic System in PCOS

links to CVD risk and to venous thrombosis (van Tilburg, Rosendaal et al. 2000, Chen, Lee et al. 2005) (Figure 2.1).

2.5.1 \(\alpha_2\) antiplasmin and \(\alpha_2\) macroglobulin

\(\alpha_2\) antiplasmin is of significance in the population of interest indirectly because of its inhibitory effects on plasmin. There have been three studies published evaluating \(\alpha_2\) antiplasmin activity in PCOS. No significant difference in comparison to controls were observed in females with PCOS (Atiomo, Fox et al. 2000, Yildiz, Haznedaroglu et al. 2002) or in obese females with the syndrome (Slopien, Lewandowski et al. 2006) versus those of controls. No correlation analyses have been completed for the marker with components of the metabolic or hormonal systems. Comments in relation to the study design of these research studies have been described earlier.

Studies that looked at \(\alpha_2\) macroglobulin levels in PCOS revealed contradictory findings with a small study of 12 PCOS and 12 controls noting reduced levels in the former versus the latter (Insenser, Martinez-Garcia et al. 2010) however a more recent study noted no significant reductions in the marker in women affected by the syndrome when compared to controls (Haoula, Shaw et al. 2014).

In conclusion, \(\alpha_2\) antiplasmin and \(\alpha_2\) macroglobulin levels do not appear to be significantly different between PCOS and controls.

2.5.2 TAFI

TAFI levels/activity in PCOS have been shown to be elevated (Karakurt, Gumus et al. 2008, Oral, Mermi et al. 2009, Guldas, Altinkaya et al. 2015) or similar to controls (Erdogan, Karadeniz et al. 2008, de Mendonca-Louzeiro, Annichino-Bizzacchi et al. 2013), whereas thrombomodulin, a cofactor responsible for activating TAFI, is also elevated in PCOS (Oral,
Mermi et al. 2009). Correlations between TAFI with insulin and HOMA-IR were observed in some studies (Oral, Mermi et al. 2009, Guldas, Altinkaya et al. 2015) but not in another (Erdogan, Karadeniz et al. 2008). A multiple regression analysis failed to find any significant effect of HOMA-IR on TAFI levels (Guldas, Altinkaya et al. 2015). On the other hand, a positive correlation between TAFI and total cholesterol and LH but a negative link with high-density lipoprotein cholesterol have been noted (Oral, Mermi et al. 2009). Inclusion of larger samples sizes would have been appropriate as well as inclusion of BMI-matched PCOS and controls in one study (Karakurt, Gumus et al. 2008).

If a hypofibrinolytic state is confirmed in PCOS, reflected by increased TAFI (and PAI-1) levels, the aetiology will need to be explored further. TAFI is synthesized in the liver (Karakurt, Gumus et al. 2008), although some reports speculate a megakaryocyte/platelet origin with subsequent release into plasma following platelet activation (Schadinger and Boffa 2009). The studies described previously indicating a potential for aberrant platelet activation/function (Zwirska-Korczala, Sodowski et al. 2008, Oktem, Ozcimen et al. 2009) in PCOS suggest a possible link to increased TAFI levels. Likewise, studies to confirm elevated TAFI levels in PCOS are required with larger sample sizes and explanations of relationships with the abnormal metabolic and hormonal systems.

2.6 Conclusions

PCOS is a highly prevalent condition in the reproductive-aged female population and is a complex disorder with significant clinical heterogeneity, making both diagnosis and treatment challenging. PCOS includes metabolic features, endothelial dysfunction, early atherosclerosis, and an increased risk of CVD development. Haemostatic abnormalities play an important role
in CVD. Exploration of the haemostatic system is therefore important in PCOS to identify its contribution to CVD.

A review of the literature has suggested abnormalities of the haemostatic system in PCOS, including abnormal platelet function/activation following observations of high sCD40L and potentially MPV. Also, endothelial dysfunction reflected mainly by elevation in ADMA and PAI-1, potential increased generation of thrombin, increased tPA levels most likely reflecting tPA–PAI-1 complexes, and enhanced inhibition of fibrinolysis reflected by high PAI-1 antigen and/or activity and potentially elevated TAFI levels.

Earlier studies included small sample sizes and lacked BMI- and age-matched controls, which are known to affect the haemostatic system, however more recent studies appear to be more robust. The existing literature however is still limited, with few studies published and contradictory findings relating to markers of haemostasis in women with PCOS remain. Correlations between haemostatic parameters and markers of the metabolic and hormonal systems also reveal inconsistent findings for most of the research completed.

Hence, the hypothesis of haemostatic abnormalities contributing to disturbed haemostasis including a hypofibrinolytic capacity and/or net prothrombotic state in PCOS remains unresolved. Further research on the haemostatic system in PCOS is critical and must comprehensively and in a systematic manner using well-designed studies assess the system in this important syndrome. Whether haemostatic abnormalities are linked to the abnormal hormonal and/or metabolic system/s in PCOS also remains to be elucidated, as these relationships have only been explored well at present with markers of endothelial function. Such research may inform on the underlying aetiology of PCOS and may lead to novel preventative or therapeutic measures targeting metabolic features of PCOS underlying CVD.
Evaluation of the effects on the haemostatic system following administration of current management strategies to modulate androgens and IR including metformin, OCP, and dietary and lifestyle modifications in PCOS may facilitate further insights into probable disturbed haemostasis and into relationships with hormonal and metabolic markers in PCOS and, in turn, with potential further CVD risk markers.
2.7 My Role

Chapters 1 and 2 contain material published as a paper in Trends in Cardiovascular Medicine, however for the purposes of this thesis, to improve structure and understanding, the material in this paper was divided into two chapters with addition of relevant information researched and acquired during my candidature. A full version of the manuscript can be found on the following pages of this thesis. As the first author of this manuscript, I contributed to the conception and design of the review that aimed to comprehensively evaluate the literature for haemostatic markers in women with PCOS. I regularly liaised with my PhD supervisors and other co-authors to develop sound literature evaluation. I independently researched, reviewed and analysed the articles to determine eligibility and risk for bias.

Following consultation I independently performed literature (and data therein) evaluation and contributed to data analysis and interpretation. I was responsible for submission for publication. This manuscript addressed key gaps relating to the haemostatic system in PCOS and relationships to hormonal and metabolic status. It informed my PhD work to address these gaps.
Hemostatic Abnormalities and Relationships to Metabolic and Hormonal Status in Polycystic Ovarian Syndrome

Genia Burchall, Matthew D. Linden, Helena Teede, and Terrence J. Piva

Polycystic ovarian syndrome (PCOS), diagnosed based on hyperandrogenism, ovulatory dysfunction, and polycystic ovaries, is one of the most common disorders of reproductive-aged females. Etiology includes both genetic and environmental/lifestyle factors contributing to both insulin resistance and hyperandrogenism. Clinically, PCOS has reproductive, psychological, and metabolic features, the latter predisposing to cardiovascular disease (CVD). Hemostatic abnormalities have an association with and a demonstrated pathophysiologic role in CVD in non-PCOS populations but have not been adequately explored in PCOS. This review focuses on the hemostatic system in PCOS, exploring also relationships to the metabolic and hormonal abnormalities of the syndrome, and aims to identify whether hemostatic abnormalities are present as potential contributors to increased cardiovascular risk. Ultimately, this area may reveal preventative and therapeutic opportunities, which could improve the cardiovascular health of women with PCOS. (Trends Cardiovasc Med 2011; 21(6–14))

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Introduction

Polycystic ovarian syndrome (PCOS) is a condition diagnosed based on ovulation, hyperandrogenism, and polycystic ultrasound appearance of ovaries (Teede et al. 2011). PCOS also presents with metabolic abnormalities, including insulin resistance (IR), hyperinsulinemia, and dyslipidemia, and increased risk for diabetes and cardiovascular disease (CVD) (Teede et al. 2011). The exact cause(s) of PCOS remains to be elucidated. It is a complex disease believed to be similar to diabetes mellitus, rheumatoid arthritis, and CVD; a combination of genetic predisposition and environmental factors contributes to the phenotypic expression of this heterogeneous disorder (Table 1) (Teede et al. 2010). PCOS is one of the most common disorders of females of reproductive age, with up to 20% of women affected (March et al. 2010).

Given its prevalence and significant clinical complications, PCOS presents a major burden to its sufferers and the health care system (Teede et al. 2011). In 2005, PCOS was estimated to cost the Australian health care system approximately $400 million per year (Aziz et al. 2005). Given the underestimated prevalence from this initial evaluation (~6%), the financial burden is currently significantly higher (March et al. 2010).

Recent data suggest an additional clinical complication of elevated cardiovascular risk in PCOS, especially relating to disturbed hemostasis. In this review, we discuss the pathogenesis of the syndrome, including a summary of the clinical presentations, diagnosis, and current treatments. We also evaluate the evidence for a relationship between PCOS and disturbed hemostasis in order to identify possible links with CVD. Relationships between hemostatic markers and metabolic and hormonal parameters are also identified in women with PCOS. This may help further our understanding of the pathophysiology of metabolic disturbance in this common and complex disease.

Pathogenesis

Hyperandrogenism

PCOS has a complex pathogenesis with a chain reaction of events that ultimately feed back to further aggravate clinical features. Hyperandrogenism, the predominant feature of the syndrome, is primarily of ovarian origin (Aziz 2009) with some contribution from the adrenal gland. Increased luteinizing hormone (LH) activity from the pituitary gland has been observed in PCOS and may be involved in the pathogenesis of the hormonal imbalance of the disease.

Insulin Resistance and Hyperinsulinemia

IR, which is observed in approximately 50–80% of women with PCOS, contributes to the pathophysiology of this syndrome (Aziz 2009). Insulin stimulates secretion of androgens by the ovarian theca cells. In PCOS, these cells appear to be hyper-responsive to insulin in secreting androgens (Aziz 2009). Reciprocally, hyperandrogenism may act on vascular tissues to induce an IR state (Carmassi et al. 2005). IR has also been linked with an abnormal lipid profile in PCOS compared to more insulin-sensitive controls. This is probably due to...
Table 1. Genes considered to play a role in PCOS

<table>
<thead>
<tr>
<th>Physiological process</th>
<th>Genes that may be affected in PCOS</th>
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<tr>
<td>Gonadotropin secretion</td>
<td>Activin receptor 1, activin receptor 2A, activin receptor 2B, follicle-stimulating hormone receptor, follistatin, inhibin subunit A, inhibin subunit Bα, inhibin subunit Bβ, inhibin subunit C, luteinizing hormone β subunit, luteinizing hormone/choriogonadotropin receptor, Müllerian inhibiting substance, sex hormone-binding globulin, mothers against DPP homolog 4</td>
</tr>
<tr>
<td>Androgen production and secretion</td>
<td>Androgen receptor; bone morphogenetic protein; growth differentiation factor 9; cytochrome P450, family 11, subfamily A polypeptides; cytochrome P450, family 17, subfamily A, polypeptide 1; cytochrome P450, family 19, subfamily A, polypeptide 1; hydroxyl-δ-5-steroid dehydrogenase; 16α- and steroid δ-isomerase 1; hydroxyl-δ-5-steroid dehydrogenase; 16α- and steroid δ-isomerase 2; hydroxysteroid (17β)-dehydrogenase 1; hydroxysteroid (17β)-dehydrogenase 2; hydroxysteroid (17β)-dehydrogenase 3; steroidogenic acute regulator</td>
</tr>
<tr>
<td>Insulin secretion and obesity</td>
<td>Adiponectin, calcitonin, insulin gene variant number of tandem repeats, insulin receptor, insulin receptor substrate 1, insulin receptor substrate 2, insulin-like growth factor 1, insulin-like growth factor 2, insulin-like growth factor 1 receptor, insulin-like growth factor 2 receptor, insulin-like growth factor binding protein 1 plus insulin-like growth factor binding protein 3, peroxisome proliferator-activated receptor γ, Leptin, insulin-like protein, leptin receptor, leptin, melanocortin-4 receptor, tumor necrosis factor, uncoupling protein 2, uncooping protein 3, proopiomelanocortin</td>
</tr>
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</table>

Adapted from Utzauw (2007).

Excessive hypersecretion of apolipoprotein B and very low-density lipoprotein from the liver following insulin stimulation, ultimately resulting in hypertriglyceridemia (Alexander et al. 2009). Low levels of high-density lipoprotein have also been reported in PCOS (Bernois et al. 2007). Insulin sensitizing agents such as metformin and lifestyle interventions that reduce IR have been shown to ameliorate the clinical findings of PCOS (Teede et al. 2011). In contrast, obesity, which increases IR, exacerbates PCOS (Teede et al. 2010).

Obesity

Women affected by PCOS are more likely to be overweight/three than non-PCOS women, and the incidence and severity of this syndrome increase with increased weight (Teede et al. 2011). Obesity exacerbates both IR and hyperandrogenism. Although some studies have found abnormal fasting insulin levels and IR only in obese PCOS females (Rajendran et al. 2000), most studies have observed these abnormal metabolic profiles in lean counterparts (Dercé et al. 2003, Ozgur et al. 2008). Two-thirds of normo-obese PCOS females are reported to have excessive body fat and central visceral adiposity inducing greater IR (Casella et al. 2008).

Clinical and Diagnostic Features

Clinical manifestations of PCOS are highly variable and may include reproductive, metabolic, as well as psychological features. PCOS features can vary significantly, creating a heterogenous condition in which diagnosis is based on the presence of only two of the following three clinical features: hyperandrogenism (clinical or biochemical), ovulatory and menstrual disturbance, and polycystic ovaries (PCO) on ultrasound (Figure 1) (Teede et al. 2011).

In the majority of women affected by PCOS, the normal menstrual cycle is disturbed. Although the etiology of PCOS is multifactorial and poorly understood, it is known that IR and potentially elevated LH induce excessive androgen production by the ovarian theca cells leading to a failed ovulation process, with a lack of full ovarian follicular maturation and rupture. The ovary then appears enlarged with multiple underdeveloped follicles. The inclusion of “poly-cystic” within the name of the syndrome is an inaccurate representation. These features are not cysts but, rather, follicles that failed to ovulate. PCO also appear secondary to hormonal imbalance rather than being a primary pathogenic mechanism in PCOS.

Treatment

The current management strategies used to ameliorate the symptoms of PCOS are highly variable, in line with the clinical heterogeneity of the syndrome (Table 2) (Teede et al. 2011). The frequently prescribed oral contraceptive pills (OCPs) present with the well-accepted risk of venous thromboembolism. This risk is further amplified because many women with PCOS are overweight (Teede et al. 2011). OCP use is also inappropriate for women wishing to conceive and those who have side effects. Metformin has been shown to improve IR and menstrual cycles and appears to improve fertility; however, further study is needed to define its role in PCOS management (Teede et al. 2011). In summary, the treatment of PCOS remains controversial, and further research is needed (Teede et al. 2010, 2011).

PCOS, CVD, and Hemostasis

PCOS is underpinned by genetic IR, environmental IR (obesity related), as well as metabolic abnormalities in the majority of cases (Teede et al. 2011). Women with PCOS also have evidence of subclinical CVD (Teede et al. 2011) and appear to...
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![Diagram](image)

Figure 1. Summary of the biology and clinical features of PCOS. Adapted from Teede et al. (2011).

have an increased risk of cardiovascular (CV) events and death (Teede et al. 2011), especially when combined with other risk factors, such as obesity and hypertension (Wild et al. 2010). Almost half of women with PCOS are affected by metabolic syndrome (Alexander et al. 2009), and diabetes, a major CV risk factor, is increased fourfold in PCOS (Moran et al. 2006). Other features associated with metabolic syndrome may occur in PCOS, including a proinflammatory and prothrombotic state, both of which have well-accepted links to CV (Alexander et al. 2009).

The underlying mechanism(s) of increased cardiometabolic risk in PCOS is unclear. Although IR is clearly a candidate, studies have shown that both obese and lean women with PCOS, both hyperinsulinemic and those with ambient insulin levels, have increased CVD risk (Macintyre et al. 2001). Hyperandrogenemia (with IR) is also linked with increased metabolic and CV morbidity in PCOS (Chartier et al. 2008). An elevated testosterone level has also been shown to be an independent risk factor for myocardial infarct (MI) and coronary atherosclerosis (Kebapci et al. 2009). Others have reported contrary findings indicating either a neutral or a beneficial effect of androgens on the vasculature (Huttunen et al. 2000, Worboys et al. 2001). There have also been reports of increased inflammatory markers in PCOS, including C-reactive protein (Boulman et al. 2004), and endothelial dysfunction, arterial stiffness, and early atherosclerosis as surrogate indicators of CV risk.

Table 2. Management therapies used to treat women with PCOS

<table>
<thead>
<tr>
<th>Clinical feature or complications observed in PCOS</th>
<th>Management strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomenorrhea/amenorrhea</td>
<td>Lifestyle change (5%-10% weight loss plus structured exercise)</td>
</tr>
<tr>
<td></td>
<td>Oral contraceptive pill (OCP) (low estrogen doses e.g. 20 μg) may have less impact on insulin resistance</td>
</tr>
<tr>
<td></td>
<td>Cyclic progesterin (eg, 10 mg medroxyprogesterone acetate 10-14 days every 2 or 3 months)</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>Metformin (improves ovulation and menstrual cyclicity)</td>
</tr>
<tr>
<td></td>
<td>Choice of options depends on patient preferences, impact on well-being, and access and affordability</td>
</tr>
<tr>
<td></td>
<td>Self-administered and professional cosmetic therapy are first line (laser recommended)</td>
</tr>
<tr>
<td></td>
<td>Ellaborate cream can be added and may induce a more rapid response</td>
</tr>
<tr>
<td></td>
<td>If cosmetic therapy is not adequate, pharmacological therapy can be considered</td>
</tr>
<tr>
<td></td>
<td>Pharmacological therapy</td>
</tr>
<tr>
<td></td>
<td>Medical therapy if patient is concerned and cosmetic therapy is ineffective/inaccessible/unaffordable</td>
</tr>
<tr>
<td></td>
<td>Primary therapy is the OCP (monitor glucose tolerance in those at risk of diabetes)</td>
</tr>
<tr>
<td></td>
<td>Anti-androgen therapy (eg, spironolactone or cyproterone acetate) should not be used without adequate contraception</td>
</tr>
<tr>
<td></td>
<td>Trial therapies for ≥6 months before changing dose or medication</td>
</tr>
<tr>
<td>Infertility</td>
<td>Combination therapy: If ≥6 months of OCP is ineffective, add anti-androgen to OCP (twice daily spironolactone &gt;50 mg or cyproterone acetate 25 mg/day, days 1-10 of OCP)</td>
</tr>
<tr>
<td></td>
<td>Lifestyle intervention (to optimize preconception health and fertility and reduce pregnancy and long term complications)</td>
</tr>
<tr>
<td></td>
<td>Advice on folate, smoking cessation, and optimal weight and exercise before conception</td>
</tr>
<tr>
<td></td>
<td>Given age-related infertility, advise women to optimize family initiation</td>
</tr>
<tr>
<td></td>
<td>Infertility therapies including clomiphene citrate, metformin, gonadotrophins, surgery, and in vitro fertilization</td>
</tr>
<tr>
<td>Cardiometabolic risk</td>
<td>Lifestyle change: ≥5% weight loss in those who are overweight reduces diabetes risk by ~50%-60% in high-risk groups</td>
</tr>
<tr>
<td></td>
<td>Optimize cardiovascular risk factors</td>
</tr>
<tr>
<td></td>
<td>Consider metformin (reduces the risk of diabetes by ~50% in adherent high-risk groups)</td>
</tr>
</tbody>
</table>

Adapted from Teede et al. (2011).
The Haemostatic System in PCOS

Figure 2. Relationships and interactions between endothelial function, platelet activation and aggregation, inflammation, coagulation, and thrombosis in PCOS. Studies have shown increased platelet numbers and abnormal function, inhibition of endothelial function, and inhibition of thrombolytic in PCOS resulting in a net prothrombotic phenotype: ADR, adenine diphosphate; ADMA, asymmetric dimethylarginine; CD40L, CD38 ligand; PPAR-α, peroxisome proliferator-activated receptor-α; PAR-1, protease-activated receptor; PROACTIN: C5a, activated protein C and protein S complex; IPA, tissue plasminogen activator; TAFI, thrombin-activated fibrinolysis inhibitor; TTM, thrombin-thrombomodulin complex; TMAp, thromboxane A2; YWPF, Von Willebrand factor.

damage (Moran et al. 2009, Teede et al. 2011). A large number of PCOS females are obese or have excess weight that can both directly and indirectly increase CV risk, with increased IR, hyperandrogenemia, dyslipidemia, and potential activation of the hemostatic system (Azziz 2009, Teede et al. 2011).

Further research is needed, but OCP may also place women with PCOS at an increased risk of developing CV problems through increased risk of arterial thrombotic events, arterial dysfunction (Meyer et al. 2007), and IR (Charniaux et al. 2008, Meyer et al. 2007), which may increase the risk of type 2 diabetes.

Potentially disturbed hemostasis may also underpin cardiometabolic abnormalities in PCOS. Although no epidemiological studies in sites demonstrate a clinically evident increased venous thrombotic risk, epidemiological studies in PCOS are limited. It was found that 20% of PCOS females have a positive family history of venous thrombosis compared to 8% of controls, but further research is needed (Aitken et al. 2000a). Shaw et al. (2008) observed that the incidence of CVD was elevated in PCOS patients compared to controls.

Arterial thrombosis is the usual cause of strokes and MI, and disturbed hemostasis may contribute to CVD risk in PCOS. The hemostatic system is also integrally linked to the endothelium and vessel wall (Figure 2). Demonstrated endothelial dysfunction and vessel wall functional and structural abnormalities seen in PCOS may also be linked pathophysio logically to disturbed hemostasis in PCOS (Moran et al. 2009).

Ultimately, a greater understanding of the potential pathophysiologic role that hemostatic factors play in the cardiometabolic features may make way for novel therapeutic measures in PCOS. This is particularly important because women are becoming increasingly obese, living longer, and experiencing higher rates of diabetes and because many women in developed countries die of CV-related diseases (Oral et al. 2008).

• PCOS and Primary Hemostasis

Assessment of primary hemostasis can be undertaken by evaluating its principal components, namely platelets and the vascular endothelium (Figure 2).

Platelet Volume, Number, and Function

Increased mean platelet volume (MPV) is considered an independent risk factor for CVD (Gursoy et al. 2006). It is also well accepted that increased platelet numbers are linked to increased CVD risks.

Two studies have demonstrated elevated platelet volume in PCOS versus controls (Gursoy et al. 2006, Kebapcilar et al. 2009). A positive correlation between MPV and IR was observed by Kebapcilar et al. but not by Gursoy et al. Differences in the relationship between MPV and testosterone levels were also observed in these studies.

Platelet numbers were shown to be high compared to those of controls in one study (Laguer-Ramirez et al. 2009) and unchanged in others (Derkil et al. 2003, Gursoy et al. 2006, Kebapcilar et al. 2009). No correlations between platelet numbers and free testosterone levels were observed in one study (Kebapcilar et al. 2009).

Investigation of platelet activation and exocytosis and abnormal thrombotic stimulus (on platelets) can be performed using soluble CD40 ligand (sCD40L), which is a thrombostimulatory marker. With 95% of its circulating levels originating from activated platelets (Oktém et al. 2008), elevated sCD40L levels represent an indirect marker of in vivo platelet activation. Elevated sCD40L levels may represent ongoing subclinical platelet-mediated thrombosis and are highly correlated with cardiovascular outcome.

sCD40L levels in PCOS were elevated compared to controls (Oktém et al. 2009, Zvirn ska-Koczala et al. 2008). Oktém et al. found that sCD40L levels were affected independently of the metabolic (fasting insulin and HOMA-IR) or hormonal (total and free testosterone) profile in PCOS. However, in another study, no correlation between sCD40L and hyperandrogenemia or IR was observed (Kebapcilar et al. 2010). Although there is disagreement in the literature, data indicate a potential role for abnormal platelet activation, suggesting disturbed hemostasis in PCOS. However, the mechanisms are unclear. The cause of the platelet abnormalities or their aberrant activation is uncertain. Further research is needed to determine the relationships of MPV and platelet numbers to metabolic and hormonal profiles in PCOS. Insulin is believed to affect platelet function, and variation in insulin sensitivity can affect platelet re-

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response to various agonists (Decrii et al. 2003). Platelet dysfunction in PCOS, following impairment of responsiveness to nitric oxide, was also shown to be independent of IR (Rajendra et al. 2009). Further research is necessary to both demonstrate the presence of abnormal platelet aggregation/activation in PCOS compared to matched controls and ascertain whether this is linked to IR. Assessment of platelet activation following OCP administration in PCOS may also identify thrombotic and CV risk factors, which are important considering the frequency of OCP use in PCOS.

