The Molecular Basis of Polycystic Ovary Syndrome: Genetics, Epigenetics and Insulin Resistance.

by

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Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is a common and complex endocrinopathy. The proposed pathophysiology of PCOS is a synergistic relationship between perturbed gonadotropin pulsatility, hyperandrogenism, insulin resistance and inflammation. However, the nuances of these relationships are yet to be fully elucidated. The biological origins of PCOS are driven by heritability and a polygenic predisposition that is exacerbated by environmental factors (e.g. obesity). Therefore, the aetiology of PCOS is considered multifactorial. While familial clustering of PCOS symptoms is well documented, providing evidence for a genetic contribution to the condition, lifestyle factors also mediate the influence of the underlying mechanism of PCOS. These mechanisms likely involve epigenetics, which are the molecular interactions between genetics and lifestyle. Epigenetic modifications, like DNA methylation alter chromatin structure and gene expression. These DNA modifications are associated with the pathogenesis of obesity-related chronic diseases, yet there is limited evidence in PCOS. Therefore, the overall aim of this thesis was to assess different molecular mechanisms that are postulated to contribute to the aetiology of the syndrome by i) conducting an overview of systematic reviews to synthesise the current evidence and quality of evidence for the relationship between candidate gene polymorphisms and PCOS, ii) investigate the differences in global DNA methylation in specific immune cell populations in women with and without PCOS, iii) identify differences in genome-wide DNA methylation patterns and gene expression in immune cells of women with and without PCOS, iv) to further explore molecular mechanisms of PCOS-specific insulin resistance. My research concluded that there is very little evidence in the literature to ascribe specific genetic variations in PCOS, clearly highlighting a need for standardisation in the design and analysis of genetic association studies in PCOS. I also report that immune cells in women with PCOS display hypo-methylation in T helper cells, T cytotoxic cells, B cells and monocytes. Furthermore, immune cells displayed genome-wide differential gene expression and DNA methylation patterns in T helper cells in women with

PCOS. Finally, I show that PCOS-specific insulin resistance may be regulated distal to Akt via interactions with the TGFβ signalling network. In summary, this thesis advances the fundamental understanding of the molecular basis of the aetiology of PCOS and offers a novel hypothesis to drive future research to better understand the syndrome and PCOS-specific insulin resistance.

STUDENT DECLARATION

"I, Danielle Hiam, declare that the PhD thesis entitled "The Molecular Basis in the Pathophysiology of Polycystic Ovary Syndrome: Genetics, Epigenetics and Insulin Resistance" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature  Date: 16/03/16

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ADDITIONAL PUBLICATIONS PRODUCED DURING CANDIDATURE

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Abell SK, Shorakae S, Harrison C.L, **Hiam D**, Moreno-Asso A, Stepto N.K, De Courten B, Teede H. The association between dysregulated adipocytokines in early pregnancy and development of gestational diabetes. *Diabetes/Metabolism Research and Reviews* (2017). doi: 10.1002/dmrr.2926.

Mousa A, Abell A, Shorakae S, Harrison C.L, Naderpoor N, **Hiam D**, Moreno-Asso A, Stepto N, Teede H, de Courten B. Relationship between vitamin D and gestational diabetes in overweight or obese pregnant women may be mediated by adiponectin. *Molecular Nutrition & Food Research* (2017). doi: 10.1002/mnfr.201700488.

Zhang X.M, **Hiam D**, Hong Y, Zulli A, Hayes A, Rattigan S, McConell G.K. Nitric oxide is required for the insulin sensitizing effects of contraction in mouse skeletal muscle. *The Journal of Physiology* (2017). doi: 10.1113/JP275133

Bescos R, Kennaway D, Boden M, Jackson M, Owens J, Trewin A.J**, Hiam D**, Marin E, Falcao F, Levinger I, Kaur G, Conte F, Konjarski L, Rattanatray L, Salkeld M, McConell G. Effects of a shift work simulation on glucose tolerance, insulin sensitivity skeletal muscle mitochondrial function and circadian rhythmicity. *Acta Physiologica* (2018).
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CONFERENCES

2017 Victoria University-ISEAL Higher Degree by Research Conference, Oral Presentation

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2017 Cell Symposia: Exercise Metabolism [*International Conference*], Poster presentation

2016 Victoria University-ISEAL Higher Degree by Research Conference, Oral Presentation

2016 Annual Meeting of AE-PCOS Society, [*International Conference*], Poster presentation

2016 Centre of Research Excellence in Polycystic Ovary Syndrome, Poster presentation

2016 Australian Diabetes Society and Australian Diabetes Educators Association Annual Scientific Conference, Oral Presentation

2015 Cell Symposia: Exercise Metabolism [*International Conference*], Poster presentation

2015 Victoria University-ISEAL Higher Degree by Research Conference, Poster Presentation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 6-10% of reproductive aged women worldwide (Bozdag, Mumusoglu et al. 2016). It has major metabolic (increased type 2 diabetes mellitus and cardiovascular risk factors) (Moran, Lombard et al. 2010), reproductive (leading cause of anovulatory infertility) (Teede, Misso et al. 2011) and psychological (increased symptoms of anxiety and depression) (Cooney, Lee et al. 2017) impacts, representing a substantial health burden. Unfortunately, it is under-recognised by health professionals leading to a delay in diagnosis (Gibson-Helm, Lucas et al. 2014, Gibson-Helm, Teede et al. 2017). This delay puts women with PCOS at higher risk of developing chronic conditions with many women not receiving adequate treatment or remaining undiagnosed (Moran, Misso et al. 2010, Deeks, Gibson-Helm et al. 2010 , Moran, Strauss et al. 2011, Teede, Misso et al. 2011, Gibson-Helm, Teede et al. 2017). PCOS is incorrectly considered an uncommon condition and therefore has been neglected in research, resulting in a lack of understanding of the biological origins and aetiology of PCOS and therefore inadequate treatments.

There is a complex interaction between genetics and environmental factors that are thought to play a role in driving both the metabolic and reproductive features of PCOS. Familial, twin, genome-wide association studies and single gene association studies certainly confirm the genetic basis for PCOS (Yildiz, Yarali et al. 2003, Vink, Sadrzadeh et al. 2006, Shi, Zhao et al. 2012, Hayes, Urbanek et al. 2015). However, there is need to synthesise the current literature to establish the consistency of the relationship between genetic variation and PCOS.

Epigenetic modifications, such as DNA methylation and histone modifications, can facilitate the environmental regulation of the gene expression (Feil and Fraga 2012), and could be a potential molecular mechanism in chronic metabolic conditions (Barres and Zierath 2011). There is emerging evidence supporting the role of inappropriate epigenetic programming in the aetiology of PCOS. However, large gaps in knowledge remain as to how epigenetic modifications relate to PCOS and the metabolic, endocrine, and reproductive sequelae.

In parallel to genetics and epigenetics, insulin resistance, has a central aetiological role in the aetiology of PCOS. Complex interactions between perturbed GnRH pulsatility, hyperandrogenism and low-grade inflammation drive PCOS (Nestler and Jakubowicz 1996, Diamanti-Kandarakis and Dunaif 2012, Shorakae, Teede et al. 2015, Cassar, Misso et al. 2016). However, the mechanisms of insulin resistance remain ill-defined and there are gaps in the understanding of improvements in insulin resistance following therapeutic treatments in PCOS. Therapeutic strategies in PCOS include medical therapy (metformin), (Naderpoor, Shorakae et al. 2015), exercise, (Harrison, Lombard et al. 2011), and diet-induced weight loss (Moran, Ko et al. 2013) which all reduce, but do not reverse insulin resistance and fail to optimally manage the symptoms of PCOS. In this context, greater insight into aetiology of insulin resistance and optimal therapies in PCOS are needed.

In this thesis I aimed to contribute further knowledge in the molecular basis in the pathophysiology of polycystic ovary syndrome. Research gaps include:

In genetics:

- What genes are implicated in PCOS, are these genes consistent across existing literature, and is there comparability between studies?