**Endothelial Function**

Endothelial dysfunction has been linked with an increased risk of CV events following its key contribution to atherosclerosis (Moran et al. 2009). Endothelial function can be assessed indirectly through measurement of its synthesized products: von Willebrand factor (VWF), asymmetric dimethylarginine (ADMA), and plasminogen activator inhibitor-1 (PAI-1). These products, along with an abnormal endothelium, have all been independently linked to type 2 diabetes, further increasing the potential for CV injury. ADMA is also an independent marker for CV morbidity and mortality (Heuilting et al. 2008).

Our group and others have shown that VWF levels in PCOS are similar to those of controls (Dahlgren et al. 1994, Kelly et al. 2002, Kuczy and Var 2009, Moran et al. 2009). One study showed a positive link between insulin and VWF antigen as well as between the latter and triglycerides, suggesting a potential link between VWF and the metabolic system (Dahlgren et al. 1994). A larger sample size and age-matched controls would have assisted in clarifying these findings (Kelly et al. 2002).

Support for endothelial dysfunction in PCOS is based on the majority of the observations in the literature noting elevated ADMA levels in PCOS (Charitidou et al. 2008, Heulting et al. 2008, Moran et al. 2009, Ostergren et al. 2008, Rajendran et al. 2009), although some have reported no significant differences in ADMA between PCOS and controls (Demir et al. 2007, Pamuk et al. 2010). Results of correlation studies are also conflicting. ADMA levels have been found to correlate with IR markers (Heulting et al. 2008, Moran et al. 2009) and androgens (Heulting et al. 2008), whereas others have not observed any significant links (Charitidou et al. 2008, Demir et al. 2007, Pamuk et al. 2010). It has been suggested that weight may affect ADMA levels (Richstein et al. 2006, Meinzer et al. 2007), but not all studies have used body mass index (BMI)-matched controls (Demir et al. 2007, Rajendran et al. 2009).


Conclusions from many studies that have assessed PAI-1 in PCOS are limited by suboptimal experimental design. Both age and weight are known to affect PAI-1 (De Pergola et al. 1997, Heinrich et al. 1992, Vague et al. 1980). Some studies were neither age nor weight matched (Carmassi et al. 2005, Glauche et al. 2004) or only matched for age (Glauche et al. 2008). In some studies, there was a significant difference in age (Yurrai et al. 2001) or in weight between PCOS and controls (Atiomo et al. 1998). Some have been supported by larger sample sizes (Atiomo et al. 1998, Oral et al. 2008, Sills et al. 2006). Some selected participants other than healthy controls (Dahlgren et al. 1994), and others indicated elevated PAI-1 levels in PCOS versus controls but did not reach statistical significance (P > 0.5) (Macor et al. 2013). One study did not mention whether the PCOS females were on medications known to interfere with PAI-1 (Lin and Yongmei 2008).

Because existing studies are generally of suboptimal quality and their results are conflicting, further research is needed to assess endothelial function and its potential link to the metabolic and/or hormonal profiles in PCOS. It is also important to determine, if a link is demonstrated, whether IR and consequent hyperinsulinemia are a result of endothelial dysfunction or insulin action. Alternatively, the disturbed metabolic system in PCOS may have caused endothelial dysfunction. Insulin induces vasodilation (Carmassi et al. 2005), and high levels may contribute to hyperstimulation of the endothelium, potentially resulting in dysfunction. Studies have shown that PAI-1 declines following metformin administration or weight loss, suggesting that IR is a contributor (Ibanez et al. 2002, Sills et al. 2003). However, intervention studies using OCP reduced ADMA levels in PCOS despite an increase in IR (Charitidou et al. 2008, Heulting et al. 2008). Insulin can also directly affect PAI-1 levels by upregulating its transcription (Sills et al. 2003). Further adequate powered studies and age-matched controls are needed to understand the relationships between endothelial functional markers and the metabolic and hormonal parameters in PCOS. Results from these studies may yield valuable etiological information, potentially providing future therapeutic targets. The relationship between vascular dysfunction and platelet activation in PCOS has not yet been explored.

**PCOS and Secondary Hemostasis**

Assessment of secondary hemostasis in PCOS can be carried out by examining significant coagulation factors within the coagulation pathways (Figure 2).

There is increasing evidence that elevated plasma levels of coagulation factor VII (FVII) are independently linked to risks for coronary heart disease (Kelly et al. 2002), cardiovascular death (Dahlgren et al. 1994), and atherosclerosis due to its affinity to lipoproteins (Dahlgren et al. 1994). Elevated fibrinogen concentrations have been linked to coronary artery disease (Dahlgren et al. 1994) and MI (Karanjuk et al. 2008, Yildiz et al. 2002), and they are a known risk factor for atherosclerosis (Nasiik et al. 2007). Fibrinogen may promote thrombosis be-
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Studies on FVII in PCOS have yielded conflicting results. One showed a significantly higher mean concentration of FVII antigen (Dahlgren et al. 1994), whereas others showed no significant difference compared to controls (Kelly et al. 2002, Yildiz et al. 2002). Positive correlations to insulin in PCOS and triglycerides in both PCOS and controls with Xa antigen were noted (Dahlgren et al. 1994).

D-dimer (DD) levels, as a marker of fibrin formation and degradation, have shown to be elevated in PCOS (Nekhbaylar et al. 2000; Oral et al. 2005; unchained (Andirin et al. 2005; Karakurt et al. 2008, Yildiz et al. 2002) compared to those in controls. Kebapcilar et al. (2005) found a correlation between DD levels and free testosterone in PCOS, whereas relationships with IR have not been explained. Further studies to confirm elevated DD levels and the clinical significance of these are warranted (Oral et al. 2005, Wernsinghoo et al. 2005). Where elevated DD levels occur in PCOS, these are likely to represent increased subclinical fibrin generation, rather than elevated fibrin degradation, because global fibrinolytic capacity is reduced.

Fibrinogen levels in PCOS were observed to be similar to (Andirin et al. 2005; Karakurt et al. 2008, Kebapcilar et al. 2009; Kelly et al. 2002, Nasek et al. 2007, Pamuk et al. 2010, Slopien et al. 2006, Yildiz et al. 2002), lower than (Dahlgren et al. 1994), or higher than (Atkinson et al. 1998, Erdogan et al. 2008, Hentling et al. 2008) those of controls. No correlation between fibrinogen and free testosterone levels in PCOS was observed (Kebapcilar et al. 2000), whereas a direct association between fibrinogen and insulin levels was noted (Dahlgren et al. 1994).

Fibrinogen studies in PCOS would also have benefited from larger sample sizes and/or weight- or age-matched controls (Andirin et al. 2005; Atkinson et al. 1998, Karakurt et al. 2008, Kelly et al. 2002, Slopien et al. 2006). Inclusion of unhealthy controls in one study may have affected outcomes (Atkinson et al. 1998). Smoking and OCPs are known to increase fibrinogen levels (Dahlgren et al. 1994). Some studies investigated only OCP effects (Karakurt et al. 2008, Slopien et al. 2006, Yildiz et al. 2002) or did not clarify confounders such as smoking status (Erdogan et al. 2008).

Further studies assessing coagulation factor VII and fibrinogen, using both weight- and age-matched controls and correlating metabolic and/or hormonal parameters, are necessary to address the inconsistency of results of studies published to date. Inclusion of markers of subclinical in vivo coagulation-mediated thrombosis, such as prothrombin fragments 1 and 2 (PF1&2) (Ota et al. 2008), would clarify the role of coagulation in PCOS-associated cardiovascular risk. To date, no studies investigating PF1&2 in PCOS have been published.

PCOS, the Fibrinolytic System, and Other Inhibitors of Coagulation

The fibrinolytic system and other inhibitors of coagulation act to control the thrombotic effects of markers with the secondary hemostatic system (Figure 2). These systems may be important in PCOS for several reasons. Suppression of the fibrinolytic system has been associated with endothelial dysfunction and with the development of CVD (Yildiz et al. 2002). If a procoagulant state is demonstrated in PCOS within the primary and/or secondary hemostatic systems, it is important to identify whether this is adequately counteracted by fibrinolytic activation and other inhibitors of coagulation.

Protein C, Activated Protein C, Protein S, and Antithrombin

Heterozygous deficiencies of protein C, protein S, and antithrombin are known to increase the risk of thrombosis (Tsiaras et al. 2002). Antithrombin levels/activity in PCOS are either similar to (Andirin et al. 2005, Yildiz et al. 2002) or higher than (Oral et al. 2009) those of controls. Protein C, protein S, and activated protein C percentages have been shown to be comparable to those of controls (Oral et al. 2009). Relationships between these hemostatic parameters and metabolic or hormonal markers have not been explored. Gaps in the literature include the need for larger sample sizes assessing these hemostatic markers in PCOS and correlations between these and hormonal or metabolic PCOS features.

Tissue Plasminogen Activator

Elevated tissue plasminogen activator (tPA) levels in PCOS have been shown to correlate with coronary artery disease and independently with chronic heart disease event rate (Kelly et al. 2002). In general, tPA levels have been shown to be elevated in PCOS (Kelly et al. 2002, Lin and Yongmei 2008, Silber et al. 2003), but one study showed no difference compared to controls, despite significant differences in IR between these groups (Carmassi et al. 2005). tPA levels have also been shown to be higher in PCOS compared to controls even after adjustment for insulin sensitivity (Kelly et al. 2002). tPA levels have also been correlated with fasting insulin (Lin and Yongmei 2008), and an inverse correlation between insulin sensitivity index and tPA has been suggested (Kelly et al. 2002). No correlation between tPA and total testosterone has been observed (Kelly et al. 2002, Lin and Yongmei 2008). Greater inclusion of both age- and BMI-matched controls in some studies (Carmassi et al. 2005, Kelly et al. 2002) as well as information on relevant medication usage (Lin and Yongmei 2008) would assist interpretation.

Although tPA levels appear elevated in PCOS, research has shown that these predominantly reflect tPA-PAI-1 complexes, formed due to elevated PAI-1 levels (Carmassi et al. 2005, Lin and Yongmei 2008). It is unclear whether elevated tPA levels are linked with elevated insulin levels. Although a link may be plausible, especially following observations of a reduction in tPA antigen in PCOS following metformin treatment (Kelly et al. 2002), the data are conflicting. Further research is thus necessary, including studies comparing tPA levels in BMI- and age-matched insulin-sensitive and IR PCOS women both pre- and post-pharmacological insulin and androgen modulation.

Global Fibrinolytic Capacity

Global fibrinolytic capacity (GFC) is a superior assay for identifying fibrinolytic potential rather than examining individual fibrinolytic components (Yildiz)
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et al. 2002). Assessment of the overall fibrinolytic system would identify the net haemostatic effect. A reduced GFC is associated with a number of CV risk factors in healthy individuals (Yildiz et al. 2002).

GFC has been shown to be significantly lower in PCOS versus controls (Yildiz et al. 2002). GFC has also been shown to be significantly lower in young girls with PCOS compared to controls (Andriman et al. 2005). Limitations included the small sample size and a lack of age and BMI-matched controls. A positive correlation between GFC level and median fasting glucose-insulin ratio was observed, with a negative correlation between GFC and both free testosterone and fasting insulin levels (Andriman et al. 2005). In contrast, no correlation between GFC and IR parameters (fasting, insulin, HOMA-IR, and fasting glucose-insulin levels) has been observed; however, a negative correlation with testosteroerone and free testosterone has been observed (Yildiz et al. 2002).

Although it is an effective and sensitive method for assessing fibrinolytic activity in large study populations (Yildiz et al. 2002), further research is needed on the accuracy and clinical relevance of GFC. More research is also needed to assess GFC in PCOS and relationships to hormonal and metabolic markers, given the small sample sizes and inconsistent findings to date.

Plasmogen

Although a significant number of the fibrinolytic system, only two studies have been published assessing plasminogen in PCOS—one showing similar (Yildiz et al. 2002) and the other showing lower activity compared to controls (Sorooga et al. 2006) with no correlation to metabolic markers completed. Additional research is needed to assess plasminogen in PCOS and should again incorporate correlation analyses to identify any links with the hormonal and metabolic systems.

• PCOS and Inhibitors of Fibrinolysis

Principal inhibition of fibrinolysis include PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI) (Figure 2). PAI-1 (discussed previously) and TAFI may be relevant in PCOS given their links to CVD risk and to various thrombosis (Chen et al. 2005, Van Tilburg et al. 2000).

TAFI levels in PCOS have been shown to be elevated (Karakurt et al. 2008, Oral et al. 2000) or similar to controls (Erdogan et al. 2005), whereas thrombomodulin, a cofactor responsible for activating TAFI, is also elevated in PCOS (Oral et al. 2000). Positive correlations between TAFI and glucose, insulin, and HOMA-IR were observed in one study (Oral et al. 2000) but not in another (Erdogan et al. 2008). A positive correlation between TAFI and total cholesterol and LH but a negative link with high-density lipoprotein cholesterol have been noted (Oral et al. 2009). Inclusion of larger sample sizes would have been appropriate as an inclusion of BMI-matched PCOS and controls (Karakurt et al. 2008).

If a hyperfibrinolytic state is confirmed in PCOS, reflected by increased TAFI and PAI-1 levels, the etiology will need to be explored further. TAFI is synthesized in the liver (Karakurt et al. 2008), although some reports speculate a megakaryocyte/placental origin with subsequent release into plasma following platelet activation (Schadinger and Iken 2009). The studies described previously indicating a potential for elevated platelet numbers (Lugo-Ramirez et al. 2009) and activation (Oktay et al. 2009, Zwińska-Kuzczala et al. 2008) in PCOS suggest a possible link to increased TAFI levels. Likewise, studies to confirm elevated TAFI levels in PCOS are required with larger sample sizes and explanations of relationships with the abnormal metabolic and hormonal systems.

• Conclusion

PCOS is a highly prevalent condition in the reproductive-aged female population. It is a complex disorder with significant clinical heterogeneity, making both diagnosis and treatment challenging. PCOS includes metabolic features, endothelial dysfunction, early atherosclerosis, and an increased risk of CVD development. Hemostatic abnormalities play an important role in CVD. Explanation of the hemostatic system is therefore important in PCOS to identify contributions to CVD.

Review of the literature suggested abnormalities of the hemostatic system in PCOS, including abnormal platelet aggregation/activation. Also, endothelial dysfunction reflected mainly by elevation in ADMA and PAI-1, high fibrinogen, increased tPA levels most likely reflecting tPA-PAI-1 complexes, and enhanced inhibition of fibrinolysis reflected by high PAI-1 antigen and/or activity and TAFI levels have been observed. The existing literature is very limited, with few studies, small sample sizes, and contradicting findings relating to markers of hemostasis in women with PCOS. Correlations between hemostatic parameters and markers of the metabolic and hormonal systems also reveal inconsistent findings. Most research studies have lacked BMI- and age-matched controls, which are known to affect the hemostatic system.

Hence, the hypothesis of hemostatic abnormalities contributing to disturbed hemostasis including a hyperfibrinolytic capacity and/or net prothrombotic state in PCOS remains unresolved. Future research on the hemostatic system in PCOS is critical and must be comprehensive and in a systematic manner using well-designed studies assessing the system in this important syndrome. Whether hemostatic abnormalities are linked to the abnormal hormonal and/or metabolic system(s) in PCOS also remains to be elucidated. Such research may inform the understanding of PCOS and may lead to novel preventative or treatment measures targeting metabolic features of PCOS underlying CVD. Evaluation of the effects on the hemostatic system following administration of current management strategies to modulate androgens and IR including metformin, OCP, and dietary and lifestyle modifications in PCOS may facilitate further insights into probable disturbed hemostasis and into relationships with hormonal and metabolic markers in PCOS and, in turn, potential further CVD risk.

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Charron N, Faure-Knotik S, Zoumatas V, et al. 2000. The administration of estrogens, combined with anti-androgens, has beneficial effects on the hormonal features and asymptomatic dimethylarginine levels in women with the polycystic ovary syndrome. Atherosclerosis 156:955–965.
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Declaration of Co-Authorship and Co-contribution: Papers incorporated in

Thesis with publications

This declaration was completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Genia Burchall
Signature: |
Date: 10/02/2018

Paper Title: Comprehensive Assessment of the Hemostatic System in Polycystic Ovarian Syndrome.

In the case of the above publication, the following authors contributed to the work as follows:

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<tr>
<th>Name</th>
<th>Contribution</th>
<th>Nature of Contribution</th>
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<tr>
<td>Genia Burchall</td>
<td>70%</td>
<td>Designed and performed the research, performed the literature search, significantly contributed to data analysis and interpretation, wrote the manuscript, constructed/edited tables and figures, provided critical review of the manuscript and approved the final version for publication.</td>
</tr>
<tr>
<td>Matthew D. Linden</td>
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<td>Undertook revision for important content and approved the final version for publication.</td>
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<tr>
<td>Terrence J. Piva</td>
<td>9%</td>
<td>Undertook critical revision for important content and approved the final version for publication.</td>
</tr>
<tr>
<td>Sanjeeva Ranasinha</td>
<td>3%</td>
<td>Assisted with guidance on the statistical requirements of the manuscript, undertook some critical revision for important content and approved the final version for publication.</td>
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Melanie E. Gibson-Helm 3 Assisted with identification, retrieval and sorting of specimens required for the study, approved the final version for publication.

Helena J. Teede 12 Undertook critical revision for important content and approved the final version for publication.

DECLARATION BY CO-AUTHORS/SENIOR SUPERVISOR

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to

a) granting bodies,

b) the editor or publisher of journals or other publications, and

c) the head of the responsible academic unit; and

5. The original data is stored at the following location(s):
Location(s): School of Health and Biomedical Sciences, RMIT University and will be held for at least five years from the date indicated below.

Date: 10/02/2018

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CHAPTER 3: Comprehensive Assessment of the Haemostatic System in PCOS

3.1 Introduction

PCOS presents with many features that increase the risks of CVD development. Metabolic abnormalities include IR, hyperinsulinaemia, dyslipidaemia and increase risks for diabetes (Teede, Misso et al. 2011). Between 75-95% of women with PCOS present with IR that is thought to contribute to the pathophysiology of this syndrome (Stepto, Cassar et al. 2013). Women affected by PCOS are more likely to be overweight/obese than those without the syndrome and the incidence and severity of the condition increases with increased weight (Teede, Misso et al. 2011). Obesity also exacerbates both IR and hyperandrogenism. Two-thirds of non-obese PCOS females are reported to have excessive body fat and central visceral adiposity inducing greater IR (Cascella, Palomba et al. 2008).

The current management strategies used to ameliorate the symptoms of PCOS are highly variable in line with the clinical heterogeneity of the syndrome and may also increase the risks of CVD (Teede, Misso et al. 2011). The frequently prescribed OCP for PCOS management present with well accepted risks of VTE as well as increased risk of arterial thrombotic events and arterial dysfunction (Meyer et al. 2007). These risks are further amplified as many of the women affected by PCOS are also overweight (Teede, Misso et al. 2011).

In the previous chapter (Chapter 2), it was noted through a review of the literature that there may be an additional cardiovascular risk factor in PCOS, potentially relating to disturbed haemostasis, through endothelial dysfunction reflected mainly by elevation in ADMA and
PAI-1. Also, potential abnormal platelet function/activation following observations of elevated sCD40L and MPV and coagulation through increased generation of thrombin; additionally enhanced inhibition of fibrinolysis, as reflected by high PAI-1 antigen and/or activity and TAFI levels as well as increased tPA levels most likely reflecting tPA – PAI-1 complexes, was also observed. It was also noted that earlier studies evaluating haemostatic factors were performed using small numbers of subjects and many of these studies lacked BMI- and age-matched controls, factors that are well known to affect the haemostatic system, potentially resulting in the contradictory findings observed amongst much of the research on the haemostatic system in women with PCOS. More recent studies however showed improved study designs, however contradictory outcomes persist. Correlations between the haemostatic parameters and markers of the metabolic and hormonal systems also revealed inconsistent findings (or were not completed).

As a consequence of the above considerations, this study aimed to comprehensively assess the haemostatic system in women affected by PCOS versus controls and adjusting for age, BMI and waist circumference, controlling also for factors such as smoking status and medications (such as the OCP) affecting haemostasis. This was achieved through evaluation of haemostatic system components including the endothelium (part of the primary haemostatic system), the coagulation cascade (secondary haemostatic system), the fibrinolytic system and relevant inhibitors as well as assessing the haemostatic system overall. The relationships and effects of haemostatic function with/from hormonal and metabolic variables associated with PCOS including insulin levels, IR and lipid markers such as cholesterol, LDL, HDL and triglycerides were evaluated in addition to correlations made with hyperandrogenism (testosterone) to identify potential underlying causes and therapeutic targets. The results from this study were published in Seminars in Thrombosis and Hemostasis.
3.2 My Role

Chapter 3 consists of a large case-controlled study investigating the haemostatic system in women with and without PCOS. I formed collaborations early in my candidature with Prof Helena Teede (Monash Centre for Health Research and Implementation, School of Public Health and Preventative Medicine, Monash University) who kindly provided me with the biobanked plasma samples needed for this study. I applied for and received an exemption from ethics review with the Science, Engineering & Health College Advisory Network of the Human Research Ethics Committee of RMIT University (refer to Appendix 1) as this study had received ethics approval from the Monash University Standing Committee on Ethics in Research Involving Humans (and I had been included as an investigator on the research teams for this project). I then selected the relevant citrate specimens (that had been stored at the School of Public Health and Preventative Medicine, Monash University, Clayton) and transported these to RMIT University (School of Medical Sciences, Bundoora) for testing. I was responsible for determining the most relevant markers to measure based on my literature review and to explore the optimal methods for assessment. I chose relevant assay kits and consumables, advising my supervisor of the ordering requirements (refer to the Materials and Methods section of the published paper on the following pages for the sources of the assay kits and consumables and methods used to analyse the haemostatic parameters of this study). I completed method validation and optimisations for all markers analysed, completed the analysis of several of the haemostatic markers including plasminogen, TAFI (results not included in final published paper; Refer to Appendix 2) as well as TG, statistically analysed and interpreted the data (following consultation with a biostatistician) and wrote the manuscript. As a result of my contribution I am first author on this manuscript, which was published in Seminars in Thrombosis and Hemostasis.
Comprehensive Assessment of the Hemostatic System in Polycystic Ovarian Syndrome

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Abstract

Polycystic ovarian syndrome (PCOS) affects 12 to 19% of women and has reproductive and metabolic features (endothelial dysfunction, increased diabetes, and cardiovascular risk factors). It also appears to have altered coagulation and fibrinolysis with a prothrombotic state with epidemiological evidence of increased venous thromboembolism. We aimed to comprehensively assess hemostasis in women with PCOS versus control women. In an established case–control cohort of lean, overweight, and obese women with (n = 107) and without PCOS (n = 67), with existing measures of plasminogen activator inhibitor-1 (PAI-1), asymmetric dimethylarginine (ADMA), hormonal, and metabolic markers, we also assessed prothrombin fragments 1 and 2 (PF1 & 2), plasminogen, tissue plasminogen activator (tPA), and thrombin generation (TG). Higher levels of ADMA (0.70 vs. 0.39 μmol/L, p < 0.01), PAI-1 (4.80 vs. 3.66 U/mL, p < 0.01), and plasminogen (118.39 vs. 108.46%, p < 0.01) were seen in PCOS versus controls, and persisted after adjustment for age and body mass index (BMI). PF1 & 2 was marginally lower (180.0 vs. 236.0 μmol/L, p = 0.05), whereas tPA and TG were not different between groups, after adjustment for age and BMI. Significant correlations were observed between hormonal and metabolic factors with ADMA and PAI-1. We demonstrate impaired fibrinolysis in PCOS. In the context of abnormal endothelial function and known hormonal and metabolic abnormalities, this finding may underpin an increased risk of cardiovascular disease and venous thrombosis in PCOS.

Keywords
• polycystic ovarian syndrome
• hemostasis
• thrombosis
• plasminogen
• PAI-1

Polycystic ovarian syndrome (PCOS) is diagnosed based on ovulatory and menstrual disturbance, hyperandrogenism, and polycystic appearance of ovaries on ultrasound. It is one of the most common endocrine disorders of females of reproductive age with 12 to 19% of women affected, depending on the population studied. The etiology of PCOS remains to be elucidated, yet it is a complex disease similar to type 2 diabetes, rheumatoid arthritis, and cardiovascular disease (CVD), underpinned by a combination of genetic predisposition and environmental factors with highly variable phenotypic expression.

PCOS metabolic abnormalities include insulin resistance (IR), hyperinsulinemia, dyslipidemia, and increased diabetes risk, all of which are cardiovascular risk factors. Between 75 and 95% of women with PCOS have IR, which occurs independent of obesity and reinforces the pathophysiology of the disease. Women with PCOS are also more likely to be overweight or obese than non-PCOS women, which appear to further increase IR and drive PCOS incidence and severity. Women with PCOS also have comparatively higher body fat and central visceral adiposity inducing...
greater IR compared with body mass index (BMI)-matched control women; however, these findings do vary. Hyperandrogenism also contributes to the pathophysiology of PCOS potentially driven by IR. Overall, PCOS is a condition with inherent IR and hyperandrogenism, causing reproductive and metabolic features.

Recently, it has been reported that women with PCOS have significantly higher risk of cardiovascular and venous thromboembolic diseases compared with their counterparts without PCOS. These risks are exacerbated by high BMI and by some current PCOS management strategies. Concerns have been raised that important therapies, namely, the oral contraceptive pill (OCP) that targets reproductive features such as menstrual cycle regulation and hirsutism management, may also increase CVD and does increase venous thromboembolic risk. PCOS combined with excess weight and the OCP renders this a high-risk group of young women.

Risks of increased arterial and venous thromboses from abnormalities in the hemostatic system are well accepted. We and others have previously noted, through a comprehensive review of the literature, that there may be additional cardiovascular risk factors in PCOS, potentially relating to disturbed hemostasis and endothelial dysfunction reflected by elevated asymmetric dimethylarginine (ADMA) and plasminogen activator inhibitor 1 (PAI-1). Potential abnormal platelet function/activation and abnormal coagulation have also been documented in PCOS. High fibrinogen levels are noted in PCOS women and enhanced inhibition of fibrinolysis reflected by high PAI-1 antigen and/or activity as well as increased tissue plasminogen activator (tPA) levels, most likely reflecting (PA-PAS-1) complexes. However, studies performed on hemostatic factors in PCOS were small and had contradictory findings. Correlations between hemostatic parameters and markers of metabolic and hormonal systems also revealed inconsistent findings. Factors contributing to the heterogeneity include that many published studies did not consider BMI or did not include age-matched controls (as both BMI and age affect the hemostatic system).

In this context, we aimed to comprehensively assess the hemostatic system in high-risk women with PCOS versus controls, adjusting for age and BMI. We aimed to evaluate components of the hemostatic system, including endothelial function, the coagulation cascade, the fibrinolytic system and relevant inhibitors, as well as assessing global markers of hemostatic function. We also investigated the relationships between hemostatic markers and hormonal and metabolic variables associated with PCOS, including IR/hyperinsulinemia and hyperandrogenism to identify potential underlying causes and future therapeutic targets.

Materials and Methods

Subjects

Citrate anticoagulated venous samples and data (endothelial and biochemical markers) from subjects collected and analyzed from the current observational study were taken from a biobank of clinical studies in women with and without PCOS. Clinical studies in women with and without PCOS were approved by the institutional review board. All participants gave written informed consent. Recruitment of participants for this case-control study was undertaken from community advertisements. Recruitment, medical assessment, and sample collection were performed in a single center (Monash University) and under the supervision of a single, expert academic clinician (H.L.). All participants were premenopausal women aged 18 to 45 years with (i.e., cases) and without PCOS (i.e., controls). All cases met the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine criteria (also known as the Rotterdam diagnostic criteria) with two of (1) irregular menstrual cycles (<21 or >35 days), (2) clinical (hirsutism, acne) or biochemical (elevation of at least one circulating ovarian androgen) hyperandrogenism, and (3) polycystic ovaries on ultrasound. Exclusion criteria were secondary causes of amenorrhea and hyperandrogenism (congenital adrenal hyperplasia, androgen-secreting tumors, Cush- ing syndrome, hyperprolactinemia, thyroid dysfunction, adrenal disorders, and pregnancy), smoking, uncontrolled hypertension, type 2 diabetes, and nonstable use of antihypertensive, lipid-lowering, or fish oil medications. In all cases, participants were required to cease OCPs, endocrine hormonal treatment, or insulin-sensitizing agents (e.g., metformin) for 3 months before clinical measurements, and all participants received standard diet and lifestyle advice. All studies were completed in the same center with the same staff and study protocol as described earlier. All controls had regular menstrual cycles (21–35 days), displayed no evidence of clinical or biochemical hyperandrogenism, and ceased medication as described earlier, 3 months before sample collection.

Clinical Measurements

All subjects were weighed in lightly weighing clothes with no shoes in the same center and the BMI was calculated by weight (kg) divided by squared height (m²). Waist circumferences were measured at the umbilicus. Measurements were performed by an experienced operator.

Biochemical, Endothelial, and Hemostatic Measurements

IR was assessed by homeostasis model assessment (HOMA score) and a 75-g oral glucose tolerance test (glucose and insulin at 0, 30, 60, and 120 minutes) as previously described. The HOMA score was calculated as fasting serum insulin (μU/mL) X fasting plasma glucose (mmol/L)/22.5. Insulin was assayed using the analyzer AsSYM that is based on the micro- particle enzyme immunoassay technology. Total testosterone was measured using a chemiluminescent immunoassay (Beckman Coulter, Fullerton, CA). Total cholesterol and triglycerides were measured using enzymatic reagents (BAX Diagnostics, Brisbane, Australia). High-density lipoprotein (HDL) cholesterol was measured by homogeneous assay techniques (HDL-C Plus, BAX Diagnostics) adapted to a BAX Dimension IXL chemistry analyzer. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (LDL cholesterol = [total cholesterol – HDL cholesterol] – [triglycerides/2.2]). Adapted to SI units. All tests were performed in the same commercial pathology laboratory at Monash Health.
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Endothelial function markers were assessed on a subset of subjects. As previously published, PAI-1 activity was assayed using the commercial chromogenic assay Berichrom PAI (Marburg, Germany)\textsuperscript{13,16,19,20} with an intra- and interassay coefficient of variation (CV) of 1.3 ± 0.6 and 4.3 ± 0.5\%, respectively. ADMA was assessed using a chromogenic immunoenzymatic assay kit from DLD Diagnostika (Hamburg, Germany) with an intraassay CV between 5.7 and 6.4\% and an interassay CV between 8.3 and 10.3\%.\textsuperscript{15,20}

Hemostatic markers were assessed on 174 citrate anticoagulated venous blood samples (107 PCOS and 67 controls). These had been centrifuged, separated, and plasma stored at -80 °C until assayed. To prevent the deteriorative effects of freeze-thaw cycles on coagulation factors, simultaneous testing of coagulation markers was undertaken. We aimed to have a systematic approach when assessing the hemostatic system. Therefore, measurement of the system’s components was undertaken as well as assessing hemostasis overall. Not all measures were completed on the full dataset because of some inadequate stored samples. Endothelial dysfunction was assessed by measuring the nitric oxide inhibitor ADMA and the endothelially synthesized product PAI-1 (as previously described).\textsuperscript{13,16,19,20} In vivo coagulation cascade activation was assessed through prothrombin fragments 1 and 2 (PF1 & 2) levels using an enzyme-linked immunosorbent assay (ELISA) method (Simens Enzygnost F1 + 2 mononalon, Marburg, Germany) with an intraassay CV between 3.6 and 5.5\% and an interassay CV between 4.4 and 11.2\%. The fibrinolytic system was assessed through plasminogen and PA. Plasminogen activity was assayed using the STA Stachrom Plasminogen colorimetric assay performed on the automated analyzer STA-1 R (Asnieres sur Seine, France) with an intraassay CV between 1.8 and 1.9\% and an interassay CV between 2.4 and 3.4\%. PA was assayed using an ELISA method (TrinitiZE PA Antigen, Toag, Bray, Ireland) with an intraassay CV between 4.9 and 5.5\% and an interassay CV between 3.5 and 5.4\%. Inhibition of fibrinolysis was assessed through PAI-1 activity. Finally, assessment of thrombin generation (TG) looked at the hemostatic system overall, evaluating the net synthesis of thrombin through coagulation activation but also evaluated the simultaneous effects of inhibitors of the coagulation cascade. TG was assessed using the calibrated automated thrombogram (CAT) method (Stago) with an intraassay CV of 4.3\% and an interassay CV of 6.1\%.

Results

Baseline, Metabolic, and Hormonal Results

Baseline characteristics of subjects (PCOS and controls) including age, BMI, waist circumference, as well as biochemical (metabolic and hormonal) markers are displayed in Table 1. Significantly higher BMI, waist circumference, cholesterol, LDL, triglycerides, insulin, HOMA-IR, and testosterone levels were observed in PCOS compared with controls.

Endothelial and Hemostasis Function: Differences between PCOS and Controls and Relationships to Hormonal and Metabolic Markers

Although there were 174 participants in this study (n = 107 PCOS and n = 67 controls), not all parameters were measured for all subjects with variable availability of adequate samples for complete testing (Table 2). With endothelial function, significantly higher levels of ADMA and increased PAI-1 activity were seen in PCOS versus controls (p < 0.001 for both parameters) (Table 1), which persisted after adjustment for age and BMI (p < 0.001 and p = 0.005, respectively). These differences also persisted after adjustment for waist circumference (p = 0.003) and p < 0.001, respectively; data not tabulated) and after adjustment for all lipid markers for which we noted differences in PCOS and controls: cholesterol (p < 0.0001 for both, respectively; data not tabulated), LDL (p < 0.001 for both; data not tabulated), and triglycerides (p = 0.009 and p < 0.001, respectively; data not tabulated).

Linear regression models using fasting insulin, HOMA score, and total testosterone demonstrated significant relationships between these metabolic and hormonal factors with both ADMA (p < 0.001 for all three factors) and PAI-1 (p < 0.01 for all three factors) after accounting for age and BMI (Table 3). A significant difference was noted between PCOS and controls in PFI & 2 levels (p = 0.028); however, this was only borderline when adjusted for age and BMI (p = 0.05), with higher levels in controls versus the PCOS group. After adjusting for age and BMI, no significant relationship was noted between PFI & 2 with fasting insulin and HOMA score (p = 0.08 and p = 0.07, respectively) and only a borderline relationship was noted with testosterone levels (p = 0.05) (Table 3). A significant difference between PCOS and controls was noted in plasminogen activity (p < 0.0001) following adjustment for age and BMI (p = 0.024) (Table 2) that also persisted following adjustment for waist circumference (p < 0.001; data not tabulated), cholesterol (p < 0.0001; data not tabulated), LDL (p < 0.0001; data not tabulated), and triglycerides (p < 0.001; data not tabulated), with higher plasminogen activity.
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Table 1  Demographics (age, BMI, waist circumference, hormonal, and metabolic parameters) for PCOS and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS</th>
<th>Controls</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>32.8±6.7</td>
<td>34.1±7.9</td>
<td>0.224</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.6±7.8</td>
<td>31.3±7.0</td>
<td>0.0004</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>104.7±16.7</td>
<td>94.9±16.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting insulin (mIU/L)</td>
<td>17.60 (11.0–23.09)</td>
<td>9.94 (5.20–16.45)</td>
<td>0.0001</td>
</tr>
<tr>
<td>HOMA score</td>
<td>3.58 (2.20–5.20)</td>
<td>2.05 (0.10–3.36)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose levels (fasting)</td>
<td>4.72±0.57</td>
<td>4.70±0.38</td>
<td>0.619</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.29±1.02</td>
<td>4.90±0.91</td>
<td>0.007</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.26±0.36</td>
<td>1.30±0.34</td>
<td>0.408</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.43±0.93</td>
<td>3.10±0.77</td>
<td>0.038</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.25±0.70</td>
<td>1.0±0.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>2.5±1.0</td>
<td>1.4±0.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; HOMA, homeostatic model assessment; LDL, low-density lipoprotein; PCOS, polycystic ovarian syndrome.
Note: Part data 3,13-17, means ± SD reported and ANOVA for age, BMI, testosterone, waist circumference, glucose levels, cholesterol, HDL, LDL, and triglycerides. Median (interquartile range) and p-values from log transformed data for insulin and HOMA score.

activity observed in the PCOS group. Linear regression models using insulin, HOMA score, and testosterone did not demonstrate significant relationships between these factors and plasmogen activity after accounting for age and BMI (p = 0.18, 0.01, and 0.13, respectively) (Table 3). Although a significant difference was noted between PCOS and controls as regard to PA antigen levels (p = 0.025), these relationships disappeared when BMI and age were taken as confounders (p = 0.55). This fibrinolytic marker did not show a significant relationship to insulin, HOMA score, or testosterone (p = 0.88, 0.88, and p = 0.88, respectively) (Table 3). No significant difference in TG was noted between these two groups.

Discussion

In the setting of increased cardiovascular risk factors and higher risk of CVD and venous thromboembolism in PCOS,11-12 limited studies have evaluated the hemostatic system and its relationship to IR/hyperinsulinemia and hyperandrogenism as the key hormonal abnormalities in PCOS. Here, we have assessed the hemostatic system, evaluating both pro- and antithrombotic elements and overall hemostatic activity and explored hemostatic relationships to metabolic and hormonal factors in women with PCOS compared with healthy controls. In this comprehensive study of the hemostatic system, our main findings include

Table 2  Comparison of hemostatic variables between PCOS and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS</th>
<th>Non-PCOS</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (µmol/L) (n = 134)</td>
<td>0.70 (0.55–1.13)</td>
<td>0.39 (0.36–0.48)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PAI-1 (µIU/mL) (n = 134)</td>
<td>4.80 (3.28–6.64)</td>
<td>3.66 (2.46–4.37)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTI &amp; 2 (µIU/mL) (n = 75)</td>
<td>180.0 (154.0–255.5)</td>
<td>236.0 (180.3–358.5)</td>
<td>0.009</td>
</tr>
<tr>
<td>TPA (nM/mL) (n = 125)</td>
<td>11.35 (8.35–14.18)</td>
<td>9.20 (7.40–10.40)</td>
<td>0.05</td>
</tr>
<tr>
<td>Plasminogen activity (%) (n = 128)</td>
<td>118.39±13.93</td>
<td>108.46±15.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TG (nmol/min) (n = 123)</td>
<td>2,179.38±533.51</td>
<td>2,071.62±546.19</td>
<td>0.271</td>
</tr>
</tbody>
</table>

Abbreviations: ADMA, asymmetric dimethyl arginine; PAI-1, plasminogen activator 1; PCOS, polycystic ovarian syndrome; PTI & 2, prothrombin fragments 1 and 2; TPA, tissue plasminogen activator; TG, thrombin generation.

Note: Means ± SD and ANOVA for plasminogen activity and TG. Median (interquartile range) and p-values from log transformed data for ADMA, PAI-1, PTI & 2, and TPA.

aPart data 13-17.

bPart data 3-17.
cPart data 51-63.
dPart data 22-31.
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Fig. 1. Significant difference in endothelial function markers (A) PAI-1 (units/ml) and (B) ADMA (μmol/l) between PCOS and controls with higher levels for both markers noted in the PCOS group. (Part data15-17). ADMA, asymmetric dimethyl arginine; PAI-1, Plasminogen activator 1; PCOS, polycystic ovarian syndrome.

Fig. 2. Significant difference in fibrinolytic marker plasminogen (%) between PCOS and controls with higher levels noted in the PCOS group. PCOS, polycystic ovarian syndrome.

Table 3. After adjustment for age and BMI, evaluation of the effects/associations of metabolic (insulin and HOMA score) and hormonal (testosterone) parameters on the hemostatic parameters (differences) between PCOS and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fasting Insulin</th>
<th>HOMA score</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β coefficient and 95% CI</td>
<td>p (R² value)</td>
<td>β coefficient and 95% CI</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.70 (0.59 to 0.86)</td>
<td>&lt; 0.01 (0.45)</td>
<td>0.70 (0.55-0.86)</td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.22 (0.06-0.37)</td>
<td>&lt; 0.01 (0.25)</td>
<td>0.22 (0.07-0.37)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>4.43 (1.10 to 9.96)</td>
<td>0.16 (0.26)</td>
<td>4.43 (1.10 to 9.92)</td>
</tr>
<tr>
<td>PF1 &amp; 2</td>
<td>−0.22 (−0.48 to 0.03)</td>
<td>0.08 (0.15)</td>
<td>−0.23 (−0.48 to 0.02)</td>
</tr>
<tr>
<td>tPA</td>
<td>0.02 (−0.15 to 0.18)</td>
<td>0.85 (0.28)</td>
<td>0.02 (−0.15 to 0.19)</td>
</tr>
</tbody>
</table>

Abbreviations: ADMA, asymmetric dimethyl arginine; CI, confidence interval; HOMA, homeostasis model assessment; PAI-1, plasminogen activator 1; PCOS, polycystic ovarian syndrome; PF1 & 2, prothrombin fragments 1 and 2; tPA, tissue plasminogen activator.

Note: Data are assessed by multivariable linear regression models including age and BMI as covariates. A p-value of < 0.05 was considered significant.
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Linear regression analysis showed that the metabolic (fasting insulin level and HOMA score) and hormonal (testosterone) abnormalities in PCOS are related to the endothelial dysfunction. Other PCOS factors that may contribute to endothelial dysfunction include excess chronic inflammation, increased visceral adiposity, or elevated plasma levels of cholesterol and/or triglycerides. Our results, however, did not indicate significant effects from increased waist circumference or lipid abnormalities on the aberrant endothelial function. The differences noted between PCOS and controls with regard to both PAI-1 activity and ADMA levels still persisted after adjustment for waist circumference (p = 0.003 and p < 0.0001, respectively; data not tabulated), cholesterol (p < 0.0001 for both; data not tabulated), LDL (p < 0.0001 for both; data not tabulated), and triglycerides (p = 0.009 and p < 0.0001, respectively; data not tabulated). Elevations in both PAI-1 and ADMA, and endothelial dysfunction pose significant CVD risks for women with PCOS, as endothelial dysfunction has been connected with an increased risk of future CVD events via contribution to atherosclerosis. ADMA is also an independent marker for CVD morbidity and mortality. Further research into the mechanisms and potential for amelioration of endothelial dysfunction in PCOS is needed.

In terms of coagulation activity, PF1 & 2 is a useful test for detecting a prothrombotic state and elevated levels of PAI-1 and 2 have been linked with a high risk of thrombosis. To our knowledge, this current study is the first to assess PF1 & 2, to evaluate the presence of subclinical in vivo coagulation activity with PCOS. After adjustment for age and BMI, the differences in PF1 & 2 levels between women with and without PCOS were only of borderline significance. HOMA score and insulin were not associated with PF1 & 2 levels with a borderline link demonstrated between the hemostatic marker and testosterone. Further study with larger sample sizes may be warranted here. Our study also investigated TG in PCOS, a test that assesses the formation of thrombin following activation of the coagulation cascade by tissue factor. TG is a global hemostatic assay that looks at both the procoagulation effects of the coagulation cascade, but also at inhibitory substances that prevent the generation of thrombin, mainly tissue factor pathway inhibitor, antithrombin (AT) and protein C. TG is very sensitive to variations of prothrombin and AT, as well as to the activity of activated protein C system. Our study showed no significant difference between PCOS and controls with regard to TG. Although the PF1 & 2 and TG results do not suggest an overall prothrombotic state, the TG assay does not take into account the effects of fibrinolysis, the markers of which mainly act upon fibrinogen and fibrin, activated downstream from thrombin in the coagulation cascade. The TG assay also does not assess the inhibitory effects of PAI-1 that counteract the effects of the fibrinolytic system. However, based on our current study, isolated coagulation activation does not appear to be increased in women with PCOS compared with healthy controls.

In terms of fibrinolysis, hypofibrinolytic capacity through net reduced fibrinolysis was demonstrated via both the plasminogen and PAI-1 results in PCOS versus controls. In the current study, we noted significantly higher plasminogen activity in women with PCOS than in controls, even after adjustment for age and BMI. Adjustments for waist circumference (p < 0.0001; data not tabulated), cholesterol (p < 0.0001; data not tabulated), LDL (p < 0.0001; data not tabulated), and triglycerides (p < 0.0001; data not tabulated) also did not affect the outcomes, and significant differences still persisted between PCOS and controls for plasminogen activity. Linear regression analysis revealed that the metabolic (insulin and IR) and hormonal (testosterone) factors do not have any significant relationship with plasminogen activity. Studies assessing plasminogen in PCOS have revealed either similar or lower plasminogen activity compared with controls, but one study observed no differences compared with controls. Elevated PAI levels predominantly reflect PAI-1 complexes, which can be generated as a consequence of elevated PAI-1 levels. It is unclear whether PAI levels are linked with insulin levels in women with PCOS. Lin and Yongmei showed that there is a relationship, but our study and others do not support this conclusion. The elevated plasminogen activity in PCOS versus controls noted in our study most likely reflects a prevention of conversion to plasmin rather than enhanced fibrinolytic capacity (though this role needs to be understood). After adjustment for age and BMI, the differences in PAI-1 levels between women with and without PCOS were only of borderline significance (-Table 2).
where increased inflammatory markers, including C-reactive protein levels, have been well documented.\textsuperscript{38,44} Future research is needed to explore the physiology of plasminogen in PCOS.

Limitations of our study include the analyses of hemostatic markers in women recruited for a study designed to address another research question. However, methods used in the original study were rigorous, women were well characterized, and samples were well prepared. We also acknowledge that not all end points were measured on all samples with inadequate sample available in some cases. Also, there was a lack of assessment of markers that may be targets for primary hemostasis, including the potential for an increased or aberrant platelet function or number. Finally, the cross-sectional design of the study precludes the establishment of any firm conclusion about causality. Strengths include a systematic approach to hemostatic assessment, a well-characterized population on no medications, adjustment for age and BMI, and prior sample size calculation to attain adequate statistical power to detect statistical significance.

In conclusion, our case-control study shows no coagulation abnormalities in PCOS compared with controls but clear evidence of impaired capacity for fibrinolysis. With increased activity of the inhibitor of fibrinolysis, PAI-1, as well as increased plasminogen (perhaps arising due to reduced conversion to active plasmin), a mild-to-moderate hypofibrinolytic state occurs. The reasons for these changes are unclear. The mechanisms driving this hypofibrinolytic state may be linked to abnormal endothelial function with significant effects from the aberrant hormonal (hyperandrogenemia) and metabolic (IR, hyperinsulinemia) changes in PCOS. Further studies are needed to confirm these cross-sectional results, including studies that therapeutically modulate the hemostatic system in PCOS. A hypofibrinolytic state in this condition represents a further CVD risk factor and risk marker in PCOS, which may contribute to the development of arterial and venous thromboembolic diseases.

Conflict of interest
All the authors have no conflict of interest to declare.

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Declaration of Co-Authorship and Co-contribution: Papers incorporated in thesis with publications

This declaration was completed for each conjoinedly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by:                                      Signature:                                        Date:
Genia Burchall                                                                                             10/02/2018

Paper Title: Differential Effects on Haemostatic Markers by Metformin and the Contraceptive Pill: A Randomised Comparative Trial in PCOS

In the case of the above publication, the following authors contributed to the work as follows:

<table>
<thead>
<tr>
<th>Name</th>
<th>Contribution %</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genia Burchall</td>
<td>72</td>
<td>Designed and performed the research, performed the literature search, independently reviewed the articles and assessed risk of bias, completed data analysis and interpretation, wrote the manuscript, constructed/edited tables, provided critical review of the manuscript and approved the final version for publication.</td>
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<tr>
<td>Terrence J. Piva</td>
<td>10</td>
<td>Assisted in design of the research, undertook critical revision for important content and approved the final version for publication.</td>
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<td>Sanjeeva Ranasinha</td>
<td>3</td>
<td>Assisted with guidance on the statistical requirements of the manuscript, undertook some critical revision for important content and approved the final version for publication.</td>
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DECLARATION BY CO-AUTHORS/SENIOR SUPERVISOR

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to

   a) granting bodies,

   b) the editor or publisher of journals or other publications, and

   c) the head of the responsible academic unit; and

5. The original data is stored at the following location(s):

   Location(s): School of Health and Biomedical Sciences, RMIT University and will be held for at least five years from the date indicated below.
Date: 10/02/2018

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CHAPTER 4: Common Pharmaceutical Interventions (OCP and Metformin) in PCOS and Haemostatic Outcomes

4.1 Introduction

PCOS presents with increased risks for CVD including metabolic abnormalities such as IR, hyperinsulinaemia and dyslipidaemia, as well as obesity (Teede, Misso et al. 2011, Teede, Joham et al. 2013). Although it remains a contentious issue, recently published large scale studies have reported that women with PCOS have significantly higher rates of CVD as well as an increase in VTE (1.5 to 2 fold increased risk) in comparison to those without PCOS (Bird, Hartzema et al. 2013, Hart and Doherty 2015). This may be exacerbated by aberrant metabolic features as well as a high BMI. However it may also be related to disturbed haemostasis. In previous chapters (Chapters 2 and 3) it was suggested that additional CVD and VTE risk factors are involved in PCOS, potentially related to aberrant haemostasis including a hypofibrinolytic state.