In epigenetics:

- How do epigenetic modifications relate to PCOS and its associated pathophysiologic sequelae?

In insulin resistance:

- What is the molecular mechanisms involved in insulin resistance in PCOS?

In therapy:

- What is the optimal exercise intervention for treating women with PCOS? How does exercise affect the molecular mechanisms of insulin resistance in PCOS?

Genetics in PCOS

In chapter 3, I conducted an overview of systematic reviews. This is new methodology that has recently been introduced due to a substantial increase in publications of systematic reviews, creating the need to assess the quality of systematic reviews on a given topic and the consistency of evidence contained in them. I was involved in all stages of this overview including; search design, article selection, data extraction, quality assessment of the included articles and wrote the manuscript. This manuscript was written to consolidate the existing literature around the candidate gene association with PCOS.

Epigenetics

I conducted a cross-sectional study to investigate the global (chapter 4) and genome-wide (chapter 5) DNA methylation patterns in immune cells of women with and without PCOS. I was involved from inception of this study to completion and will be the first author for the manuscripts that arise from this study. I co-designed this study with assistance from my supervisor (Professor Nigel Stepto), wrote and sought ethics approval, recruited the participants and gained informed consent from all participants. I co-ordinated the trial including running of the oral glucose tolerance tests, the body composition measures and lifestyle monitoring. I extracted the immune cells from the baseline blood for global, and genome-wide DNA methylation and transcriptome analysis. I independently analysed all the hormones measures via ELISA or radio-immunoassays. With expert assistance from my co-supervisor (Dr David Simar) and an external collaborator (Professor Romain Barres) I counted, FACS sorted cells, stained cells, extracted RNA and DNA for analysis of DNA methylation patterns (both global and genome-wide) in the immune cells of women with and without PCOS. I statistically analysed all the data and interpreted the data in chapter four. In chapter five I examined the transcriptome and DNA methylome in one population of immune cells from this cross-sectional study, for this I travelled to Copenhagen where I could learn and run next generation sequencing including RNA sequencing and reduced representation bisulphite sequencing for the transcriptome and DNA methylome analysis respectively. Due to the specialised nature of the data analysis, a bioinformatician was involved with the statistical analysis of data generated by next generation sequencing for which I interpreted findings and constructed the conclusions in chapter 5.

Insulin signalling in skeletal muscle

This cross-sectional study and randomised control trial (chapter 6) was a study undertaken by my supervisor (Professor Nigel Stepto) and was a collaboration between Adelaide, Monash and Victoria Universities. My role was conducting all protein analysis (immunoblotting), data analysis, statistical analysis and interpretation. I co-wrote the manuscript and therefore as a major contributor I am able to include this in my thesis, with permission of all authors. This chapter was written an extended manuscript and will be shortened and submitted to the Journal of Endocrinology and Metabolism.

Exercise therapy- Randomised control trial

As indicated above there is a fundamental gap in the clinical knowledge where optimal exercise intensities for management and treatment of PCOS remain. I therefore lead the development (in collaboration with Professor Nigel Stepto) of the pilot randomised control trial to explore the efficacy of two different exercise intervention in PCOS. In this trial I was responsible for the study design, methodology development, data collection, I wrote the ethics and handled any amendments. I was the main co-ordinator for this on-going trial and this involved screening and recruitment of participants, I liaised with multiple staff including doctors, exercise physiologists and other staff members involved in the running of the trial to ensure a smooth progression of trial. I ran the trials and co-ordinated exercise training with the exercise physiologist. This trial has successfully completed (n=8) as of March 2018 and is expected to be completed in 2019. This trial is being run in accordance with the CONSORT guidelines in which the SPIRIT initiative (Standard Protocol Items: Recommendations for Interventional Trials) recommends the publishing of the protocol of randomised control trials. Therefore, while only a limited amount of preliminary data will be presented in this thesis I included the protocol (chapter 7). I will lead the submission of this chapter as a protocol paper in BMC

Trials. Post PhD I will still be involved in the analysis of the DNA methylation analysis from the trials and qualify as an author in most publications that arise from this study.