The increased risks for CVD and VTE may be further exacerbated by current PCOS management strategies. First line therapy for PCOS includes lifestyle intervention if overweight to combat menstrual disturbances, infertility and CVD risks. Additional pharmaceutical therapy includes the OCP, anti-androgens, or metformin to target metabolic and reproductive features. Whilst the OCP is indicated for contraception and PCOS treatment, concerns have been raised about increased risks of VTE, which are well accepted in the general population. Women taking the OCP are 3-5 times more likely to have a VTE event than those that do not (Soares, Vieira et al. 2009, van Hylckama Vlieg, Helmerhorst et al. 2009, Stewart and Black 2015). The risks however vary depending on oestrogen dose and progesterone type, with lower oestrogen
dosages and second generation progesterone preparations having lower risks (Soares, Vieira et al. 2009, van Hylckama Vlieg, Helmerhorst et al. 2009, Stewart and Black 2015). A twofold increased risk of VTE among women with PCOS taking combined OCP compared to those unaffected by the syndrome taking the same drug therapy has been observed (Bird, Hartzema et al. 2013). Oestrogen and the OCP have been shown to increase the risks of VTE in the general population by affecting many/most coagulation factors and increasing prothrombin (factor II) levels, fibrinogen (factor I) and factors VII, VIII and X and by decreasing AT and protein S levels; increasing TAFI activity and activated protein C (APC) resistance (Rosing, Curvers et al. 2001, van Hylckama Vlieg, Helmerhorst et al. 2009, Szabo and Schaff 2013). While OCP may affect some haemostatic factors that exert anticoagulant effects including increasing levels of plasminogen and protein C, an overall net pro-coagulant effect is still believed to be present (Rosing, Curvers et al. 2001). A net thrombotic effect is noted with use of the combined OCP from the oestrogen component but the type of progesterone used may also affect haemostatic outcomes. Some third generation progesterone combinations (containing desogestrel or gestodene) seem to have greater effects on prothrombin and factor VII as well as greater resistance to APC than second generation preparations (levonorgestrel) (Rosing, Curvers et al. 2001, van Hylckama Vlieg, Helmerhorst et al. 2009). A recent review on the pharmacodynamics of combined oral contraceptives (COC) and effects on the haemostatic system has confirmed that a dose related effect of the oestrogen component of COC was noted on the haemostatic system with an overall pro-coagulatory effect and parameters, that is only party counteracted by fibrinolytic system action, with a net increase in fibrin generation (following observations of increased PF1 & 2 and DD levels) (Farris, Bastianelli et al. 2017). The progestin component of COC can modulate the actions of oestrogen and the haemostatic effects noted however the exact mechanisms by which they do this and ultimate haemostatic outcomes are still unclear (Farris, Bastianelli et al. 2017). Research in this area is contentious
and ongoing and large studies are needed to determine any definitive outcomes. Increased risks of arterial disease including myocardial infarct and ischaemic stroke have also been noted in women taking the OCP (Stewart and Black 2015). In women with PCOS, OCP may increase VTE risks (Bird, Hartzema et al. 2013) as well as leading to arterial dysfunction, altered glucose metabolism and IR (Meyer, McGrath et al. 2007), yet studies on the impact of the OCP on the haemostatic system in PCOS are limited with key evidence gaps remaining (Burchall, Piva et al. 2017).

Metformin is also used to treat PCOS (Teede, Misso et al. 2011), where it improves IR, menstrual cycles and prevents weight gain (Naderpoor, Shorakae et al. 2015). It increases pregnancy rates and may impact on live birth rates, however further study is needed to define its exact role and mechanism of action/s in infertility management in PCOS (Teede, Misso et al. 2011, Naderpoor, Shorakae et al. 2015). Metformin has been shown to have positive effects on the haemostatic system in diabetic patients, a population with known increased VTE risk. Metformin reduces the level of coagulation factors VII and XIII, PAI-1 and von Willebrand factor (Belfiore and Mogensen 2000, Rojas and Gomes 2013). In a high-risk population, metformin may offer additional benefits through positive effects on the haemostatic system, however few published studies have examined the impact of metformin on this system.

To date, studies evaluating the effects of OCP and metformin in PCOS have predominantly looked at the efficacy on reproductive and hormonal features, but not on the haemostatic system. In this context, this study investigated and compared the haemostatic impact of common pharmacological therapies for PCOS, specifically higher-dose OCP (35µg ethinyl estradiol [EE]/2mg cyproterone acetate), low-dose OCP with a second generation progestin (20 µg EE/100 µg levonorgestrel) and an anti-androgen (spironolactone 50 mg b.d.) and metformin therapy (1 g b.d.). Similar to the earlier study above (Chapter 3), the present study aimed for a
systematic and comprehensive evaluation of the haemostatic system in women with PCOS on the aforementioned treatments. Similar haemostatic markers were assessed here as in the previous study (see Chapter 3). It was aimed to evaluate the components of the haemostatic system including endothelial function (part of primary haemostasis/haemostatic response), the coagulation cascade (secondary haemostasis), the fibrinolytic system and relevant inhibitors as well as assess global markers of haemostatic function. Finally, relationships between haemostatic markers and clinically relevant hormonal and metabolic variables associated with PCOS including markers of insulin resistance and hyperandrogenism were also investigated to help identify potential aetiological factors of the syndrome as well as assist with improvement in current treatment choices.
4.2 My Role

Chapter 4 consists of a large cross-sectional study investigating the haemostatic system in women with PCOS on three interventions, namely low- and higher-dose OCP and metformin. I initially reviewed the literature which revealed the limited knowledge available on the haemostatic effects of the frequently prescribed pharmacological interventions in PCOS. I received an exemption from ethics review from the Science, Engineering & Health College Advisory Network of the Human Research Ethics Committee of RMIT University (refer to Appendix 1) as this study had received ethics approval from the Monash University Standing Committee on Ethics in Research Involving Humans. I selected the appropriate citrate specimens (that had been stored at the School of Public Health and Preventative Medicine, Monash University, Clayton) and transported these from Monash University to RMIT University (School of Health & Biomedical Sciences, Bundoora). I was responsible for determining key endpoints and assays and sourcing these as well as choosing any relevant consumables including advising my supervisor of the ordering requirements (refer to the Materials and Methods section of the published paper on the following pages for the sources of the assay kits and consumables and methods used to analyses the haemostatic parameters of this study). I performed any relevant method validations and optimisations and analysed several of the haemostatic markers including plasminogen, TAFI and TG, statistically analysed and interpreted the data (following consultation with the biostatistician) and wrote the manuscript. As a result of my contribution I am first author on this manuscript, which was published in Thrombosis and Haemostasis in 2017.
Differential Effects on Haemostatic Markers by Metformin and the Contraceptive Pill: A Randomised Comparative Trial in PCOS

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Abstract

Background Polycystic ovarian syndrome (PCOS) affects up to 18% of reproductive-aged women with increased risks of cardiovascular disease and venous thromboembolic disease, related to metabolic and hormonal features, obesity and an apparent hypofibrinolytic state, possibly exacerbated by current PCOS treatments.

Objective To investigate and compare haemostatic impacts of common pharmacological treatments and explore relationships with hormonal and metabolic variables in PCOS.

Patients/Methods This mechanistic sub-study using biobanked samples from a 6-month randomised comparative trial of pharmacological treatments assessed pro- and anti-thrombotic markers and overall haemostatic activity. Overweight women of mean age 33.9 ± 6.7 years and mean BMI (body mass index) of 36.5 ± 7.0 kg/m² with PCOS (n = 60) were randomised to either metformin, higher-dose oral contraceptive pill (OCP) or low-dose OCP + spironolactone (OCP + S). Primary outcome measures included changes in plasminogen activator inhibitor 1 (PAI-1), asymmetric dimethylarginine (ADMA), prothrombin fragments 1 and 2 (PF1 and 2), plasminogen, tissue plasminogen activator (tPA), thrombin activatable fibrinolysis inhibitor (TAFI) and thrombin generation (TG).

Results PAI-1 activity fell in all groups, ADMA fell in higher-dose OCP, PF1 and 2 increased with metformin and higher-dose OCP, TG rose and tPA fell in both OCP groups, plasminogen increased in all and TAFI increased after higher-dose OCP. Conclusion Endothelial function (primary haemostasis) improved with higher dose with some improvement in low-dose OCP + S and metformin. Aberrant coagulation was noted in both OCP groups, but not with metformin. Fibrinolysis was reduced with higher-dose OCP. Our work suggests an additional dimension of treatment (haemostatic system effects) that favours metformin treatment over the OCP in PCOS.

Keywords fibrinolysis, haemostasis, metformin, oral contraceptives, polycystic ovary syndrome

Introduction

Polycystic ovarian syndrome (PCOS) is a condition diagnosed based on ovulatory and menstrual disturbance, hyperandrogenemia and polycystic appearance of ovaries on ultrasound. It is one of the most common disorders of females of reproductive age, affecting 12-18% of women. PCOS is a complex disease where a combination of genetic predisposition and environmental factors contribute to the aetiology and phenotypic expression of the disorder.
PCOS has many features that increase the risks of cardiovascular disease (CVD), including metabolic abnormalities such as insulin resistance (IR), hyperinsulinaemia and dyslipidaemia, as well as obesity.\textsuperscript{8,10} Although it remains controversial, recently large-scale studies have reported that women with PCOS have significantly higher rates of CVD and recent studies show an increase in venous thromboembolic disease (VTE) (1.5- to 2-fold increased risk) in comparison to those without PCOS.\textsuperscript{5,5} This may be exacerbated by aberrant metabolic factors and high body mass index (BMI). However, it may also be related to disturbed haemostasis. In our previous work, we have suggested that additional CVD and VTE risk factors are involved in PCOS, potentially related to a hypo- 
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The increased risks for CVD and VTE may be further exacerbated by current PCOS management strategies that include the oral contraceptive pill (OCP), anti-androgens or metformin to target metabolic and reproductive features. While the OCP is indicated for contraception and PCOS treatment, concerns have been raised about increased risks of VTE, which are well accepted in the general population. Women taking the OCP are three to five times more likely to have a VTE event than those who do not.\textsuperscript{12-14} The risks, however, vary depending on estrogen dose and progesterone type, with lower estrogen dosages and second-generation progesterone preparations having lower risks.\textsuperscript{12-14} Bird et al observed a twofold increased risk of VTE among women with PCOS taking combined OCP compared with those unaffected by the syndrome taking the same drug therapy.\textsuperscript{9} Increased risks of arterial disease including myocardial infarction and ischemic stroke have been observed in women taking the OCP.\textsuperscript{13} In women with PCOS, OCP may increase VTE risks\textsuperscript{5,9,10} as well as lead to arterial dysfunction, altered glucose metabolism and IR,\textsuperscript{10} yet studies on its impact on the haemostatic system in these women are limited with key evidence gaps remaining.\textsuperscript{11}

Metformin is also used to treat PCOS,\textsuperscript{2} where it improves IR and menstrual cycles, which assists in infertility management, and can prevent weight gain.\textsuperscript{15-18} It has been shown to have positive effects on the haemostatic system in diabetes, a population with known increased VTE risk. Metformin reduces the levels of coagulation factors VII and XIII, plasminogen activator inhibitor 1 (PAI-1) and von Willebrand factor.\textsuperscript{12,18} In a high-risk population, metformin may offer additional benefits through positive effects on the haemostatic system; however, few published studies have examined its impact on this system.

To date, few studies evaluating the effects of OCP and metformin on the haemostatic system in PCOS have been undertaken. In this context, we investigated and compared the haemostatic impact of common pharmacological therapies for PCOS, specifically (1) higher-dose OCP (35-μg ethinyl estradiol [EE]/2-mg cyproterone acetate), (2) low-dose OCP with a second-generation progesterin (20-μg EE/100-μg levonorgestrel) and an anti-androgen (spironolactone 50 mg twice a day) (referred to herein as OCP + S) and (3) metformin therapy (1 g twice a day). We aimed to evaluate the components of the haemostatic system including endothelial function (part of the primary haemostatic system/response), the coagulation cascade (secondary haemostasis), the fibrinolytic system and relevant inhibitors as well as assessing global markers of haemostatic function. Our hypothesis was that women with PCOS taking higher-dose OCP and low-dose OCP + spironolactone will have a shift in the balance of haemostasis towards that of thrombosis with a more marked impact observed following higher-dose OCP usage, while metformin use would not have a negative impact.

Materials and Methods

Participants

Analysis of biobanked samples obtained from an existing intervention study was undertaken in this current study.\textsuperscript{15} Data on metabolic (area under the curve [AUC], oral glucose tolerance test [OGTT], fasting insulin, AUC Insulin and homeostasis model assessment, HOMA-IR) and hormonal parameters (testosterone)\textsuperscript{15} as well as endothelial function (PAI-1 and asymmetric dimethylarginine, ADMA)\textsuperscript{15} have been previously published. The Southern Health Research Advisory and Ethics Committee approved the original study,\textsuperscript{15} and participants gave written informed consent. Recruitment of participants was undertaken from community advertisements. Eligibility for the study was assessed initially by telephone screening followed by a thorough medical examination of the participants.\textsuperscript{15} All participants were overweight and obese women aged 33.9 ± 6.7 years (BMI 36.5 ± 7.0 kg/m²) with PCOS diagnosed after medical review by the study team. PCOS was diagnosed based on the National Institutes of Health (NIH) criteria that includes irregular menstrual cycles (<21 or >35 days) and clinical (hirsutism, acne) or biochemical (elevation of at least one circulating ovarian androgen) hyperandrogenism.

Exclusion criteria were secondary causes of amenorrhea and hyperandrogenism (congenital adrenal hyperplasia, androgen-secreting tumours, Cushing’s syndrome, hyperprolactinaemia, thyroid dysfunction, adrenal disorders and pregnancy), smoking and diabetes. For 3 months prior to measurements, participants were required to cease oral contraceptives and any medication affecting IR including metformin. Participants received standard diet and lifestyle advice at the screening visit.\textsuperscript{15} Three months later, women were randomly allocated (based on computer-generated random numbers) to one of three interventional groups: (1) higher-dose OCP (35-μg EE/2-mg cyproterone acetate), (2) low-dose OCP (20-μg EE/100-μg levonorgestrel) combined with an anti-androgen (spironolactone 50 mg twice a day) (OCP + S) or (3) metformin (1 g twice a day with dose titrated up over 4 weeks starting at 500 mg twice a day).\textsuperscript{15}

This was an open-label study.

Clinical Measurements

All participants were weighed in lightly weighing clothes with no shoes in the same centre and the BMI was calculated by weight (kg) divided by squared height (m²). Measurements were performed by an experienced operator.

Haemostatic Measurements

Haemostatic markers were retrospectively assessed on 60 citrate-anticoagulated venous blood samples: 21 women on
higher-dose OCP. 16 women on low-dose OCP + 5 and 23 women on metformin therapy. Samples were centrifuged and the plasma stored at 80°C until assayed. To prevent the deteriorative effects of freeze-thaw cycles on coagulation factors, simultaneous testing of coagulation markers was undertaken. We aimed to comprehensively assess the haemostatic system in women with PCOS in response to the interventions. We assessed the components within the haemostatic system as well as evaluate global markers of haemostasis. Initiation of thrombus formation within the blood vasculature follows a disturbed endothelium with subsequent activation of platelets (primary haemostatic system/response). Endothelial function was assessed by measuring the nitric oxide inhibitor ADMA and the endothelially synthesized product PAI-1. The initial thrombus formed through the primary haemostatic response needs further stabilization by fibrin. Fibrin is formed following activation of the coagulation cascade (secondary haemostasis). In vivo coagulation cascade activation was assessed through prothrombin fragments 1 and 2 (PF1 and 2) levels. The fibrinolytic system acts to counteract the effects of the coagulation cascade by activating plasmin, which then acts to break down fibrin. The fibrinolytic system was assessed by measuring plasminogen activity and tissue plasminogen activator (tPA) levels. Finally, inhibition of fibrinolysis, which supports thrombosis formation, was assessed through PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI) Assesment of thrombin generation (TG) looked at the haemostatic system overall, evaluating the net synthesis of thrombin (which acts to convert fibrinogen into insoluble fibrin) through coagulation activation, but also evaluates the simultaneous effects of inhibitors of the coagulation cascade. All these analyses were measured as described previously.18 Metabolic, hormonal and haemostatic markers were assessed at baseline and after 6 months of intervention. Participants were also assessed by the same investigator at baseline and 6 months after the commencement of medication(s). End-point sample collection was completed by a research nurse who was blinded to the treatment allocation.

Results
At screening, there were 110 women eligible to participate in the study, of which 37 women were randomized to receiving intervention with metformin, 35 higher-dose OCP and 38 low-dose OCP + 5. There were 10 women who withdrew from the study (1 from the metformin group, 4 from the higher-dose and 5 from the low-dose OCP + 5 groups). Most withdrawals were for personal reasons (8/10), while for one participant, who had been allocated to the higher-dose OCP group, this was because she experienced mood swings. After withdrawal, 100 participants remained, 36 on metformin, 31 on higher-dose OCP and 33 on low-dose OCP + 5.13 Not all participants had samples available for haemostatic measurements. Haemostatic assessments were performed retrospectively on biobanked samples from 21 PCOS women in the higher-dose OCP group, 16 in the low-dose OCP + 5 group and 23 from the metformin group.

Baseline, Metabolic and Hormonal Results
The baseline characteristics of participants including age and BMI as well as metabolic (AUC OGTT, insulin, HOMA-IR and AUC insulin) and hormonal (testosterone) markers for the three interventional groups conducted over a 6-month period are shown in – Table 1.

As previously reported, testosterone dropped in the low-dose (p = 0.004) and higher-dose OCP groups (p = 0.005),15,18 AUC OGTT increased significantly with both higher-dose (p = 0.001) and low-dose OCP + 5 (p = 0.030) (15). AUC insulin increased significantly after 6 months of intervention with higher-dose OCP (p = 0.003), but not with metformin (p = 0.009).15 There was no significant difference in age between the three interventional groups.
Table 1 Demographics (age, BMI, hormonal and metabolic parameters) for the three interventional groups at baseline and 6 months for the 60 participants involved in this study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1: higher-dose OCP (n = 21)</th>
<th>Group 2: low-dose OCP + S (n = 16)</th>
<th>Group 3: metformin (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>34.41 ± 6.73</td>
<td>35.44 ± 6.91</td>
<td>32.16 ± 6.52</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>35.91 ± 8.11</td>
<td>35.25 ± 5.71</td>
<td>37.79 ± 6.81</td>
</tr>
<tr>
<td>6 mo</td>
<td>35.99 ± 7.96</td>
<td>35.61 ± 5.39</td>
<td>36.81 ± 6.93</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>0.08 (-0.56 to 0.72)</td>
<td>0.07 (-0.74 to 0.87)</td>
<td>-0.98 (-2.08 to 0.13)</td>
</tr>
<tr>
<td>AUC OGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>741.00 ± 166.69</td>
<td>885.90 ± 182.35</td>
<td>824.59 ± 151.90</td>
</tr>
<tr>
<td>6 mo</td>
<td>850.88 ± 149.21</td>
<td>1,003.89 ± 148.36</td>
<td>805.84 ± 165.24</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>109.66 (54.92 to 164.40)</td>
<td>132.20 (40.61 to 223.80)</td>
<td>-23.00 (-77.57 to 31.57)</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.81 (2.33–3.13)</td>
<td>2.70 (2.46–3.03)</td>
<td>2.87 (2.33–3.57)</td>
</tr>
<tr>
<td>6 mo</td>
<td>2.88 (2.22–3.30)</td>
<td>2.88 (2.36–3.14)</td>
<td>2.37 (2.10–2.33)</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>0.08 (0.15–0.30)</td>
<td>-0.06 (0.33 to 0.21)</td>
<td>-0.28 (0.61 to 0.06)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.10 (0.69–1.53)</td>
<td>1.16 (0.89–1.47)</td>
<td>1.29 (0.70–1.79)</td>
</tr>
<tr>
<td>6 mo</td>
<td>1.17 (0.54–1.63)</td>
<td>1.27 (0.87–1.58)</td>
<td>0.77 (0.40–1.53)</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>0.05 (0.20 to 0.30)</td>
<td>-0.08 (-0.37 to 0.21)</td>
<td>-0.33 (-0.66 to 0.01)</td>
</tr>
<tr>
<td>AUC Insulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>10.568 ± 4.820</td>
<td>11.750 ± 4.838</td>
<td>8.142 ± 4.301*</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>2.343 (802 to 3,885)</td>
<td>-36.00 (-4,096 to 4,024)</td>
<td>-4.055 (-7,361 to -749)</td>
</tr>
<tr>
<td>Testosterone (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.07 ± 0.66</td>
<td>2.83 ± 1.02</td>
<td>2.34 ± 0.72</td>
</tr>
<tr>
<td>6 mo</td>
<td>1.60 ± 0.62</td>
<td>2.11 ± 1.21*</td>
<td>2.10 ± 1.06</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>-0.46 (-0.80 to -0.12)</td>
<td>-0.72 (-1.22 to -0.21)</td>
<td>-0.28 (-0.83 to 0.27)</td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under the curve; BMI, body mass index; CI, confidence interval; HOMA, homeostasis model assessment; IR, insulin resistance; OCP, oral contraceptive pill; OCP + S, oral contraceptive pill + spironolactone; OGTT, oral glucose tolerance test.

Note: Part data:15 mean ± SD reported for age, BMI, AUC OGTT, AUC Insulin and testosterone. Median (interquartile range) from log-transformed data for Insulin and HOMA. Mean change = change from baseline to 6 months ± 95% confidence interval (CI).

*Within group change, p ≤ 0.05, comparing baseline to 6 months using paired Student’s t-test.

Between group change, p ≤ 0.05, comparing baseline results for higher-dose OCP and metformin.

Haemostasis Results

**Within-group change.** Changes in the haemostatic variables in the three treatment groups (higher-dose OCP group, n = 21; low-dose OCP + S group, n = 16; and metformin group, n = 23) following 6 months of intervention are shown in Table 2. PAI-1 activity fell in all three groups after treatment (p = 0.001, 0.0007 and 0.014, respectively). ADMA levels significantly dropped after treatment with higher-dose OCP (p = 0.0125). PF1 and 2 increased with higher-dose OCP and metformin intervention (p = 0.017 and 0.043, respectively). TG levels rose with intervention with higher-dose OCP (p = 0.0005) and low-dose OCP + S (p = 0.00089), while IPA levels fell with higher- and low-dose OCP + S therapy (p = 0.004 and 0.012, respectively). Platelet activity increased in all three interventions after treatment (p < 0.0001 for higher-dose OCP, p = 0.0004 for low-dose OCP + S and p = 0.037 for metformin). TAFI activity rose significantly after higher-dose OCP (p = 0.0004).

**Between-group change.** Comparing higher-dose OCP, low-dose OCP + S and metformin treatments, only changes to PF1 and 2 showed a significant difference between hormonal treatments (p = 0.023), with a significant increase in PF1 and 2 observed in the higher-dose OCP group following treatment (mean change = -150.79) but not in the low-dose group (mean change = -13.30) (Table 2). Changes in TG levels were significantly different in both the higher- and low-dose OCP groups compared with metformin (p < 0.0001 and 0.0002, respectively). TG levels significantly increased after treatment with higher-dose (mean change = 446.44) and low-dose OCP + S (mean change = 348.59) but not with metformin (mean
# The Haemostatic System in PCOS

## Aberrant Haemostasis from OCP but Not Metformin in PCOS

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### Table 2: Baseline, 6 months and change in haemostatic variables in the three interventional groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Higher-dose OCP (n = 21)</th>
<th>Low-dose OCP + S (n = 16)</th>
<th>Metformin (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary haemostasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1&lt;sup&gt;a,b&lt;/sup&gt; (U/mL)</td>
<td>5.52</td>
<td>5.62</td>
<td>6.24</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>3.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.37&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>-1.91 (-2.70 to -1.12)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-1.49 (-2.22 to -0.75)</td>
<td>-0.87 (-1.54 to -0.19)</td>
</tr>
<tr>
<td><strong>ADMA</strong> (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.06</td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>6 mo</td>
<td>0.78&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>1.02</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>-0.28 (-0.50 to -0.07)</td>
<td>-0.06 (-0.18 to -0.06)</td>
<td>-0.11 (-0.25 to -0.03)</td>
</tr>
<tr>
<td><strong>Secondary haemostasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PF1 and 2 (pM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>168</td>
<td>234</td>
<td>178</td>
</tr>
<tr>
<td>6 mo</td>
<td>318&lt;sup&gt;e&lt;/sup&gt;</td>
<td>221</td>
<td>272&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>150.79 (31.89 to 269.68)</td>
<td>-13.30 (-55.78 to 29.18)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>93.63 (3.39 to 183.89)</td>
</tr>
<tr>
<td><strong>TG (nM/min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1,508</td>
<td>1,522</td>
<td>1,546</td>
</tr>
<tr>
<td>6 mo</td>
<td>1,957&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1,871&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1,422</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>446.44 (223.09 to 666.80)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>348.59 (102.78 to 594.41)</td>
<td>-120.69 (-298.01 to 56.63)&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fibrinolytic system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tPA (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11.52</td>
<td>11.71</td>
<td>11.35</td>
</tr>
<tr>
<td>6 mo</td>
<td>8.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.74&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.23</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>-3.22 (-5.28 to -1.16)</td>
<td>-1.97 (-3.58 to -0.36)</td>
<td>-1.12 (-2.60 to -0.37)</td>
</tr>
<tr>
<td><strong>Plasminogen (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>116.24</td>
<td>121.07</td>
<td>122.87</td>
</tr>
<tr>
<td>6 mo</td>
<td>143.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>150.40&lt;sup&gt;e&lt;/sup&gt;</td>
<td>129.96&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>27.38 (18.15 to 36.61)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>29.33 (15.80 to 42.87)</td>
<td>7.09 (0.47 to 13.70)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Inhibition of fibrinolysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TAFI (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>119.62</td>
<td>122.57</td>
<td>128.87</td>
</tr>
<tr>
<td>6 mo</td>
<td>135.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>141.2</td>
<td>124.39</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>16.05 (8.09 to 24.00)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.71 (-4.38 to 41.81)</td>
<td>-4.48 (-15.07 to 6.11)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>PAI-1&lt;sup&gt;a,b&lt;/sup&gt; (U/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.52</td>
<td>5.62</td>
<td>6.24</td>
</tr>
<tr>
<td>6 mo</td>
<td>3.61&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.37&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean change (95% CI)</td>
<td>-1.91 (-2.70 to -1.12)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-1.49 (-2.22 to -0.75)</td>
<td>-0.87 (-1.54 to -0.19)</td>
</tr>
</tbody>
</table>

**Abbreviations:** ADMA, asymmetric dimethylarginine; CI, confidence interval; PF1 and 2, prothrombin fragments 1 and 2; OCP, oral contraceptive pill; OCP + S, oral contraceptive pill + spironolactone; PAI-1, plasminogen activator inhibitor 1.

**Note:** Mean change = change from baseline to 6 months ± 95% confidence interval (CI).

<sup>a</sup>|<sup>b</sup>PAI-1 results reflect both endothelial dysfunction/function and inhibition of fibrinolysis.

<sup>d</sup>Within group change, p ≤ 0.05, comparing baseline to 6 months.

<sup>f</sup>p ≤ 0.05, comparing higher-dose OCP and metformin.

<sup>g</sup>Between group change, p ≤ 0.05, comparing higher-dose OCP and low-dose OCP + S.

<sup>h</sup>TG reflects net secondary haemostasis (global haemostatic marker).

<sup>i</sup>p ≤ 0.05, comparing low-dose OCP + S and metformin.

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change = -120.69) (Table 2). The change in plasminogen activity over the 6-month period was significantly different in women taking either higher-dose or low-dose OCP + S compared with metformin (p = 0.001 and 0.001, respectively). The change in plasminogen activity in both higher- and low-dose OCP treatment groups (mean change = -27.38 and 29.33, respectively) was significantly higher than that observed in the metformin group (mean change = 7.09) (Table 2). TAFI activity was significantly different in women taking higher- or low-dose OCP compared with those on metformin (p = 0.003 and 0.035, respectively). TAFI activity rose significantly in those women on higher-dose OCP (mean change = 16.05%) with no significant changes recorded for low-dose OCP + S (mean change = -4.98%) (Table 2). While all three of the ADMA levels showed a significant reduction in PAI-1 activity, the greatest change was recorded in women receiving higher-dose OCP (1.91 U/ml), which was significantly greater than that recorded for metformin (p = 0.04), where it only fell by 0.87 U/ml (Table 2).

Regression Analyses
Regression analyses in the higher-dose OCP, low-dose OCP + S, metformin and the entire group between changes in hormonal measures and changes in haemostatic parameters show a significant relationship between changes to PAI-1 and testosterone in the higher- and low-dose OCP groups (p = 0.001, r = 0.704 and p = 0.046, r = 0.523, respectively) as well as for the entire cohort (p = 0.002, r = 0.398); however, no relationship between changes in ADMA and testosterone levels was observed. No relationships were noted for changes in either PAI-1 or ADMA with that of the metabolic markers assessed (insulin, IR and AUC Insulin) in all analyses.

A significant relationship between changes in plasminogen and testosterone levels were noted for higher-dose OCP (p = 0.007, r = 0.567) and the entire cohort (p = 0.001, r = 0.430), where a relationship was also noted between changes in plasminogen and AUC Insulin (p = 0.022, r = 0.323). A relationship between changes in tPA and AUC Insulin (p = 0.002, r = 0.674) was noted only in the higher-dose OCP intervention. A significant relationship was noted for changes to TAFI and AUC Insulin for the entire cohort only (p = 0.045, r = 0.288).

Discussion and Conclusion
Women with PCOS have increased cardiovascular risk factors, apparent higher rates of CVD and VTE. Here, we report a novel and comprehensive randomized comparative trial on the impact on pro- and anti-thrombotic markers and overall haemostatic activity of PCOS therapies, including the OCP and metformin. In a three-arm randomized clinical trial intervention with (1) higher-dose OCP, (2) low-dose OCP + an anti-androgen spironolactone or (3) metformin, we have observed an improvement in primary haemostasis (through improvement in endothelial function) with higher-dose OCP and some improvement with low-dose OCP + S and metformin. Both the low-dose OCP + S and higher-dose OCP with cyproterone acetate increased coagulation and induced a potential hypofibrinolytic state in the higher-dose form, which may further increase CVD and VTE risk in women with PCOS. Metformin, on the other hand, did not show an adverse (net) haemostatic effect.

Within-Group Outcomes
Effects on the Primary Haemostatic System/Response (Endothelial Function)
It is well established that endothelial dysfunction can predispose an individual to CVD. Endothelial dysfunction is present in PCOS, reflected by elevated levels of ADMA and PAI-1 antigen/activity. Here, 6 months of intervention with higher-dose OCP appeared to improve endothelial function and reduced both PAI-1 activity and ADMA levels in women with PCOS. This is consistent with prior studies in women with PCOS showing reduced ADMA levels after higher-dose OCP therapy (30- to 35-μg EE) (32,34,35) low-dose OCP + S and metformin interventions may also have potential benefits on endothelial function in PCOS with a fall in PAI-1 activity, but not in ADMA levels. Most studies conducted on metformin use in PCOS have shown a reduction in both markers. These changes in PAI-1 activity and ADMA levels indicate a potential beneficial effect of higher-dose OCP, low-dose OCP + S and metformin on endothelial function and therefore in preventing initiation of an aberrant primary haemostatic response in PCOS.

Factors that contribute to endothelial dysfunction in PCOS include both metabolic (fasting insulin level and HOMA score) and hormonal (testosterone) abnormalities. We have shown a reduction in testosterone levels after intervention with both higher- and low-dose OCP with an increase in IR based on AUC Insulin level in the higher-dose group only. In this study, we observed a strong relationship between PAI-1 and testosterone with both OCP treatments and the entire cohort. A significant reduction in AUC Insulin after 6-month intervention with metformin with no changes in testosterone levels has been observed. In this study, we did not reveal a relationship between either of the endothelial function markers and AUC Insulin with metformin use. Oestrogens is well known to improve endothelial function, yet in PCOS potential beneficial effects of the OCP (±spironolactone) may also be mediated by lowering testosterone levels. Metformin’s mechanisms of action on the endothelium are uncertain, yet they may relate to metabolic improvements seen with metformin use in PCOS.

Effects on Coagulation (Secondary Haemostasis)
PF1 and 2 are markers of in vivo coagulation-mediated thrombosis. PF1 and 2 levels appear similar between PCOS and controls. In our study, PF1 and 2 levels significantly increased in the higher-dose OCP group but not in the low-dose group. We also investigated TG, a global haemostatic assay that looks at both the pro-coagulation effects of the coagulation cascade, but also at inhibitory substances that prevent the generation of thrombin, mainly tissue factor pathway inhibitor, anti-thrombin and protein C. TG levels are also similar in women with PCOS compared with controls. Here, TG was significantly
increased after higher- and low-dose OCP in women with PCOS. These results indicate increased generation of thrombin and net coagulation activity in both the higher- and low-dose OCP groups as reflected by TG as well as PF1 and 2 in the former group. It is also well accepted that thrombin is a strong platelet agonist, while our earlier results showed improved in endothelial function through reductions in PAI-1 in both OCP groups and ADMA with higher-dose OCP treatment, increased generation of the powerful platelet activator, thrombin, may potentially counteract these positive effects and trigger activation of the primary haemostatic response/system.

After 6 months of metformin, PF1 and 2 levels significantly increased from baseline; however, no differences were noted for TG in women with PCOS. While no studies have been published assessing PF1 and 2 or TG in women with PCOS on metformin therapy, Luque-Ramirez et al noted an increase in coagulation after metformin use through a decrease in prothrombin time and an increase in prothrombin activity. The PF1 and 2 results indicate increased coagulation cascade activity with metformin use, yet the net coagulation effect reflected by TG was not significantly increased. Differential effects on overall coagulation are likely to be related to impacts on inhibitors of coagulation; however, further research is needed to confirm this.

**Effects on Fibrinolysis**

A hypofibrinolytic capacity as a result of net reduced fibrinolysis due to elevated inhibitory activities of PAI-1 with a subsequent prevention of conversion of plasminogen to active plasmin has been observed in women with PCOS. With 6 months of higher- or low-dose OCP and metformin, plasminogen levels significantly increased in all groups, whereas tPA fell only after the higher- and low-dose OCP. PAI-1 activity fell in all three groups, while fibrinolytic inhibitor TAFI only increased following treatment with higher-dose OCP. No studies have been published assessing plasminogen or tPA in PCOS following OCP and only one study assessed TAFI that evaluated antigen levels rather than activity after 6 months of higher-dose OCP, revealing no significant change with therapy. Studies assessing tPA levels following metformin therapy in PCOS did not show any significant differences after intervention with the insulin-lowering agent, PAI-1, as previously mentioned, fell in most cases after metformin therapy.

The fall in tPA noted with hormonal treatments most likely reflects a drop in PAI-1 levels, rather than a fall in the plasminogen activator, as tPA travels in circulation linked to the fibrinolytic inhibitor. Elevated plasminogen activity may represent increased synthesis of the marker following an increased need for fibrinolysis (due to increased coagulation), but may also represent potential inhibitory effects and prevention of conversion to active plasmin. A significant relationship between changes in plasminogen and that of testosterone was noted with higher-dose OCP and with AUC Insulin in the entire cohort, suggesting a potential link between the fibrinolytic marker and both hormonal (testosterone) and metabolic (AUC insulin) markers, although larger sample sizes are needed to confirm this. Increased TAFI activity noted with higher-dose OCP may diminish fibrinolytic capacity in women with PCOS. TAFI cleaves lysine residues from partially degraded fibrin, reducing the conversion of plasminogen into active plasmin. Higher activity of the fibrinolytic inhibitor may be related to increased coagulation as TAFI is activated by thrombin. Changes in TAFI are related to AUC Insulin, in the entire cohort, suggesting a link between the two. The increase in plasminogen activity (noted for all treatments) needs to be investigated to identify if this is a result of increased synthesis to enhance fibrinolytic effects, related to metabolic and hormonal changes from these interventions and/or related to a prevention of conversion to functional form. Prevention of conversion to active plasmin may be related to increased TAFI levels in the higher-dose OCP group.

Overall, despite an improvement in primary haemostasis, through improvement in endothelial function, and reduction in testosterone levels, we noted increased coagulation with both OCP as well as deterioration of the metabolic profile and the potential for a hypofibrinolytic state in women with PCOS following intervention with OCP with 35 mg of EE and cyproterone acetate. Additionally, increased TG with both OCP may result in increased platelet activation and initiation of the primary haemostatic response. Further studies on the effects on platelets from OCP use in PCOS are needed to confirm this. The strong relationships noted between PAI-1 and testosterone as well as between plasminogen and both testosterone and AUC insulin suggest androgens and the metabolic profile may influence the coagulation status in PCOS. Overall, metformin therapy appears to have beneficial haemostatic effects, a potential improvement in primary haemostasis (through improved endothelial function) and no (net) effects on coagulation, although the increased PF1 and 2 levels noted here would need to be investigated further.

**Between-Group Outcomes**

Only changes in PF1 and 2 levels were significantly different between higher- and low-dose OCP groups, suggesting a benefit of low-dose OCP + 5, consistent with evidence in non-PCOS women of a dose-related effect of OCP on thrombosis risk. The higher-dose OCP used in our study was combined with a third-generation progesterin, cyproterone acetate, with a second-generation progesterin preparation, levonorgestrel, in the low-dose oestrogen pill. A meta-analysis that compared the venous thrombosis risk of combined oral contraceptives reported that the 20-mg EE pill combined with the second-generation progesterin levonorgestrel was associated with a relative risk of VTE of 0.6 (95% confidence interval, 0.3–1.0) when compared with the 30-mg oestrogen dose in combination with cyproterone acetate. However, a recent literature review that assessed studies which evaluated the venous thrombosis risk of combined oral contraceptives noted that there are no high-quality studies comparing the different progestins (in combination with oestrogen-containing pills), and if there is an increased risk of venous thrombosis in third-generation progestins versus second-generation progestins, these risks are only minor. Furthermore, studies that reviewed the haemostatic/coagulation effects of the different combined OCP have revealed equivocal results, with no
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Our study, including the small samples sizes in each group, the age of participants (some in the limit of receiving OCP) and that the original design assessed metabolic outcomes, given the apparent high risk of VTE and CVD in women with PCOS, the known adverse impact of the OCP on thrombosis risk and the current haemostatic system findings, our work suggests an additional dimension of treatment (haemostatic system effects) that favours metformin treatment over the OCP in PCOS.

What Is Known on This Topic

- Women with PCOS have higher rates of CVD and VTE related to metabolic and hormonal features and obesity.
- Additional CVD and VTE risk factors are involved in PCOS, potentially related to a hypofibrinolytic state.
- Increased rates for CVD and VTE may be further exacerbated by current and frequently prescribed management strategies for PCOS, including the oral contraceptive pill (OCP), anti-androgens or metformin.
- Increased risks of VTE from OCP use are well accepted in the general population.

What This Paper Adds

- We report a novel comprehensive randomized comparative trial on impact on pro- and anti-thrombotic markers of common PCOS therapies including higher- and low-dose OCP and metformin, where studies on the impact on the haemostatic system in these women are limited with key evidence gaps remaining.
- Both higher-dose and low-dose OCP increased coagulation and induced a potential hypofibrinolytic state in higher-dose form, inducing an increased risk of thrombosis.
- Metformin did not elicit an adverse (net) haemostatic effect.
- In PCOS, a higher risk group for VTE and CVD, our work suggests an additional dimension of treatment (haemostatic system effects) that favours metformin treatment over the OCP in PCOS.

Work Performed at
School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria 3083, Australia.

Disclosure Statement
The authors have nothing to disclose.

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Conflict of Interest
All the authors on this manuscript have no conflict of interest to declare.

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References


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Declaration of Co-Authorship and Co-contribution: Papers incorporated in thesis with publications

This declaration was completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by:                                        Signature:                                        Date:

Genia Burchall                                                                                                10/02/2018

Paper Title: The Plasminogen System in the Polycystic Ovary Syndrome (PCOS) Mouse Ovary.

Note: Refer also to Appendix 3: Collaborative Research Agreement & Memorandum of Understanding (MOU) for this project that was created at the conception of this study to ensure appropriate understanding of collaborators throughout and at the completion of this project (including outcomes derived from this in the manuscript submitted for publication). The MOU also includes relevant information on ethics approval.

In the case of the above publication, the following authors contributed to the work as follows:

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<td>Genia Burchall</td>
<td>70</td>
<td>Designed and performed the research, performed the literature search, independently reviewed the articles and assessed risk of bias, performed the data analysis and interpretation, wrote the manuscript, constructed tables and figures, provided critical review of the manuscript and submitted the final version for publication.</td>
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Kristy Walters 8 Contributed to the design of the project, developed and provided the PCOS (DHT postnatal induced) and control mice ovarian tissues for the project, including sending the (paraffin) embedded blocks of these to Melbourne (RMIT), and reviewed the manuscript (for publication).

Dodie Pouniotis 8 Assisted GB with immunohistochemistry (IHC) troubleshooting needs of markers tested, contributed to the design of the project, commented on distribution of immunohistochemical staining of all markers assessed, reviewed the manuscript for publication.

Helena J. Teede 3 Assisted in design of the research, undertook revision for important content of the manuscript.

Terrence J. Piva 11 Assisted in design of the research, undertook critical revision for important content of the manuscript.

DECLARATION BY CO-AUTHORS/SENIOR SUPERVISOR

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to

a) granting bodies,

b) the editor or publisher of journals or other publications, and
c) the head of the responsible academic unit; and

5. The original data is stored at the following location(s):

Location(s): School of Health and Biomedical Sciences, RMIT University and will be held for at least five years from the date indicated below.

Date: 10/02/2018

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CHAPTER 5: The Fibrinolytic/Proteolytic System in the PCOS Mouse

Ovary

5.1 Introduction

Women with PCOS have increased risks for CVD and VTE likely relating to the aberrant metabolic features and high BMI as well as an abnormal haemostatic system. A hypofibrinolytic state is present in women with PCOS, relating to increased PAI-1 which prevents conversion of pro-enzyme plasminogen to active plasmin. The fibrinolytic system components and relevant inhibitors however play a number of roles, apart from their function in blood haemostasis and thrombosis. Of importance to research into PCOS is their role in the ovulation processes.

Plasmin has been detected in both the follicular fluid (FF) and granulosa cells (GC) of the ovary at the time of or just before rupture of the ovarian follicle in porcine models (Politis, Srikandakumar et al. 1990). This suggests that plasminogen is converted to active plasmin at the time of ovulation (follicular rupture). To achieve this increased plasmin activity, a decrease in inhibitor PAI-1 and an increase in plasminogen activators (tissue plasminogen activator, tPA and/or urokinase plasminogen activator, uPA) is needed. Changes in the levels of these markers were also observed just before follicular rupture in female pigs (Politis, Srikandakumar et al. 1990). In addition to time of ovulation/cyclical period, evaluation of areas within the ovary has revealed that PAI-1 levels are at their highest immediately prior to ovulation in the centre of the ovary where the follicles that are less likely to ovulate reside (smaller follicles including primordial and primary follicles) whereas tPA levels at the same stage (in the ovulation
process) are highest at the surface of the ovary where pre-ovulatory or Graafian (mature) follicles reside (Devin, Johnson et al. 2007) (Figure 5.1).

**Figure 5.1** Internal structure of the ovary and the ovulation process (taken from http://catalog.flatworldknowledge.com/bookhub/reader/12013?e=tye_1.0-ch03_s02 Last accessed 20/10/2017).

The plasminogen system at an ovarian level is under gonadotropin control, primarily LH (Devin, Johnson et al. 2007). Early research showed that each type of plasminogen activator involved in the ovulation process is species-specific and cell-specific, with tPA thought to be primarily secreted by the GC of rats and pigs in pre-ovulatory/mature follicles and uPA as the main plasminogen activator secreted by the GC of mice ovaries just before ovulation (Canipari, O’Connell et al. 1987, Liu, Cajander et al. 1987, Politis, Srikandakumar et al. 1990). Subsequent studies have shown that both plasminogen activators (PA) are of importance in ovulation within a species, however with likely differing actions (Macchione, Epifano et al.
tPA is thought to be primarily involved in rupture of the follicular wall whereas uPA is thought be involved in the tissue remodeling process and in early follicular development (Lafrance, Zhou et al. 1993, Cao, Sahmi et al. 2004). A recent review by Liu et al have concluded that the serine protease tPA and inhibitor PAI-1 both play the most significant roles in oocyte maturation and in ovulation processes (Liu, Liu et al. 2013). They note that differences in PA between species do exist with tPA as the only plasminogen activator secreted by rats and monkeys (Rhesus) while uPA as the principal plasminogen activator of mice. Secretion of tPA is mainly from GC and the oocyte (of rats and mice) while PAI-1 predominantly form follicular walls (theca cells, TC) (Liu, Liu et al. 2013). In the rat only tPA and PAI-1 are under gonadotropin control at an ovarian level, and not uPA (Liu, Liu et al. 2013). Both a cell-specific and time-dependent response are noted in the rat ovary in expression of mRNA and protein levels of tPA and PAI-1, including the area of the ovary where these markers are expressed. An overall proteolytic activity just prior to ovulation following an increase in tPA and decrease in regulator PAI-1 (both mRNA and protein levels) localized at the surface of the ovary was observed (Liu, Liu et al. 2013). Blocking tPA action in rats, following administration of α2 antiplasmin, significantly reduces ovulation in this species (Tsafriri, Bicsak et al. 1989). In mice however the blocking effects of either tPA or uPA alone do not significantly impact ovulation, and only when both plasminogen activators are lost, mice display reduced ovulation outcomes (through the reduced fertility rates noted)(Leonardsson, Peng et al. 1995).

The importance of the plasminogen system has also been noted in the regulation of oocyte maturation and not just in follicular wall degeneration (Liu, Liu et al. 2013). tPA mRNA is secreted by GC of developing follicles however this nuclear product cannot be translated to its protein form due to the effects of GC secreted inhibin subunits. It is only when the follicles approach ovulation, following a LH surge, inhibin levels decrease and the translation of tPA
mRNA occurs. Following the production of tPA protein, germinal vesicle breakdown follows to allow the oocyte to resume meiosis (Liu, Liu et al. 2013). It has also been noted that tPA plays a role in oocyte release by its effects on the cumulus oophorus and assisting in detachment of this from the stratum granulosum prior to ovulation in both rats and mice (Liu and Hsueh 1987, Liu, Liu et al. 2013). Research in this area however is still unclear and requires further investigation.

Studies in humans have identified the presence of tPA in GC and within the FF and PAI-1 also in TC (Beers 1975, Piquette, Crabtree et al. 1993, Atiomo, Hilton et al. 2000). Studies that have evaluated these fibrinolytic/proteolytic system markers and inhibitors within the PCOS ovaries in either humans or in animal models are scarce. Ambekar et al found that plasminogen is degraded within the FF of the ovary in women with PCOS (Ambekar, Kelkar et al. 2015). Atiomo et al, using IHC, found that there was no significant difference in PAI-1 antigen levels within the ovaries of PCOS women and controls (Atiomo, Hilton et al. 2000). Their sample size however was quite small and the women from whom the samples were taken were at various stages of the menstrual cycle. In contrast, Devin et al compared ovarian tissue from women with PCOS and unaffected women’s ovaries and found PAI-1 to be prevalent in the PCOS ovary but lacking in normal ovaries (Devin, Johnson et al. 2007). They also performed a small sub-study on transgenic female mice which constitutively secrete PAI-1 and found the latter to contain many large cystic structures within their ovaries and had plasma testosterone levels nearly twice as high as observed in control mice (Devin, Johnson et al. 2007). These researchers did not evaluate metabolic markers in these mice however Ma et al showed that mice which lacked PAI-1, unlike their wild-type counterparts, did not go on to develop obesity or IR despite being on a high fat/high carbohydrate diet(Ma, Mao et al. 2004).
While PAI-1 is believed to play a physiological role within the ovary in preventing ovulation of immature follicles, persistent elevation of this fibrinolytic/proteolytic inhibitor, as observed in the plasma of women with PCOS, may potentially contribute to a lack of ovulation. By preventing conversion of plasminogen to active plasmin, these high PAI-1 levels will in turn prevent follicular wall breakdown of mature pre-ovulatory follicles, the result of which may ultimately give rise to the ovarian architecture currently observed in the ovaries of women with PCOS. Additionally, the inhibitory effects of PAI-1 on tPA may prevent the latter in participating in oocyte maturation events.