Thesis Format and publication

This is a traditional thesis and therefore remains unpublished. I have written all chapters in manuscript form to highlight the publishable quality of my thesis. Chapters (3-7) in this thesis are either in review or being prepared as manuscripts for publication, with chapters 3, 6 and 7 in submission to the Journal of Metabolism (Clinical and Experimental), Journal of Clinical Endocrinology, and Metabolism and BMC Trials respectively. Chapters 4 and 5 will be condensed as a single comprehensive manuscript to be submitted to a reputable epigenetic, endocrine or reproductive journal dependent on the additional analysis required.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Prevalence and burden of PCOS

Polycystic ovary syndrome (PCOS) is a multifactorial disorder that has significant metabolic, reproductive and psychological consequences (Moran, Misso et al. 2010, Teede, Misso et al. 2011, Dokras, Clifton et al. 2012, Teede, Joham et al. 2013). It is a major public health concern affecting 6-10% of reproductive aged women worldwide (Bozdag, Mumusoglu et al. 2016). However, in Australia, the prevalence is higher when considering the Aboriginal and Torres Strait Islander populations with reported rates of up to 21% of reproductive aged women being diagnosed with the syndrome (March, Moore et al. 2010, Boyle, Cunningham et al. 2012).

The high prevalence places a significant economic burden on health-care systems worldwide. it has been estimated that PCOS costs up AUD$400 million annually in Australia,USD$4 billion annually in the US, and £237 million per year in diabetes related treatment in the UK (Azziz, Marin et al. 2005, Teede, Deeks et al. 2010, Teede, Misso et al. 2011, Ding, Hardiman et al. 2018). The treatment of metabolic dysfunction, specifically PCOS associated diabetes, accounts for approximately 40% of total costs followed by treating menstrual dysfunction (31% of total costs) and then fertility treatment accounting for 12% of total costs (Azziz, Marin et al. 2005). This indicates that health care costs associated with metabolic function places the most significant burden on costs associated with PCOS. These estimates are based on women who were diagnosed by the National Institute of Health (NIH) diagnostic criteria, generally attributed to the more severe phenotypes (due to a more pronounced metabolic profile) in PCOS (Moran and Teede 2009). Using different criteria such as the Rotterdam/ESHRE diagnostic criteria these estimate of costs to the health care system would be significantly higher as it includes the milder phenotypes of the syndrome that are still considered to have an adverse metabolic profile when compared to women without PCOS.

2.2 Diagnostic Criteria

PCOS was first described in medical literature in 1935 as Stein-Leventhal syndrome (Stein and Leventhal 1935) and described as a reproductive condition (amenorrhoea and polycystic ovaries). More recently it has been defined as a hyperandrogenic condition (Azziz, Carmina et al. 2006). The PCOS diagnostic criteria is evolving and there has been much controversy over the optimal diagnostic criteria. The three most recognised diagnostic criteria are the Rotterdam/ESHRE criteria, the NIH criteria and the androgen excess (AE)-PCOS society criteria (Table2.1). At present the internationally accepted criteria for diagnosis of PCOS is the revised Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group 2004) which requires presence of two of three characteristics: oligo-or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism. It must be noted that the NIH criteria was created in 1990 and is generally recognised to diagnose the most severe phenotypes (A and B) of PCOS (Panidis, Tziomalos et al. 2012).

The phenotypes of PCOS (Table 2.1) are based on the presence or absence of oligo/anovulation, hyperandrogenaemia, hirsutism, and polycystic ovaries and a comparison of the different diagnostic criteria (Moran and Teede 2009).

Table 2.1: The phenotypes of PCOS

Features	Phenotypes			
	A	**B**	**C**	**D**
Hyperandrogenism (clinical and biochemical)	+	+	+	
Oligo- or anovulation	+	+		+
Polycystic Ovaries	+		+	+
Diagnostic criteria				
NIH	✓	✓		
AE-PCOS	✓	✓	✓	
Rotterdam criteria	✓	✓	✓	✓

AE-PCOS, Androgen Excess and Polycystic Ovary Syndrome Society; NIH, National Institute of Health.

While PCOS is diagnosed on endocrine and reproductive features, PCOS is known to have additional clinical features including metabolic dysfunction and psychological implications. The Rotterdam criteria encompasses those at greatest risk of reproductive and metabolic complications (Moran and Teede 2009, Moran, Norman et al. 2015) including the mild non-hyperandrogenic anovulatory phenotype. As the Rotterdam criteria is now the internationally accepted and recommended diagnostic criteria, the use of this criteria for the women with PCOS was used in the studies contained in this thesis (Teede, Misso et al. 2018).

2.3 Pathophysiology of PCOS

PCOS involves multiple systems of the body and has significant metabolic, reproductive and psychological features (Teede, Deeks et al. 2010, Teede, Misso et al. 2011, Dokras, Clifton et al. 2012). Figure 2.1 highlights the complexity between the features and drivers of PCOS and the synergistic relationships between them.

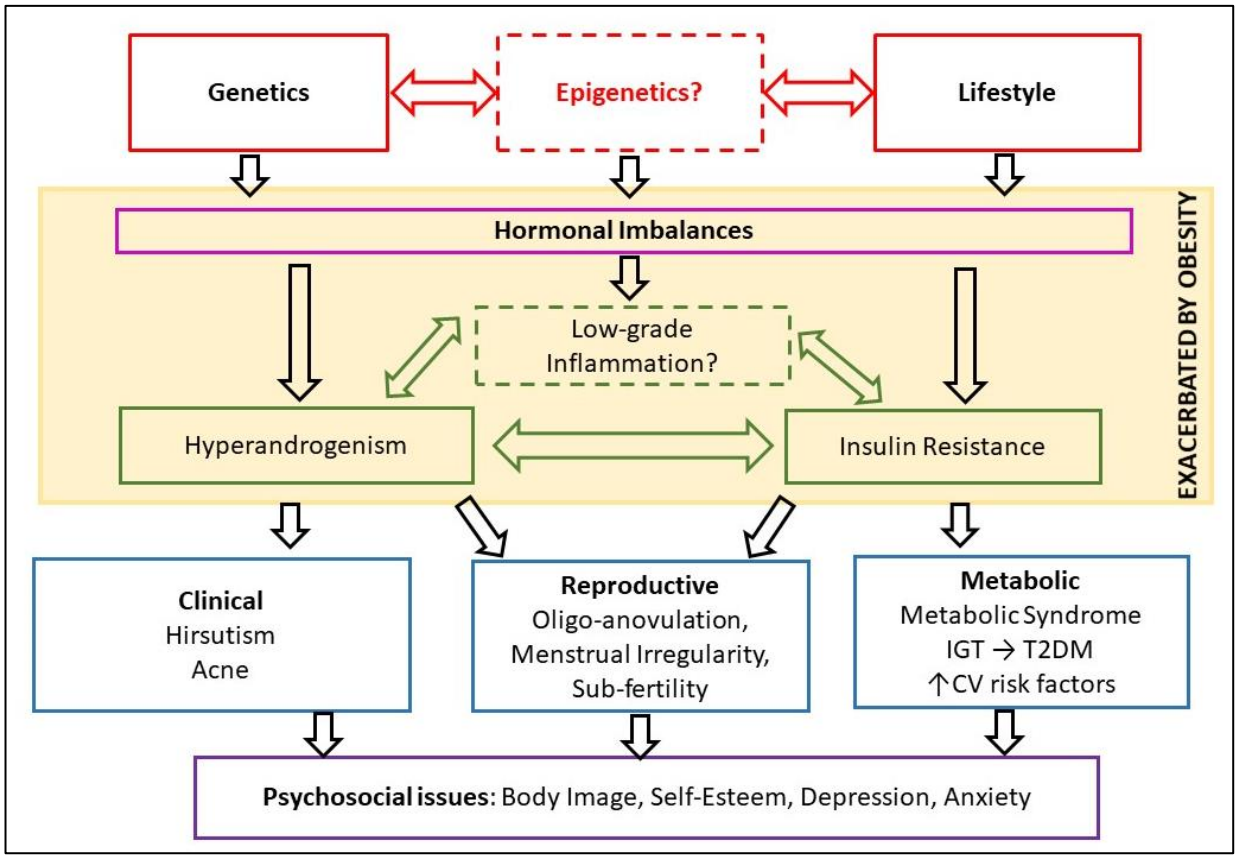

Figure 2.1: Proposed pathophysiology and aetiology of Polycystic Ovary Syndrome. Adapted and reproduced with permission (Teede, Misso et al. 2011). CV, cardiovascular; IGT, impaired glucose tolerance; TD2M, type 2 diabetes.