This study (Chapter 5) is the first, that I am aware of, that comprehensively evaluates the fibrinolytic/proteolytic system in the PCOS ovary. In this study the expression of fibrinolytic/proteolytic markers tPA, uPA, plasminogen, plasminogen/plasmin and regulator PAI-1 were investigated in control and PCOS mouse ovaries to ascertain the presence and distribution of these markers in these tissues. Experiments described in this chapter aimed to identify if these markers may potentially play a role in ovulation and folliculogenesis processes and if these processes are disturbed in the PCOS ovaries following observations of aberrations or differences in normal levels and/or distributions of these markers in the tissues assessed. As PCOS is the leading cause of anovulatory infertility in women in many countries, research into this area could potentially provide significant breakthrough as well as support further study into the infertility treatment options for women with this very complex and common disorder.
5.2 My role

Chapter 5 is based on the results obtained from an observational study using an animal model assessing the fibrinolytic/proteolytic markers plasminogen, plasminogen/plasmin, tPA, uPA and PAI-1 in PCOS and control mice ovaries. I initiated a collaboration with Dr Kirsty Walters (School of Women’s & Children’s Health, University of New South Wales, Sydney) who kindly provided me with paraffin-embedded ovarian tissues from PCOS and control mice. I researched appropriate methods for preparation and set these up at RMIT in collaboration with Assoc Prof Ian Darby and Dr Dodie Pouniotis. I prepared the required sections from six PCOS and six control mice ovaries. I immunohistochemically stained these sections for markers of interest (plasminogen, plasminogen/plasmin, tPA, uPA and PAI-1) once I completed any method validation and optimisation steps. I then completed training on the Olympus Microscope and Slide Scanner (Olympus VS-ASW 2.9, Tokyo, Japan) and was able to scan the slides of interest. Further to receiving training on the Olympus cellSense software (Olympus cellSense Dimension Desktop 1.16, Tokyo, Japan) I completed image analyses of slides of interest once I was competent in this method, which included performing a number of troubleshooting requirements. I was responsible for the initial selection of consumables and all reagents required for the study and advised my supervisor of the ordering requirements (please refer to the Materials and Methods section of the submitted manuscript on the following pages for the sources of consumables). I statistically analysed and interpreted the data (following consultation with the biostatistician) and wrote the manuscript which has been submitted for publication to Biology of Reproduction (manuscript number: BIOLRE-2018-0123). As a result of my contribution I am first author on this manuscript.
The Plasminogen System in the Polycystic Ovary Syndrome (PCOS)

Mouse Ovary

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ABSTRACT

The fibrinolytic system and its relevant inhibitors play a number of roles, apart from their function in blood haemostasis, namely in ovulation. Plasminogen is converted to active plasmin at the time of follicular rupture through a decrease in plasminogen activator inhibitor-1 (PAI-1) and an increase in plasminogen activators. Oligo-/anovulation and follicle arrest are key characteristics of PCOS, but few studies have evaluated fibrinolytic/proteolytic markers within human PCOS ovaries or PCOS animal models. We used a PCOS mouse model to investigate and compare the presence and distribution of fibrinolytic/proteolytic markers plasminogen, plasminogen/plasmin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and inhibitor PAI-1 in PCOS and control ovaries. A hyperandrogenised PCOS mouse model was used that mimics a breadth of ovarian, endocrine and metabolic features of the human condition. Immunohistochemical examination revealed differences in the ovarian distribution of PAI-1 that was localised throughout the PCOS ovary unlike peripheral distribution of controls and plasminogen that presented in small follicles only in PCOS ovaries and not in small follicles of control ovaries. While no differences were noted in the overall expression (mean total percentage and mean colour intensity) of ovarian staining of markers assessed, our findings show a potential role for the plasminogen system in both the physiological mouse ovary and in PCOS. Further studies evaluating these markers at different time-points of ovulation may help to further clarify both physiological and potential pathological actions these markers play in ovulation processes distorted in PCOS.
INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is a common condition, affecting 12-18% of reproductive aged women and is diagnosed based on ovulatory and menstrual disturbance, hyperandrogenism and polycystic appearance of ovaries on ultrasound (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004, March, Moore et al. 2010, Teede, Misso et al. 2011, Yildiz, Bozdag et al. 2012). Women with PCOS have increased risk for cardiovascular disease (CVD) and venous thromboembolism (VTE) likely relating to the aberrant metabolic features and high BMI, frequently present in these females, but also to the recently identified abnormal haemostatic and fibrinolytic systems (Burchall, Linden et al. 2011, Burchall, Piva et al. 2016). A likely hypofibrinolytic state is present in women with PCOS, relating to increased plasminogen activator inhibitor 1 (PAI-1) which prevents conversion of the pro-enzyme plasminogen to its active form of plasmin (Burchall, Piva et al. 2016). The fibrinolytic system and its inhibitors play a number of roles, apart from their function in blood haemostasis and thrombosis. Plasmin has been detected in both the follicular fluid (FF) and granulosa cells (GC) of ovarian follicles at the time of or just before rupture in porcine models. The study suggested that plasminogen is converted to functional plasmin at the time of follicular rupture. This is achieved following a decrease in PAI-1 and an increase in plasminogen activators (PA) observed just before follicular rupture in female pigs (Politis, Srikandakumar et al. 1990). PAI-1 levels are at their highest immediately prior to ovulation in the centre of the ovary where immature follicles reside whereas tissue plasminogen activator (tPA) at the same stage is highest at the surface where pre-ovulatory follicles are localised (Devin, Johnson et al. 2007). Both plasminogen activators tPA and uPA were believed to be important in ovulation within a species, though with likely differing actions (Macchione, Epifano et al. 2000, Dow, Bakke et al. 2002). A recent review by Liu et al concluded that the serine protease tPA and inhibitor PAI-1 play the most significant role in oocyte maturation and
in ovulation processes (Liu, Liu et al. 2013). They note however that differences in plasminogen activators between species do exist with tPA as the only plasminogen activator secreted by the GC and oocytes of rats while uPA as the principal plasminogen activator secreted by these same cells in mice. Both a cell-specific and time-dependent response are noted in the expression of both tPA and PAI-1, with the latter predominantly expressed by the theca cells (TC) of follicular walls in the rat ovary. Liu et al confirmed that literature to date notes an overall proteolytic activity just prior to ovulation following an increase in tPA and decrease in regulator PAI-1 at the surface of the ovary (Liu, Liu et al. 2013). The importance of the plasminogen system has also been noted in oocyte maturation and release (Liu, Liu et al. 2013).

Ambekar et al found that plasminogen is degraded within the FF of ovaries in women with PCOS (Ambekar, Kelkar et al. 2015). However Atiomo et al did not find a significant difference in PAI-1 antigen concentrations within the ovaries of PCOS women compared to controls (Atiomo, Hilton et al. 2000). Both studies however included small sample sizes. In contrast, Devin et al found PAI-1 to be prevalent in the PCOS ovary but not in non-PCOS ovaries (Devin, Johnson et al. 2007). They also performed a small sub-study on transgenic female mice which constitutively secrete a stable variant of active human PAI-1 and found the latter to contain many large cystic structures within their ovaries and had plasma testosterone levels nearly twice as high as in control mice, concluding that overexpression of PAI-1 promotes the development of PCO in female mice. These researchers did not evaluate metabolic or fibrinolytic markers in these mice. However Ma et al showed that mice which lacked PAI-1, unlike their wild type counterparts, did not go on to develop obesity and insulin resistance, which are key clinical features of PCOS, despite being on a high fat/high carbohydrate diet (Ma, Mao et al. 2004). While PAI-1 is believed to play a physiological role within the ovary in preventing ovulation of immature central follicles, persistent elevation of
the fibrinolytic/proteolytic inhibitor, as is noted in the plasma of women with PCOS, may potentially contribute to a lack of ovulation. PAI-1 may prevent follicular wall breakdown of more mature pre-ovulatory follicles and may contribute to the ovarian architecture currently observed in the ovaries of women with PCOS. PCOS is also a leading cause of infertility (Homburg 2003, Azziz, Carmina et al. 2016). Oligo-/anovulation and follicle arrest are key characteristics of PCOS, associated with infertility in this group of women, with the exact mechanisms driving this still as yet unclear (Pierre, Peigne et al. 2013). In this context, we aimed to investigate and compare the presence and distribution of fibrinolytic/proteolytic markers plasminogen, plasmin, tPA and uPA and inhibitor PAI-1 in control and PCOS ovaries. We used a PCOS mouse model treated with dihydrotestosterone (DHT) that display extensive ovarian, endocrine and metabolic features of humans affected by PCOS including oligo- or anovulation, irregular menstrual cycles, polycystic ovaries, obesity and dislipidaemia. We hypothesized that all fibrinolytic/proteolytic markers investigated in this study will be detected in both the PCOS and control mice ovaries, however they will vary in their expression and distribution within the tissues. In general PAI-1 expression will be elevated in the PCOS mouse ovaries compared to that of controls and this marker will be distributed throughout the tissue, however in controls it will be localised mainly centrally. The ovarian expression and distribution of PA in PCOS and control mice will be similar, but differences may be noted for plasmin/plasminogen between the two experimental models.
MATERIALS & METHODS

Mice

As previously reported, the mice used in this study were maintained under standard housing conditions (ad libitum access to food and water in a temperature- and humidity controlled, 12-hour light cycle environment) at the ANZAC Research Institute [Concord, Australia] (Caldwell, Middleton et al. 2014). All mice had a wild-type AR genotype and were taken from a colony used to generate AR-knockout mice (Walters, Allan et al. 2007, Simanainen, Gao et al. 2012). This colony has been backcrossed onto a C57Bl/6J background for at least 10 generations prior to use in experiments. In all experiments littermate controls were used. All procedures were performed under ketamine/xylazine anesthesia. All procedures were approved by the Sydney Local Health District Animal Welfare Committee within NHMRC guidelines for animal experimentation (Caldwell, Middleton et al. 2014).

Development of PCOS mouse model

The PCOS mice were generated by implanting female mice with 1 cm SILASTIC brand implant (id, 1.47 mm; od, 1.95 mm, Dow Corning Corp, catalog no. 508-006) containing approx. 10 mg DHT at 21 days of age and for a period of 90 days (Caldwell, Middleton et al. 2014). Controls were administered a blank (oil) 1cm SILASTIC implants also at 21days and for the same time period as PCOS mice. The experimental strategy chosen to generate our PCOS mice was based on our previous study where we identified the optimal approach for experimental PCOS studies using a mouse model (Caldwell, Middleton et al. 2014). Mice were
collected after 13 weeks of drug administration (control, n = 8; DHT, n = 9) when the mice were 16 weeks of age (Caldwell, Middleton et al. 2014).

Assessment of Estrous Cycle

The stage of the estrous cycle of mice was identified on a daily basis using light microscopy examination of vaginal epithelial cell smears, as previously described (Caldwell, Middleton et al. 2014). The stage of the estrous cycle was determined based on the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells. Proestrous was characterized by the presence of mostly nucleated and some cornified epithelial cells; at the estrous stage mostly cornified epithelial cells were present; at metestrus both cornified epithelial cells and leukocytes were present; and at diestrus primarily leukocytes were visible (Caldwell, Middleton et al. 2014). Mice were anesthetized (at the diestrus stage of the estrous cycle of control mice & stage of cycle was unascertainable for PCOS mice as these mice did not cycle) using ketamine/xylazine and the ovaries removed.

Collection and processing of ovaries

Ovaries were weighed and fixed in 4% paraformaldehyde, stored at 4°C overnight after which they were put into 70% alcohol (Caldwell, Middleton et al. 2014).

The present study was completed based on biobanked ovarian tissue samples from control and PCOS mice from a prior study (Caldwell, Middleton et al. 2014). Data on body weight/fat, metabolic (cholesterol, triglycerides, insulin tolerance test), hormonal (FSH, LH, testosterone) and reproductive/ovarian parameters have been previously published (Caldwell, Middleton et al. 2014).
Paraffin-wax embedded ovarian tissue from the DHT induced PCOS mice and control mice was sectioned at 4 μm thickness and mounted onto 3-aminopropyltricttiosilane-coated glass slides, then dried overnight in a 50°C incubator or incubated at 60°C for 2 hours. The rabbit antibodies used for the study were directed towards the fibrinolytic/proteolytic markers and inhibitor of interest.

*Expression of PAI-1, tPA, uPA, plasminogen and plasmin by immunohistochemistry*

We used immunohistochemical detection of PAI-1, tPA, uPA, plasminogen/plasmin and plasminogen only in sectioned tissue of both PCOS and control mice. We were unable to identify a plasmin only anybody. Immunohistochemistry (IHC) was initially performed on sections of normal mouse tissue known to contain PAI-1, tPA, uPA, plasmin and plasminogen; namely liver for PAI-1, plasminogen/plasmin and plasminogen only, brain for tPA and kidney for uPA (positive control tissues) to determine ideal working conditions. A negative control was also tested with each marker by omitting to add the primary antibody. PCOS and control sections were stained simultaneously for each marker in order to avoid variability due to staining techniques. Commercially available primary antibodies that have been shown to work in IHC applications on paraffin-embedded sections (IHC-P) were used for all markers with the exception of the plasminogen only antibody that was shown to work in immunocytochemistry techniques and it was hypothesized that it would also work in IHC. We confirmed this through initial IHC staining of positive and negative controls followed by PCOS and control ovarian tissue.

Slides were deparaffinised in xylene (thrice for 5 min) and rehydrated in graded alcohol (100%, 100%, 70%). Antigen retrieval was performed in a pressure cooker containing sodium citrate buffer (pH 6.0) for 20 min for all markers except for PAI-1 where only 10 min incubation was
used and uPA where TrisEDTA buffer (pH 9.0) was used instead of sodium citrate. Endogenous peroxide activity was quenched by 3% H$_2$O$_2$ in water for 15 min. Non-specific binding was reduced using 1% normal goat serum (ABC Kit VECTASIN). The slides were incubated with the primary antibody at room temperature for either 90 min for PAI-1 (rabbit polyclonal antibody to mouse PAI-1, ABCAM: ab28207) (1:250), uPA (rabbit monoclonal antibody to mouse uPA, ABCAM:ab133563) (1:150), tPA (rabbit polyclonal antibody to mouse tPA, ABCAM: ab28208) (1:200) and plasminogen/plasmin antibodies (rabbit polyclonal antibody to mouse plasminogen/plasmin, Novus Biologicals: NBP2-19859) (1:300) or overnight at 4°C for the plasminogen only antibody (rabbit polyclonal antibody to mouse plasminogen, Novus Biologicals: NB300-544) (1:100). Slides were then incubated with the secondary antibody (ABC Kit VECTASIN Anti-rabbit IgG biotinylated, affinity purified anti-immunoglobulin) for 30 min for all antibodies except tPA where this step was omitted (as the primary antibody to tPA was biotinylated). 3,3'-diaminobenzidine, DAB (Vector ImmPACT DAB) was used as a chromagen for 120-180 sec and haematoxylin was used for counterstaining.

**Image analysis**

Sections were scanned into digital format using an Olympus Microscope and Slide Scanner (Olympus VS-ASW 2.9, Tokyo, Japan) keeping the (light) exposure time constant for all slides (930 μs). Digital image analysis and processing of each scanned slide was performed using Olympus cellSens software (Olympus cellSens Dimension Desktop 1.16, Tokyo, Japan), Count & Measure functionality that allows selection of a region of interest and cell/region counting capabilities. Intensity analysis was used to determine intensity of (brown) staining in each of the sections. Positive thresholds (of intensity of staining) were set for each marker on the positive control tissues. The positive thresholds for each marker were then applied to the
negative control tissues to ensure negative/no detection of staining for the thresholds set. Mouse PCOS and control ovarian tissues were evaluated with the set positive intensity threshold of staining of each marker. The whole ovary for both PCOS and controls was selected as the region of interest (ROI). Overall ovarian staining (Sum Area, \( \mu \text{m}^2 \)), percentage of ovarian staining (Area Fraction ROI, \%) and intensity of this staining (Mean Colour Intensity) was investigated and compared in PCOS and control ovaries. Sections were also examined and the distribution of staining in the ovary (PCOS or control) for each marker was determined. The small follicles described typically represent primordial and primary follicles while the large follicles include secondary and Graafian follicles.

Sections were examined and characterized by two operators blinded to the identity of the ovarian sample/tissue type.

**Statistics**

Image analysis parameters are presented as mean values \( \pm \)SD. Statistical analysis was performed using SPSS for Windows 22.0 software (SPSS, Inc., Chicago, USA) with statistical significance \( p \leq 0.05 \). Data were assessed for normality and log transformed where appropriate. Results are presented for a total of 12 ovarian sections/mice ovaries (Controls = 6 ovarian sections/mice ovaries & PCOS = 6 ovarian sections/mice ovaries). Differences between PCOS and control IHC staining for all markers were assessed using the student’s t test for normally distributed data and the Wilcoxon-Mann Whitney test for non-normally distributed data.
RESULTS

As previously published, PCOS mice have DHT concentrations 8-fold higher than that of control mice with the former having a mean ± SEM of 1.64 ± 0.32 ng/mL compared to the latter of 0.19 ± 0.07 ng/mL (p<0.05) (Caldwell, Middleton et al. 2014).

Localisation of Fibrinolytic/Proteolytic markers in murine PCOS and Control ovaries

Expression and localization of PAI-1, tPA, uPA, plasminogen/plasmin and plasminogen only in both DHT postnatally inducted PCOS and control murine ovaries was investigated. In the control ovaries, PAI-1 was predominantly found in the GC, with highest expression present in large follicles located mainly in the periphery of the ovary (Table 1 & Figure 1). The marker was also present in the FF and in the stroma with only scant amounts in the small (central) follicles and TC. In the PCOS ovaries, PAI-1 was localised throughout the ovary. Similar to controls, it was also abundant in the GC, and noted in the FF, stroma and large follicles with low amounts noted in the small follicles and TC of PCOS ovaries.

uPA was present mainly in the periphery of control ovaries as well as in the stroma and GC (Table 1 & Figure 2). This marker was also detected in large and some in small follicles but only in scant amounts in the TC and FF. A similar pattern was observed in the PCOS ovaries, where uPA was predominantly present in the periphery; it was abundant in the GC, present in the stoma (mainly in the centre of the ovary), in the large and small follicles with little amounts observed in the TC and FF.

In general tPA was not observed in either the PCOS or control ovaries (Table 1 & Figure 3), light staining was noted in the centre of the control ovaries and the stroma, small follicles and GC.
As there was not a specific plasmin antibody that could be used for IHC staining of the ovaries, an antibody which detected both plasminogen/plasmin as well as one which only detected plasminogen was used. Differences in the staining between these two antibodies would be most likely due to the expression of plasmin itself. Plasminogen/plasmin (NBP2-19859) was present mainly in follicles in the periphery of the control ovaries, with lower levels of staining observed in central follicles (Table 1 & Figure 4). It was present in abundance in GC and FF and also in the stroma. Low levels were detected in blood vessels as well as in the antrum and TC of control ovaries. In the PCOS ovaries plasminogen/plasmin was also mainly observed in the periphery with a similar pattern of distribution in the GC, FF, stroma, large follicles, antrum, blood vessels and TC to that seen in controls.

The plasminogen antibody NB300-544 only detects plasminogen but not plasmin (Table 1 & Figure 5). Plasminogen was detected throughout the ovaries of controls, mainly present in the GC and FF. Its presence was detected also in large follicles and sometimes also noted in TC; low levels were observed in small follicles, the stroma, antrum and cumulus oophorus of control ovaries. In the PCOS ovaries plasminogen was abundantly present in the FF, noted in the TC and GC, as well as in small and large follicles, with low levels in the stroma.

**IHC comparisons of fibrinolytic/proteolytic markers in murine PCOS versus control ovaries**

It is well accepted that in human PCOS ovaries are generally larger in size/volume in comparison to control ovaries. We noted a significant difference in the average area ROI (average ovarian size) between PCOS and control mice ovarian sections (p=0.01). As a result of this we then investigated the % of staining of the ovaries in PCOS and controls (Area Fraction ROI) of each marker. Overall we did not observe any significant difference in the staining for any of the 5 markers evaluated for either mean total area of ovarian IHC staining.
(Sum Area), Area Fraction ROI or average colour intensity of overall ovarian staining (Mean Colour Intensity Value) between PCOS and control ovaries (Table 2).

**DISCUSSION**

This is the first study that has comprehensively looked into the expression and distribution of fibrinolytic/proteolytic markers and the plasminogen system in the polycystic ovary.

We observed that four (PAI-1, uPA, plasminogen/plasmin, plasminogen only) out of the five markers assessed were expressed in both the PCOS and control mouse ovaries. The exception being tPA which was observed in low levels in controls and absent in PCOS mouse ovaries. This agrees with that seen in early literature where each type of plasminogen activator involved in the ovulation process is species-specific, where uPA is the main plasminogen activator secreted by the GC of healthy mice ovaries just before ovulation while tPA is absent (it is thought to be primarily secreted by the same cells in pre-ovulatory follicles in rats and pigs) (Canipari, O'Connell et al. 1987, Liu, Cajander et al. 1987, Politis, Srikandakumar et al. 1990). However, it should be noted that the stage of the oestrous cycle at which the mice were assessed may have affected the outcomes, as our control mice were at the diestrus stage of this cycle, with few/little presence of pre-ovulatory/large follicles. Our novel data has evaluated the presence and distribution of tPA in the PCOS mouse ovary and demonstrates the presence and potential role of four of the fibrinolytic/proteolytic markers and of the plasminogen system in physiological and/or pathological actions within the ovaries of healthy control mice, and also in PCOS mice models, noting the lack of tPA involvement (at this stage of the oestrous cycle) in both groups of mice.
In the control ovary PAI-1 was predominantly observed in the GC, but also present in large follicles mainly in the periphery of the ovary as well as in FF and in the stroma with only low levels noted in the small (central) follicles and TC. In the rat, the TC are predominantly responsible for the production of PAI-1 with no synthesis from the oocyte (into the FF); the GC may also express PAI-1 however this will correlate with the stage of the ovulation cycle to ensure appropriate proteolytic (plasmin) activity with each stage (Liu, Peng et al. 1991, Liu, Liu et al. 2013). In our study, at the diestrus stage of the cycle, the GC seem to be involved in the production of PAI-1 in mice. In prior studies in pigs and rats the distribution of PAI-1 immediately prior to ovulation is lowest in peripheral/large follicles and highest in small/central follicles to assist in ovulation and follicular wall rupture of mature follicles (Liu, Liu et al. 1997, Devin, Johnson et al. 2007). As the control mice in our study were in the diestrus stage of the cycle a different pattern was noted, which may be related to differential proteolytic needs at this stage of the cycle (i.e. less needs for high plasmin activity) or the differences noted may be related to inter-species differences. If ovulation/fertilization does not take place, PAI-1 levels regress, leaving an excess of plasminogen activators to ensure the formation and maintenance of the corpus luteum to assist with tissue remodeling processes to form luteal tissues under the control of the plasminogen system (Liu, Liu et al. 1997, Devin, Johnson et al. 2007). In the PCOS ovary PAI-1 was found throughout the ovary unlike the peripheral distribution observed in the controls. It was abundant in the GC, and noted in FF, stroma and large follicles with trace amounts in the small follicles and TC. This pattern of distribution noted for PCOS mice may relate to the acyclical nature of these mice and the presence of predominantly ‘arrested’ follicles that do not go on to ovulate. As PAI-1 is present in follicles approaching ovulation, and its levels drop immediately prior to ovulation in pre-ovulatory follicles, even distribution of PAI-1 in ovaries of PCOS mice may relate to the presence of follicles in various stages of development along with a reduced number of large/pre-ovulatory follicles. Our results are
similar to that reported by Devin et al who assessed transgenic PAI-1 overexpressing mice that went on to develop multicystic ovaries, in contrast to the wild-type controls (Devin, Johnson et al. 2007). In this study PAI-1 was abundant throughout the ovaries of the transgenic mice, and highly expressed in the GC of developing follicles, thickened tunica and cyst lining and in the hypertrophied theca (interstitium). These researchers also noted little evidence of ovulation in the PAI-1 overexpressing transgenic female mice, in comparison to their wild-type counterparts, where the various stages of follicular development and the corpora lutea could rarely be identified (Devin, Johnson et al. 2007). Atiomo et al observed the presence of PAI-1 in the granulosa and theca cell compartments of both control and PCOS ovaries with weak expression in endothelial cells and serosal surface of humans (Atiomo, Hilton et al. 2000). Devin et al observed PAI-1 to be localized on the GC lining cystic structures and in atretic follicles and not detected in the TC, stroma, corpora lutea or corpora albicans of females with PCOS and was absent or observed in scant amounts in human controls (Devin, Johnson et al. 2007). Neither research groups stated the stage of the oestrous cycle of control women.

We selected the whole ovary, as the region of interest when completing image analysis investigations and comparisons between PCOS and controls for all five markers evaluated, as there have been few or no published studies assessing the plasminogen system in the PCOS ovary, in order to investigate if this system, all relevant markers part thereof, is expressed, in general, in the PCOS mouse ovary. When we compared the mean total amount of ovarian IHC staining, the average intensity of IHC staining and the mean total percentage of ovarian IHC staining for PAI-1 in PCOS and controls, we found no statistically significant differences for these measurements between the two groups. This however does not exclude a potential difference in the expression of the marker between PCOS and controls. However, our results are consistent with the study by Atiomo et al that also noted overall more PAI-1 was detected in PCOS but not statistically different to controls (Atiomo, Hilton et al. 2000).
We noted similar patterns of distribution of uPA in PCOS and controls with the marker being predominantly present in the periphery and in the GC and stroma of both control and PCOS ovaries. It was observed in large and small follicles and some also detected in FF and TC in both ovaries. No significant differences in the mean total amount of ovarian IHC staining, the average intensity of IHC staining and the mean total percentage of ovarian IHC staining were noted for uPA for these two groups. Here we present novel data on the presence and distribution of uPA in the PCOS mouse ovary and demonstrate the presence and potential role of this plasminogen activator in normal physiological folliculogenesis and tissue remodeling processes in mouse ovaries; we also demonstrate no distorted presence/distribution in PCOS. Under normal physiological function, uPA (and other plasminogen activators) are thought to play a role in the formation and maintenance of the corpus luteum and tissue remodeling processes post-ovulation in rhesus monkeys and may do so in mice as well (Liu, Liu et al. 1997, Liu, Feng et al. 2003, Devin, Johnson et al. 2007).

A similar pattern of distribution of the plasminogen antibody used in the study (which does not distinguish between plasminogen and active plasmin), was noted in PCOS and control ovaries. Plasminogen/active plasmin was predominantly present in the peripheral follicles of controls and in the periphery of PCOS ovaries. A similar pattern of distribution of this marker was noted for other remaining ovarian structures assessed between PCOS and controls. When we used the plasminogen-specific antibody we observed this to be expressed throughout the control ovaries with strong staining noted in the FF and also observed in the GC and large follicles of both control and PCOS ovaries. TC and small follicles of PCOS ovaries seem to express plasminogen however this was only sometimes observed or noted in low levels in controls.

No significant differences were noted between PCOS and controls for mean total amount of IHC staining, the average intensity of IHC staining and the mean total percentage of ovarian
IHC staining for either the plasminogen/active plasmin or plasminogen only antibody. This was novel data showing plasminogen and/or plasmin levels in mice ovaries. In humans, Ambekar et al noted that plasminogen was degraded in the FF of the ovary in women with PCOS (Ambekar, Kelkar et al. 2015). While we had hypothesized that the differences in staining between the plasminogen/active plasmin and plasminogen only antibodies would reveal the activity of plasmin only in the ovarian tissues, we actually noted overall stronger ovarian staining with the latter when compared to the former antibody. This may be related to differences in the activity of each antibody type to its antigen/s of interest. We therefore cannot identify and comment on specific plasmin activity and distribution in the PCOS or control ovaries and have therefore discussed the activity and distribution of plasminogen and plasmin interchangeably/collectively.

Overall our results demonstrate the expression inferring active role in ovarian function including in ovulation and follicular development processes of the plasminogen system in the functional mouse ovary and also in PCOS. The results however do not show a statistically significant difference between markers assessed in PCOS versus controls for overall ovarian expression. These may be related to the small sample size assessed and the stage of the ovulation cycle of mice (controls). We noted however differences in the distribution of ovarian immunohistochemical staining for PAI-1 and plasminogen only antibodies between PCOS and controls indicating potential differences in actions of these markers or underlying causes between ovarian physiological and pathological (PCOS) states.

While not all features of humans affected by PCOS are displayed by the mouse model used, no mouse (or other animal) model has been developed to date which does. Additionally as PCOS females present with a heterogeneity of clinical features, not all humans affected by the syndrome will present with all features listed. The long-term postnally treated DHT PCOS
mouse provides the best mouse model for experimental studies of PCOS pathogenesis through replicating extensive ovarian (irregular cycles/acyclicity, oligo-/anovulation, multicystic ovaries, antral follicle arrest, increased follicle atresia, reduced granulosa and increased theca cell layer thickness), endocrine (reduced progesterone and increased DHT) and metabolic (obesity, adipocyte hypertrophy, dyslipidaemia and presence of steanosis) features of humans affected by the syndrome (Caldwell, Middleton et al. 2014). Strengths of our study include a comprehensive analysis of the fibrinolytic/proteolytic markers and the plasminogen system in the control and PCOS mouse ovaries, with limited or no studies previously published assessing these markers in either physiological or pathological (PCOS) states. Further studies with larger samples sizes may further clarify outcomes. Additionally evaluating these fibrinolytic/proteolytic markers at different time-points of the oestrous cycle, particularly immediately preceding ovulation, or assessing the plasminogen system in individual ovarian follicles of varying sizes from small to pre-ovulatory follicles, may help to further clarify physiological and any potential pathological roles of these markers in the ovulation process and in aberrant folliculogenesis in PCOS. Such studies may significantly improve our understanding of this very complex condition as well as assist with treatment options for the frequent infertility noted for women with this common syndrome.
DECLARATION OF INTEREST

All the authors on this manuscript have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported to declare.
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urokinase plasminogen activator) and the urokinase plasminogen activator receptor within bovine periovulatory follicular and luteal tissue. *Biol Reprod* **66**(5): 1413-1421.


**Liu YX, Liu XM, Nin LF, Shi L & Chen SR** 2013 Serine protease and ovarian paracrine factors in regulation of ovulation. *Front Biosci (Landmark Ed)* **18**: 650-664.


FIGURE LEGENDS

Figure 1: Expression of PAI-1 in normal and PCOS mouse ovaries. Representative histological images are from normal (control) (A, B) or PCOS ovaries (C, D) stained with an anti-PAI antibody. The scale bar represents either 100 μm (A, B & D) or 200 μm (C).

Figure 2: Expression of uPA in normal and PCOS mouse ovaries. Representative histological images are from normal (control) (A,B) or PCOS ovaries (C,D) stained with an anti-uPA antibody. The scale bar represents either 200 μm (A), 100 μm (C & D) or 50 μm (B).

Figure 3: Expression of tPA in normal and PCOS mouse ovaries. Representative histological images are from normal (control) (A,B) or PCOS ovaries (C,D) stained with an anti-tPA antibody. The scale bar represents either 200 μm (A, C & D) or 100 μm (B).

Figure 4: Expression of plasminogen/plasmin (NBP2-19859) in normal and PCOS mouse ovaries. Representative histological images are from normal (control) (A,B) or PCOS ovaries (C,D) stained with an anti-plasminogen/plasmin antibody. The scale bar represents 100 μm.

Figure 5: Expression of plasminogen (NB300-544) in normal and PCOS mouse ovaries. Representative histological images are from normal (control) (A,B) or PCOS ovaries (C,D) stained with an anti-plasminogen only antibody. The scale bar represents either 100 μm (A, B & C) or 200 μm (D).
### TABLES

**Table 1:** Expression of the plasminogen system in the granulosa and theca cells, large and small follicles and general distribution in control and PCOS mice ovaries.

<table>
<thead>
<tr>
<th></th>
<th>Granulosa cells</th>
<th>Theca cells</th>
<th>Large follicles</th>
<th>Small follicles</th>
<th>General distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>PCOS</td>
<td>Cont</td>
<td>PCOS</td>
<td>Cont</td>
</tr>
<tr>
<td>PAI-1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>tPA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>uPA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Plasminogen/Plasmin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Plasminogen only</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+* = expression of marker; *-* = marker absent or in low levels. PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; Cont, controls; P, peripheral; C, central; T, throughout; A, absent.
Table 2: Comparisons of mean total area of ovarian IHC staining (Sum Area), percentage of ovary stained (Area Fraction ROI) and average intensity of overall ovarian staining (Mean Colour Intensity Value) of markers assessed in PCOS and control mice ovaries.

<table>
<thead>
<tr>
<th>Marker</th>
<th>PCOS/Cont</th>
<th>Sum Area (x10³, μm²)</th>
<th>Area fraction ROI (%)</th>
<th>Mean (Colour) Intensity Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1</td>
<td>Controls</td>
<td>281±264</td>
<td>7.86±5.96</td>
<td>178.27±6.79</td>
</tr>
<tr>
<td></td>
<td>PCOS</td>
<td>323±266</td>
<td>13.90±10.57</td>
<td>177.07±8.40</td>
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<tr>
<td>tPA</td>
<td>Controls</td>
<td>17±22</td>
<td>0.63±0.79</td>
<td>172.90±6.02</td>
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<tr>
<td></td>
<td>PCOS</td>
<td>8±10</td>
<td>0.29±0.38</td>
<td>169.73±4.20</td>
</tr>
<tr>
<td>uPA</td>
<td>Controls</td>
<td>227±174</td>
<td>7.61±5.72</td>
<td>164.58±6.57</td>
</tr>
<tr>
<td></td>
<td>PCOS</td>
<td>155±125</td>
<td>6.86±5.37</td>
<td>163.43±4.04</td>
</tr>
<tr>
<td>Plasminogen/Plasmin</td>
<td>Controls</td>
<td>271±183</td>
<td>7.21±4.09</td>
<td>178.95±3.18</td>
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<tr>
<td>NBP2-19859</td>
<td>PCOS</td>
<td>193±79</td>
<td>8.64±3.53</td>
<td>180.70±6.25</td>
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<tr>
<td>Plasminogen</td>
<td>Controls</td>
<td>1,147±1,092</td>
<td>38.93±23.14</td>
<td>165.16±5.09</td>
</tr>
<tr>
<td>NB300-544</td>
<td>PCOS</td>
<td>936±646</td>
<td>41.10±23.38</td>
<td>162.72±8.75</td>
</tr>
</tbody>
</table>

Mean±SD are represented for all values. PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; ROI, region of interest.
FIGURES

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) [Image]

(B) [Image]

(C) [Image]

(D) [Image]
CHAPTER 6: Conclusions and Future Directions

6.1 Final Conclusions

Relevant discussions and conclusions of the work presented in the body of this thesis have been presented in each chapter, centred around published or submitted manuscripts. I will conclude with a summary of the original contribution my work has made to the knowledge and understanding of the haemostatic system in women with PCOS. I have also incorporated future directions, limitations and overall conclusions in separate sections below.

This thesis comprised a series of related studies that aimed to enhance our understanding of the haemostatic system in women with PCOS, including relationships with the commonly aberrant hormonal and metabolic systems and also aimed to identify the potential for further CV and venous thrombosis risk factors in an already high-risk group. Following a review of the literature (Chapters 1 and 2) it was shown that an aberrant primary haemostatic response was associated with PCOS, with abnormal platelet activation/function with studies showing increased sCD40L (Zwirska-Korczala, Sodowski et al. 2008, Oktem, Ozcimen et al. 2009, Kebapcilar, Kebapcilar et al. 2011, El-Mesalla my, Abd El-Razek et al. 2013) and elevated MPV (Gursoy, Ertugrul et al. 2006, Kebapcilar, Taner et al. 2009, Cakiroglu, Vural et al. 2016, Yilmaz, Duran et al. 2016); also, endothelial dysfunction was noted in females with the syndrome, as reflected mainly by elevations in ADMA (Charitidou, Farmakiotis et al. 2008, Heutling, Schulz et al. 2008, Ozgurtas, Oktenli et al. 2008, Moran, Hutchison et al. 2009, Rajendran, Willoughby et al. 2009, Moran, Cameron et al. 2011, Toulis, Goulis et al. 2011, Bayrak, Dursun et al. 2012, Choi, Yang et al. 2012) and PAI-1 (Sampson, Kong et al. 1996, Atiomo, Bates et al. 1998, Macut, Micic et al. 2001, Sills, Drews et al. 2003, Diamanti-Kandarakis, Palioniko et al. 2004, Glueck, Wang et al. 2004, Orio, Palomba et al. 2004,
Carmassi, De Negri et al. 2005, Lin and Yongmei 2008, Moran, Hutchison et al. 2009, Oral, Mermi et al. 2009, Manneras-Holm, Baghai et al. 2011, Toulis, Goulis et al. 2011, Koiou, Tziomalos et al. 2012, Moran, Noakes et al. 2012, Aziz, Sidelmann et al. 2015, Elci, Kaya et al. 2017). The potential for an abnormal coagulation or a secondary haemostatic response was also suggested with some studies reporting high fibrinogen levels (Atiomo, Bates et al. 1998, Erdogan, Karadeniz et al. 2008, Heutling, Schulz et al. 2008, Bagir, Bakiner et al. 2016). Increased tPA levels (Kelly, Lyall et al. 2002, Sills, Drews et al. 2003, Lin and Yongmei 2008, Lindholm, Bixo et al. 2010), most likely reflecting tPA–PAI-1 complexes, indicating enhanced inhibition of fibrinolysis confirmed by studies showing high PAI-1 antigen and/or activity as well as some studies showing elevated TAFI levels (Karakurt, Gumus et al. 2008, Oral, Mermi et al. 2009, Guldas, Altinkaya et al. 2015). Global haemostatic tests showed increased generation of thrombin, through the TG assay (de Mendonca-Louzeiro, Annichino-Bizzacchi et al. 2013, Glintborg, Sidelmann et al. 2015), and a reduced fibrinolytic state through a decreased GFC (Yildiz, Haznedaroglu et al. 2002, Andiran, Yordam et al. 2005), where further detrimental effects on the haemostatic system in women with the syndrome may be observed with increased fibrin formation, potential further activation of platelets (and therefore further negative outcomes on the primary haemostatic system/response) and a reduced capacity to counteract coagulation effects.

At the time the review was published (2011) it was also noted that many of the existing studies were suboptimal and that more robust study designs were needed to accurately evaluate the haemostatic system in women with PCOS. This included adequately powered studies, participants matched for age, BMI and confounding factors such as relevant medication usage (that can affect haemostasis) and assessment for smoking status (Burchall, Linden et al. 2011). More recent studies however (completed in the past 5-7 years) have shown improved study
protocols in general. The literature evaluating the haemostatic system in PCOS however is still limited and heterogeneity in quality and outcome persist (Burchall, Linden et al. 2011, Toulis, Goulis et al. 2011) (see Chapter 2). Overall the published review of the literature showed the lack of available data that had comprehensively, accurately and systematically evaluated the haemostatic system in women with PCOS, including a lack of assessment of global markers of haemostatic function, such as the TG test, as well as relevant coagulation factors and coagulation markers that evaluate for \textit{in vivo} haemostatic effects including PF1 & 2 and urinary TXM (Burchall, Linden et al. 2011). The literature had therefore failed to adequately explain both the true state of the haemostatic system in women with PCOS and the mechanisms by which many of the haemostatic effects were noted. This however did reveal a need for further research to address these key gaps in our knowledge, potentially also through use of interventions that modulate the abnormal hormonal and metabolic features and observation of the haemostatic effects/outcomes in these contexts. While existing literature failed to adequately assess and address the state of the haemostatic system in women with the syndrome, it did however suggest a pro-thrombotic state as a potential CV and venous thrombosis risk. This review of the literature was published in a reputable scientific journal to ensure dissemination of outcomes to other researchers in the field (Burchall, Linden et al. 2011).

Building on my extensive literature review, I wanted to address the gaps highlighted. The cross-sectional study completed in Chapter 3 aimed for a systematic, comprehensive and reliable assessment of the haemostatic system; both pro- and anti-thrombotic markers of haemostasis were evaluated as well as markers of global assessment. Markers that assessed components of the haemostatic system in women with PCOS were measured including those of the primary haemostatic system (through evaluations of principal endothelial function markers PAI-1 and ADMA), while secondary haemostasis was evaluated through markers of \textit{in vivo}
coagulation cascade activation and of *in vivo* mediated thrombosis, namely PF1 & 2. The TG test was used as a global marker of haemostasis measurement, which evaluates the net coagulation effects following coagulation cascade activation but also accounts for the simultaneous effects of coagulation inhibitors. tPA and plasminogen were measured to identify the state of the fibrinolytic system and PAI-1 activity was also determined, as a principal inhibitor of fibrinolysis. I followed a robust study design, completed pre-study power calculations to ensure adequate statistical power, considered factors that could interfere with haemostatic function including age, BMI and smoking status and medications that could interfere with study outcomes (such as OCP). I also completed relevant correlation studies between the haemostatic system markers measured and those of the metabolic (HOMA-IR and insulin level) and hormonal (testosterone levels) systems. I explored for potential mechanisms of action and relationships between these systems. Further to this, I explored the effects of other markers frequently aberrant in PCOS including lipids such as LDL, triglycerides and cholesterol as well as assessed for effects of waist circumference on haemostasis and endothelial function.

I noted endothelial dysfunction indicating a potentially aberrant primary haemostatic system/response in women with PCOS in comparison to controls, and a further CV risk marker, through increased ADMA and PAI-1, regardless of age, BMI, waist circumference or lipid profile. I was also able to demonstrated that endothelial dysfunction was related to both the aberrant metabolic (HOMA-IR) and hormonal (testosterone levels) systems through regression analyses. No coagulation cascade (secondary haemostasis) abnormalities were noted in women with the syndrome compared to controls (including the function/effects of inhibitors of coagulation), and no relationships with metabolic factors (IR and insulin levels) and hormone relationships. Clear evidence of impaired capacity for fibrinolysis was detected and age, BMI,
waist circumferences and the lipid profile did not affect these outcomes. With increased activity of inhibitor of fibrinolysis, PAI-1, as well as increased plasminogen (likely arising due to reduced conversion to active plasmin), a hypofibrinolytic state arises, with further CV and VTE risks. While the reasons for this are somewhat unclear, my research supported potential mechanisms driving this hypofibrinolytic state; reduced fibrinolytic effects may be linked, at least in part, to abnormal endothelial function with significant relationships noted to the aberrant hormonal (hyperandrogenaemia) and metabolic (IR/hyperinsulinaemia) systems. The outcomes of this research were published to ensure broad dissemination of these important findings (Burchall, Piva et al. 2016).

Abnormal endothelial function and a hypofibrinolytic capacity was demonstrated in my first study (see Chapter 3), and women with PCOS have higher risks for and apparent higher rates of CVD and VTE. Additionally, the frequently prescribed management strategies to women with PCOS including the OCP present with well accepted risks of VTE in the general population. In the second study (Chapter 4) I explored the haemostatic impact of current treatments for PCOS to identify if these treatment strategies may exacerbate the cardiovascular and venous thrombotic risks noted. OCP have haemostatic effects with a net pro-coagulant outcome for those in the general population while metformin some positive haemostatic results, yet neither treatment option was adequately explored nor the haemostatic impacts documented in women with the syndrome. Furthermore, varying haemostatic and thrombotic effects have been noted from use of differing preparations of combined oral contraceptives in the general population and therefore it was also important to explore the effects of oestrogen dosage, progesterone preparation and use of an anti-androgen component and haemostatic impacts in PCOS.

In my second study I assessed haemostatic outcomes following commonly prescribed medical therapies in women with PCOS, including low oestrogen dose OCP of 20μg EE (combined
The Haemostatic System in PCOS

with a second generation progesterone, levonorgestrel and an anti-androgen, spironolactone), higher-dose OCP of 35μg EE (combined with a third generation progestin component, cyproterone acetate) or metformin. Correlations between haemostatic markers with hormonal and metabolic markers both within and between intervention groups were also explored to investigate potential underlying mechanisms of action for the OCP. I noted beneficial effects from all interventions on the endothelium, particularly following higher-dose OCP, through a reduction in either or both endothelial function markers (PAI-1 and ADMA). Both OCPs however demonstrated an aberrant coagulation profile (following increased TG with both hormonal treatments as well as an increase in PF1 & 2 levels in the higher-dose group) and a hypofibrinolytic state with higher-dose OCP (following observations of increased TAFI activity in this group). As increased coagulation and generation of thrombin was noted, this may serve to counteract some of the positive effects noted on the primary haemostatic system/response, following potential increased activation of platelets by OCPs, as thrombin is a powerful platelet agonist (McNicol and Robson 1997). Overall, metformin therapy appeared to have beneficial haemostatic effects, a potential improvement in primary haemostasis and no (net) effects on coagulation. Potential mechanisms of action included improved endothelial function, which is likely related to improved androgen (testosterone) levels with OCP use and from a better metabolic (AUC Insulin) profile with metformin administration. Androgens and the metabolic profile appear to be able to influence coagulation status in PCOS. When comparing the three interventions, metformin had overall favorable haemostatic outcomes and may reduce risks of CVD and VTE when compared to both the higher- or low-dose OCP therapies. Outcomes of this study were published (Burchall, Piva et al. 2017) in order to better inform clinical practice of the haemostatic system effects of common management strategies used in women with PCOS.
As PCOS is the leading cause of anovulatory infertility and as the fibrinolytic system has demonstrated both a fibrinolytic but also a proteolytic role including in a number of ovarian functions, the third study (Chapter 5) aimed at investigating the expression and distribution of the fibrinolytic/proteolytic markers and the plasminogen system in PCOS and control mouse ovaries. A PCOS mouse model (long-term postnally treated DHT) that displayed extensive features of humans with this syndrome was used in this study. These mice have been shown to provide for the best mouse model for experimental studies of PCOS pathogenesis (Caldwell, Middleton et al. 2014). Little or no evidence has been published on the state of the plasminogen system in the PCOS ovary in animals or humans and as my earlier investigations demonstrated a hypofibrinolytic state in women with the syndrome (see Chapter 3), I aimed to identify if these fibrinolytic alterations contributed to the anovulation and ovarian architecture seen in PCOS. The results of this study demonstrated involvement of the plasminogen system in both the physiological control mouse ovary and in PCOS, and potentially in ovulation and follicular development processes. All the fibrinolytic markers (uPA, plasminogen and plasminogen/plasmin) and inhibitor (PAI-1) assessed were shown to be expressed in both PCOS and control mouse ovaries, except for tPA. While I did not observe any significant differences in overall ovarian expression (mean total percentage and mean colour intensity) of markers assessed between PCOS and controls, differences were noted in the ovarian distribution of PAI-1 (that was localised throughout the PCOS ovary unlike peripheral distribution seen in control ovaries) and plasminogen (that presented in small follicles only in PCOS but not in these same follicles of control ovaries), indicating potential differences in ovarian physiology in PCOS. While further research is still needed in this novel area of study, the outcomes from this study provide the foundation for further studies. They enhance our understanding of this very complex condition and may assist with therapeutic options for anovulation in PCOS.
6.2 Limitations

Whilst these studies have made important contributions to the literature, it must be acknowledged that both the first and second study were completed on biobanked plasma samples from earlier studies (Meyer, McGrath et al. 2007, Hutchison, Stepto et al. 2011, Moran, Strauss et al. 2011, Stepto, Cassar et al. 2013), which addressed different end-points. However haemostatic assessment was part of the original study protocols and appropriate samples were collected and processed. Original study methods were rigorous and the participants well characterized. Additionally these first two studies only assessed one arm of the primary haemostatic response, namely endothelial function and could have benefited from assessment of platelet functionality/activation studies in this group of women. As only platelet-poor plasma (stored frozen at -80°C) was available, it was difficult to complete this assessment, as fresh whole blood or platelet-rich plasma (for platelet functional studies), serum (for measurement of sCD40L levels) or urine (for TXM analyses) would have been required (Paniccia, Priora et al. 2015). Results presented are from cross-sectional studies, rather than longitudinal research. Not all markers were measured on all parameters due to lack of availability of sufficient plasma for some samples. Differing progestin components were used in the second study as part of the combined oral contraceptives as preparations of differing oestrogen doses with the same progesterone component were unavailable at the time of study.

Limitations of the final study include a lack of assessment of ovarian plasminogen system markers and inhibitors at differing time-points of the oestrous cycle, particularly immediately preceding ovulation. Only mouse (control) ovarian tissue at the diestrus stage of the cell cycle was available. Evaluation of plasmin in PCOS (and control) ovarian tissue would have
greatly assisted in interpretation of outcomes in this final study however this was not possible due to lack of a suitable commercial plasmin only antibody.

### 6.3 Key Future Research Goals

The findings of this thesis have enhanced our knowledge of the haemostatic system in women with PCOS, yet they have also unearthed a number of questions and areas for future research including a need for further evaluation of the primary haemostatic system platelet functionality and potential aberrant activation in females with the syndrome. Insufficient research has been completed to date on this branch of the haemostatic system and enhanced platelet aggregability may also increase the risk of thrombosis and of CVD. Further studies assessing platelet function following use of OCPs (and other common treatments) that pose increased CV or VTE risks is also pertinent and should be investigated further, especially considering the frequency of use in women with PCOS and the outcomes noted in Chapter 4.

Due to the heterogeneity of clinical features that females with PCOS can present, four PCOS phenotypes are currently described (by the Rotterdam and Androgen Excess Society) including (1) Frank PCOS (oligomenorrhoea, hyperandrogenism, and PCO), (2) Ovulatory PCOS (hyperandrogenism, PCO, and regular menstrual cycles), (3) Non-PCO PCOS (oligomenorrhoea, hyperandrogenism, and normal ovaries) and (4) mild or normoandrogenic PCOS (oligomenorrhoea, PCO, and normal androgens) (Clark, Podolski et al. 2014). As the prevalence of CV risk varies with different PCOS phenotypes (Tziomalos 2016), studies assessing the haemostatic system in women with PCOS using different diagnostic criteria (NIH, Rotterdam and AES) should be also be completed to identify if any significant
differences are noted in haemostatic outcomes and to determine if the haemostatic system may be associated with these differing risks for the four PCOS phenotypes.

Additionally, as age is an important consideration by clinicians prescribing the OCP, large studies should be carried out assessing the haemostatic effects of different OCPs on different age groups in women with PCOS, as the studies completed to date assessing these pharmacological effects in this group of women are limited and include small sample sizes and as increasing age is well known to increase cardiovascular risk.

Studies comparing haemostatic impacts of common pharmacological treatments such as OCPs in women not affected by PCOS versus females with the syndrome should also be undertaken as well as comparisons made to women in the general population not on hormonal therapies. Such studies are limited and are needed to accurately ascertain the cardiovascular effects and risks of these management strategies in women with this syndrome, which are likely to show the significant CV and/or VTE risks posed with such therapies in women with PCOS.

Evaluation of the plasminogen system and relevant inhibitors in the human ovary in PCOS at different time points of the menstrual cycle, particularly immediately preceding ovulation, as well as evaluation of the markers in different sized ovarian follicles, from small to large/Graafian follicles, would also be critical, to review for potential links to aberrant ovulation and folliculogenesis. This may help to determine aetiological factors and/or pathological pathways contributing to the reduced fertility in females with this syndrome.

Pregnancy is considered a hypercoagulable state (Shan, Wang et al. 2013) and females with PCOS have been observed to have increased risks for pregnancy associated complications, including increased rates for miscarriage (Kamalanathan, Sahoo et al. 2013). Additionally, as aberrant haemostasis was demonstrated (in Chapter 3), evaluation of haemostatic markers in
pregnant females with PCOS, and correlating to infant outcomes, would also be very important and should be considered in future research studies. Such studies may help to identify a potential for high risk of thrombosis in pregnant females with PCOS and may unravel aetiological factors linked to early foetal loss potentially following from placental insufficiency.

6.4 Overall Conclusions

Given the high prevalence of PCOS with strong links to CVD and VTE risk factors, few published studies had been completed on the haemostatic system in PCOS. Yet this system is a key contributor to cardiovascular and thrombotic outcomes. The findings presented in this thesis have made a significant contribution to the understanding of the haemostatic system in women with PCOS and demonstrate a potential further CV and venous thrombosis risk factor in this already high-risk group, exacerbated by some of the common treatments used. Involvement of the fibrinolytic/proteolytic system in the PCOS ovary and in potential aberrant ovarian functions has also been shown which may contribute to anovulation and infertility. Overall the findings of this thesis have helped improve our understanding of this very complex condition and may guide development of future treatments.
APPENDIX 1

Exemption from Ethics Review (Outcome)

6th July 2010

Genia Burchall
School of Medical Science
Building 223 Level 2, Room 19
RMIT University

Dear Genia

ASEHAPP 62 – 10 BURCHALL, Haemostatic System in Patients with Polycystic Ovarian Syndrome (PCOS)

Thank you for submitting your application for an exempt for review project for consideration by the Science, Engineering & Health College Advisory Network of the Human Research Ethics Committee of RMIT University.

Your application was considered out of session by the Chair and the following is attached for your information.

4.1.7 ASEHAPP 62 – 10 BURCHALL, Haemostatic System in Patients with Polycystic Ovarian Syndrome (PCOS)

It is noted:

1. You have joined the Jean Hailes Clinical Research Unit as a PhD research student under the supervision of Professor Helena Teede

2. Your role is to receive unidentifiable coded serum samples with metabolic and reproductive data for laboratory and data analyses

3. Samples and data are from participants who were recruited during three projects for which Professor Teede is the Principal Researcher; the projects have the ethical approval of the Monash University Standing Committee on Ethics in Research Involving Humans (SCERH); you have been included as an investigator on the research teams for the three projects.

4. You are performing specific tests and are unable to diagnose diseases

5. Any samples remaining after analysis will be returned to the Jean Hailes Clinical Research Unit

6. A copy of approval from Monash University SCERH to include you as an investigator has been provided.

Thank you for advising the CHEAN of your project to receive and analyse coded samples and data from the Jean Hailes Clinical Research Unit. The CHEAN has determined that your project is Exempt from Review as you are an investigator in research with the ethical approval of Monash University SCERH, the samples and data to be analysed will be provided anonymously, and remaining samples will be returned to the Jean Hailes Clinical Research Unit. The
responsibility for ethical conduct of the research rests with Jean Hailes Clinical Research Unit as part of their normal obligations to participants.

For your information your application number is A5EHAPP62 - 10 please refer to this number in any future correspondence to the CHEAN.

Yours sincerely

Diana Donohue  
Chair, Science Engineering & Health  
College Human Ethics Advisory Network ‘A’  

Cc: Other Investigators: Assoc Prof Terry Piva School of Medical Science  
Professor Helena Terde Monash University  
Dr Matthew Linden School of Medical Science
APPENDIX 2

TAFI Activity in PCOS and Controls

Table A2.1 displays the TAFI activity results for PCOS and control participants part of the first study (Chapter 3) not included in the final published paper (Burchall, Piva et al. 2016) due to the low control (non-PCOS) sample numbers available.

Table A2.1: TAFI activity in PCOS and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS (n=51)</th>
<th>Non-PCOS (n=17)</th>
<th>p-value</th>
<th>p-value adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAFI Activity (%)</td>
<td>121.0 (103.0-137.0)</td>
<td>116.0 (99.0-141.5)</td>
<td>0.854</td>
<td>0.720</td>
</tr>
</tbody>
</table>

Median (interquartile range) and P values from log transformed data. p-value adjusted = p value adjusted for age and BMI. TAFI = thrombin activatable fibrinolysis inhibitor.
APPENDIX 3

Collaborative Research Agreement and Memorandum of Understanding

Project title

Plasminogen System in the PCOS Mouse Ovary: An observational study looking into the fibrinolytic/proteolytic markers Plasminogen, Plasmin, tPA, uPA and inhibitor PAI-1 in the ovaries of PCOS mice versus ovaries of control mice.

Researchers / investigators

<table>
<thead>
<tr>
<th>School of Health &amp; Biomedical Sciences, RMIT University</th>
<th>MCHRI, SPHPM, Monash University</th>
<th>ANZAC Research Institute, University of Sydney</th>
<th>Robinson Research Institute, University of Adelaide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assoc Prof Terrence Piva</td>
<td>Prof Helena Teede</td>
<td>Dr Kirsty Walters</td>
<td>Prof Ray Rogers</td>
</tr>
<tr>
<td>Mrs Genia Burchall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assoc Prof Ian Darby</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conception date

Memorandum drafted and sent to all collaborators for review and appoval on the 1st of June 2016.
Background

Polycystic Ovarian Syndrome (PCOS) is a condition which affects 12-18% of reproductive aged women and is diagnosed based on ovulatory and menstrual disturbance, hyperandrogenism and polycystic appearance of ovaries on ultrasound (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004, Teede, Misso et al. 2011)(March, Moore et al. 2010, Yildiz, Bozdag et al. 2012). PCOS also presents with aberrant metabolic features including hyperinsulinaemia, insulin resistance (IR) and dislipidaemia. Women with PCOS have increased risks for cardiovascular disease (CVD) and venous thromboembolism (VTE) likely relating to the aberrant metabolic features and high BMI as well as an abnormal haemostatic system (Burchall, Piva et al. 2016). A likely hypofibrinolytic state is present in women with PCOS, relating to increased plasminogen activator inhibitor 1 (PAI-1) which prevents conversion of the pro-enzyme plasminogen to its active form, plasmin (Burchall, Piva et al. 2016). The fibrinolytic system components and relevant inhibitors however play a number of roles, apart from their function in blood haemostasis and thrombosis. Of importance to research into PCOS is their role in the ovulation process.

Plasmin has been detected in both the follicular fluid and granulosa cells of the ovary at the time of or just before rupture of the ovarian follicle in porcine models (Politis, Srikandakumar et al. 1990). This suggests that plasminogen is converted to plasmin at the time of follicular rupture (ovulation). To achieve this increased plasmin activity, a decrease in inhibitor PAI-1 and an increase in plasminogen activators (tissue plasminogen activator, tPA and/or urokinase plasminogen activator, uPA) is needed, also observed just before follicular rupture in female pigs (Politis, Srikandakumar et al. 1990). In addition to time of ovulation/cyclical period, evaluation of areas within the ovary has revealed that PAI-1 levels are at their highest immediately prior to ovulation in the centre of the ovary where the follicles that are less likely to ovulate reside whereas tPA levels at the same stage (in the ovulation
process) are highest at the surface of the ovary where pre-ovulatory or Graafian (mature) follicles reside (Devin, Johnson et al. 2007) (Figure A3.1).

Figure A3.1 Internal structure of the ovary and the ovulation process (taken from http://catalog.flatworldknowledge.com/bookhubreader/12013?e=tye_1.0-ch03_s02 Last accessed 25/05/2016).

The plasminogen system at an ovarian level is under gonadotropin control, primarily luteinizing hormone (LH) (Devin, Johnson et al. 2007). Early research has shown that each type of plasminogen activator involved in the ovulation process is species-specific and cell-specific, with tPA thought to be primarily secreted by the granulosa cells of rats and pigs in pre-ovulatory/mature follicles and uPA as the main plasminogen activator secreted by the granulosa cells of mice ovaries just before ovulation (Canipari, O'Connell et al. 1987, Liu, Cajander et al. 1987, Politis, Srikandakumar et al. 1990) (Figure A3.2). Subsequent studies have shown that both plasminogen activators are of importance in ovulation within a species, however with likely differing actions (Macchione, Epifano et al. 2000, Dow, Bakke et al. 2002); tPA is thought to be primarily involved in rupture of the follicular wall whereas uPA is thought be involved in the tissue remodeling process and in early follicular development (Lafrance,
Studies in humans have identified the presence of tPA in granulosa cells and within the follicular fluid and PAI-1 also in theca cells (Beers 1975, Piquette, Crabtree et al. 1993, Atiomo, Hilton et al. 2000). Few studies have evaluated these fibrinolytic/proteolytic system markers and inhibitors within the PCOS ovaries in either humans or in animal models. Ambekar et al found that plasminogen is degraded within the follicular fluid of the ovary in women with PCOS (Ambekar, Kelkar et al. 2015). Atiomo et al, using immunohistochemistry (IHC), found that there was no significant difference in PAI-1 antigen levels within the ovaries of PCOS women and controls (Atiomo, Hilton et al. 2000). Their sample size however was quite small and the women from whom the samples were taken were at various stages of the menstrual cycle. In contrast, Devin et al compared ovarian tissue from women with PCOS and
unaffected women’s ovaries and found PAI-1 to be prevalent in the PCOS ovary but lacking in normal ovaries (Devin, Johnson et al. 2007). They also performed a small sub-study on transgenic female mice which constitutively secrete PAI-1 and found the latter to contained many large cystic structures within their ovaries and had plasma testosterone levels nearly twice as high as in control mice (Devin, Johnson et al. 2007). These researchers did not evaluate metabolic markers in these mice however a paper published by Ma et al showed that mice which lacked PAI-1, unlike their wild type counterparts, did not go on to develop obesity and insulin resistance despite being on a high fat/high carbohydrate diet (Ma, Mao et al. 2004).

While PAI-1 is believed to play a physiological role within the ovary in preventing ovulation of immature follicles, persistent elevation of this fibrinolytic/proteolytic inhibitor, as observed in the plasma of women with PCOS, may potentially contribute to a lack of ovulation. By preventing conversion of plasminogen to active plasmin, the high PAI-1 levels will in turn prevent follicular wall breakdown of more mature pre-ovulatory follicles, the result of which may ultimately give rise to the ovarian architecture currently observed in the ovaries of women with PCOS.

**Objectives and hypotheses**

We aim to investigate the presence and distribution of fibrinolytic/proteolytic markers plasminogen, plasmin, tPA and uPA and inhibitor PAI-1 in PCOS ovaries compared to ovaries from control mice. We will use dihydrotestosterone (DHT)-treated mice that display extensive ovarian, endocrine and metabolic features of humans affected by PCOS including oligo- or anovulation, irregular menstrual cycles, polycystic ovaries, obesity and dislipidaemia (Caldwell, Middleton et al. 2014). We also aim to correlate the presence/absence of the aforementioned fibrinolytic/proteolytic markers and inhibitors within the PCOS and control mouse ovaries with that of hormonal and metabolic markers available for the rodents. We hypothesize that PAI-1 is elevated in the PCOS mouse ovary in comparison to controls.
and the high levels/activity of this marker is distributed throughout the ovary and not restricted to immature follicles in the central region of the ovary. In the controls however PAI-1 is restricted to the centre of the ovary with scant or no detectable levels/activity in mature pre-ovulatory follicles on the surface of the ovary. tPA, uPA and/or plasminogen may show strong staining/presence in both the PCOS and control mice, particularly at the surface of the ovary (in pre-ovulatory follicles) however plasmin activity should only be evident/significantly increased in the control mice but not in the PCOS mice ovarian tissue.

**Materials and methods**

The present study will be completed based on bio-banked ovarian tissue samples from control and PCOS mice from a prior study lead by Dr Kirsty Walters (Caldwell, Middleton et al. 2014). They have published data on metabolic, hormonal and reproductive/ovarian parameters of these mice (Caldwell, Middleton et al. 2014). The Sydney Local Health District Animal Welfare Committee approved the original study as being within NHMRC guidelines for animal experimentation. The PCOS mice were generated by implanting female mice with 1-cm Silastic brand implant (id, 1.47 mm; od, 1.95 mm, Dow Corning Corp, catalog no. 508-006) containing approx. 10 mg DHT at 21 days of age. PCOS mice displayed DHT levels 8x higher than that of control mice. The stage of the estrous cycle was identified on a daily basis using light microscopy examination of vaginal epithelial cell smears, as previously described (Caldwell, Middleton et al. 2014). Mice were anesthetized using ketamine/xylazine and the ovaries removed, weighed and fixed in 4% paraformaldehyde and stored refrigerated overnight after which they were put into 70% alcohol. Histological processing, follicular classification, enumeration and health status assessment were undertaken as previously described (Caldwell, Middleton et al. 2014).
For the present study, paraffin-wax embedded ovarian tissue from the DHT-induced PCOS mice and control mice will be sectioned at 4 μm in thickness and mounted onto glass slides, then dried overnight in a 37°C incubator. These slides will be posted to Melbourne (RMIT University, Plenty Rd, Bundoora, VIC) for immunohistochemical staining for the following fibrinolytic/proteolytic markers: plasminogen, plasmin, tPA and uPA and inhibitor PAI-1.

At RMIT, the glass slides will be stored securely prior to IHC testing. The antibodies used for IHC will be of monoclonal or polyclonal specificity, raised in rabbits and directed towards the fibrinolytic/proteolytic markers and inhibitor of interest. We will use Polymer based-IHC for immunohistochemical detection of PAI-1, tPA, uPA, plasmin and plasminogen of sectioned tissue in both control and PCOS mice (at various stages of the estrous cycle in PCOS mice and at the diestrus stage of the cycle in control mice). IHC will be performed on mice tissues know to contain PAI-1, tPA, uPA, plasmin and plasminogen (positive control tissues). A negative control will also be tested with each marker by omitting to add the primary antibody in the immunohistochemical procedure.

PCOS and control sections will be stained simultaneously for each marker in order to avoid variability due to staining techniques. Sections will then be examined microscopically (Leica DMD 108) and photographed (under x100, x400 & x1000 magnification) with a digital camera (Leica). All images will be processed at the same time. Sections/images will be examined and characterized by three operators (Dr Dodie Pouniotis, A/Prof Ian Darby, Genia Burchall), blinded to the type of tissue (PCOS or control) viewed. Digital image analysis and processing of each photograph may be performed (if required).
**Primary outcome measures**

Our main focus of this study is to assess the expression of the fibrinolytic/proteolytic markers plasminogen, plasmin, tPA and uPA and inhibitor PAI-1 within the PCOS and control mouse ovaries.

**PAI-1**: PAI-1 primary antibody (rabbit polyclonal) from ABCAM (ab28207) can be used in paraffin embedded sections (IHC-P). The antibody will initially be tested in a positive control (in mouse lung and/or carotid know to contain PAI-1) according to the manufacturer’s instructions and on a negative control (also in mouse lung and/or carotid) with the primary antibody omitted.

**tPA**: tPA primary antibody (rabbit polyclonal) from ABCAM (ab28208) can be used in paraffin embedded sections (IHC-P). As per the protocol stated for PAI-1 above, positive and negative controls will be tested for tPA (in mouse normal kidney tissue know to contain tPA).

**uPA**: uPA primary antibody (rabbit monoclonal) from ABCAM (ab133563) can be used in paraffin embedded sections (IHC-P). As per the protocol stated for PAI-1 above, positive and negative controls will be tested for uPA (in mouse normal kidney tissue know to contain uPA).

After confirmation that the antibodies for PAI-1, tPA and uPA have stained the positive control tissue appropriately (with no/scant background staining) and no staining observed in the negative control tissues, the antibodies will be used on the ovarian mouse PCOS and control tissue slides (received from Dr K Walters). Images of the slides will be taken using a digital camera.

**Plasminogen**: Plasminogen primary antibody (rabbit polyclonal) from NOVUS (NB300-544) has been tested and shown to work in immunocytochemical (ICC) staining and therefore likely to also work in
IHC however this will need to be demonstrated/proven before testing on the PCOS and control ovarian tissue. As per the protocol stated for PAI-1 above, positive and negative controls will be tested for plasminogen (on mouse liver and/or adrenal gland known to contain plasminogen). After confirmation that the antibody has stained the positive control tissue appropriately (with no/minimal background staining) and no staining observed in the negative control tissue, the antibody will be tested (via IHC-P) on the ovarian mouse PCOS and control tissue slides received from Dr K Walters on one slide only (from each control and PCOS tissue). Images of slides will be taken with a digital camera. Further ovarian mouse PCOS and control tissue slides will be stained after initial/preliminary testing/staining. Plasminogen primary antibody (rabbit polyclonal) NB300-544 is specific for plasminogen and will not recognise plasmin.

Plasminogen/Plasmin: Plasminogen/Plasmin primary antibody (rabbit polyclonal) from NOVUS (NBP2-19859) has been tested and shown also to work in ICC and therefore likely to also work in IHC however this will need to be demonstrated/proven before testing on the PCOS and control ovarian tissue. Testing/confirmation procedures will be as for that indicated for the plasminogen antibody above (NB300-544). Images of all slides will be taken with a digital camera. Plasminogen primary antibody (rabbit polyclonal) NBP2-19859 will recognise both plasminogen and plasmin.

Significance of project

This will be the first study that we are aware of that comprehensively evaluates the fibrinolytic/proteolytic system in the PCOS ovary. We will compare the expression of these markers between control and PCOS ovaries to observe what effect these may have on the latter. Outcomes from this study will support further research to be undertaken in this area as well as potentially lead to research into treatment modalities for overcoming the anovulation noted in many/most women with PCOS. As PCOS is the leading cause of anovulatory infertility in women in many countries, research
into this area could potentially provide significant breakthrough in the fertility treatment options for women with PCOS. Correlations between hormonal and metabolic markers with fibrinolytic markers in the ovary may also inform potential mechanisms of action in the anovulation process, follicular development and ovarian architecture in the PCOS ovary in this very complex and common disorder.

**Expected outputs**

It is expected this work will yield at least one journal publication and several conference abstracts. Outputs will include:

- At least one manuscript and two or more conference (national and international) oral presentations and posters on the Fibrinolytic/Proteolytic Markers (plasminogen, plasmin, tPA & uPA) and inhibitors (PAI-1) in the PCOS Ovary

Authorship will be decided according to the guidelines set out in the “Authorship and funding” section below.

**Ethics approval**

Ethics approval for the original study carried out on the control and PCOS mice in question has already been sought (by Dr Kirsty Walters, University of New South Wales) and approved by The Sydney Local Health District Animal Welfare Committee (as being within NHMRC guidelines for animal experimentation). The trial has been registered on clinicaltrials.gov and the Australian New Zealand Clinical Trials Register.
**Statistical analyses plan**

Statistical analysis will not be required for this study.

**Intellectual property**

It will be acknowledged that this work was completed in an intellectual collaboration between the School of Health and Biomedical Sciences, RMIT University, Monash Centre for Health Research and Implementation (MCHRI), Monash University, ANZAC Research Institute, University of Sydney and the Robinson Research Institute, University of Adelaide. All intellectual property for this project is managed by the School of Health and Biomedical Sciences.

The investigator roles are as follows:

- Assoc Prof Terrence Piva (CIA) – School of Health & Biomedical Sciences, RMIT University
  - Contribution to the design of the project
  - Principal supervisor of PhD student (Genia Burchall)
  - Reviewing manuscript (for publication)

- Mrs Genia Burchall (CIB) – School of Health & Biomedical Sciences, RMIT University
  - Designing the original project and adjusting this based on feedback provided by other collaborators on the project
  - Training in IHC techniques and IHC testing of all control and test tissues of all markers of interest (as specified above)
  - Data analysis
- Writing the first draft (and all subsequent drafts) of the manuscript (following feedback received)
- Writing and presenting outcomes of study at conferences (oral presentations and posters)

- Prof Helena Teede (CIC) – MCHRI, SPHPM, Monash University
  - Contribution to the design of the project
  - Co-supervisor of PhD student (Genia Burchall)
  - Reviewing manuscript (for publication)

- Assoc Prof Ian Darby – School of Health & Biomedical Sciences, RMIT University
  - Contribution to the design of the project
  - Training of PhD student (Genia Burchall) in IHC techniques
  - Reviewing manuscript (for publication)

- Dr Kirsty Walters – ANZAC Research Institute, University of Sydney
  - Contribution to the design of the project
  - Provision of PCOS and control mice ovarian tissues
  - Embedding (paraffin) and cutting into sections PCOS and control mice ovarian samples, then placing on slides and posting to Melbourne
  - Reviewing manuscript (for publication)

- Prof Ray Rogers – Robinson Research Institute, University of Adelaide
  - Contribution to the design of the project
  - Reviewing manuscript (for publication)
Authorship and funding

Funding for this project will be obtained from The Helen MacPherson Smith Trust Grant, and the School of Health and Biomedical Sciences (RMIT). Publications costs as well as costs associated with travel to conferences will be covered by the School of Graduate Research, RMIT University. Authorship will be based on intellectual contribution throughout the project, including analysis and interpretation, and manuscript preparation. All CIs will be included as co-authors on all outputs and other team members will be included on publications for which they have made a significant contribution. CIA Piva will be last author on output (manuscript), unless otherwise agreed upon by the CIs. The first author of the article will be CIB Burchall (makes the largest contribution to data analysis and manuscript preparation). Those who are engaged in the project but not meeting authorship requirements will be acknowledged in the manuscript/s. The outcomes of the collaboration are anticipated to be presented at both national and international meetings.

Agreement and signatures

We the researchers agree to work collaboratively and collegially and in good faith, and to abide by the terms stated in this agreement/memorandum:
A/Prof Terrence Piva*

01/06/2016

Mrs Genia Burchall

01/06/2016

Prof Helena Teede

01/06/2016

A/Prof Ian Darby

01/06/2016

Dr Kirsty Walters

01/06/2016

Prof Ray Rogers

01/06/2016

*CIA/Principal PhD supervisor of GB signed on behalf of some of the collaborators.
References


Karakurt, F., A. Carlioglu, I. Kaygusuz, Gumus, II, B. Uz and D. Akdeniz (2014). "Effect of ethinyl estradiol-cyproterone acetate treatment on asymmetric dimethyl-arginine levels in women with polycystic ovary syndrome." Arch Gynecol Obstet\textsuperscript{289}(1): 135-140.


Polycystic Ovarian Syndrome Association of Australia (POSAA), Jean Hailes Foundation for Women’s Health (JHF), University of Adelaide, Robinson Institute, Keogh Institute for Medical Research, Monash University, The Liggins Institute, Melbourne IVF, Prince Henry’s Institute, Royal Hospital for Women, Royal Women’s Hospital, School of Women’s and Infant’s Health, Southern Health, University of Adelaide and Victoria University (2009). PCOS Australian Alliance: A single voice for Polycystic Ovary Syndrome 2009 Report, The Jean Hailes Foundation for Women’s Health.


The Haemostatic System in PCOS


The Haemostatic System in PCOS