2.3.1 Reproductive Complications

Hyperandrogenism is present in 80-85% of women diagnosed with PCOS (O'Reilly, Taylor et al. 2014, De Leo, Musacchio et al. 2016). It originates predominantly from the ovaries with contribution from the adrenals and minor contributions from adipose tissue (De Leo, Musacchio et al. 2016). Hyperandrogenism is clinically and mechanistically determined from

197 elevated serum levels of androgens: total testosterone (TT), free testosterone (fT),
198 androstenedione, 17-hydroxy progesterone and/or dehydroepiandrosterone sulphate or by the
199 free androgen index (FAI = (TT/ sex hormone binding globulin [SHBG]) X 100). SHBG is a
200 glycoprotein that regulates bioavailability of sex steroids therefore plays a significant role in
201 regulating the level of free circulating testosterone (Moran, Teede et al. 2013, Thaler, Seifert-
202 Klauss et al. 2015). It is regulated by a variety of hormonal (insulin) and non-hormonal factors
203 such as inflammatory cytokines and liver fat content and BMI is also negatively associated
204 with SHBG (Tsai, Wang et al. 2013, Simó, Sáez-López et al. 2015). In PCOS, SHBG is
205 commonly low, increasing the FAI and contributing to hyperandrogenism but is also
206 associated with insulin resistance and the associated compensatory hyperinsulinemia
207 (Diamanti-Kandarakis and Dunaif 2012, Moran, Teede et al. 2013, Cassar, Teede et al. 2015).
208 Oligo-anovulation, menstrual irregularities and resulting sub-fertility are unsurprisingly
209 common reproductive complications in women with PCOS. In an Australian community-
210 recruited study, 72% of women with PCOS reported infertility while only 16% of women
211 without PCOS reported infertility (Joham, Teede et al. 2015). The study highlighted that
212 women with PCOS are fifteen times more likely to suffer from infertility than women without
213 PCOS and this was independent of BMI. (Joham, Teede et al. 2015).
214
215 Hyperandrogenism may in part be caused by neuroendocrine dysfunction in PCOS
216 (Waldstreicher, Santoro et al. 1988, Hayes, Urbanek et al. 2015, Rosenfield and Ehrmann
217 2016). Hypothalamic-pituitary axis (HPA) is a complex feedback loop comprising of the
218 hypothalamus (containing gonadotropin releasing hormone [GnRH] neurons), pituitary gland
219 (responsible for the secretion of luteinising hormone [LH] and follicle stimulating hormone
220 [FSH]) and the ovary which responds to changes in gonadotropin concentrations (Doi, Towers
221 et al. 2005, Roland and Moenter 2014). In a normal menstrual cycle, the frequency of the

GnRH pulses undergo cyclical changes (Doi, Towers et al. 2005, Roland and Moenter 2014). LH and FSH are in a tightly controlled relationship by GnRH pulsatility, which transiently balances the conversion of androgens to oestrogens throughout the menstrual cycle (Blank, McCartney et al. 2006) Androgens are produced by theca cells in the ovary and are regulated by LH. While FSH regulates the conversion of androgens to oestrogens in the granulosa cells of the ovary (Blank, McCartney et al. 2006). Women with PCOS are thought to have an elevated GnRH pulse frequency that favours the release of LH, results in a deficiency of FSH and an elevated LH: FSH ratio. The elevated LH frequency promotes the theca cells to produce androgens and the deficiency of FSH contributes to the failure of the follicles to mature resulting in the polycystic morphology demonstrated in Figure 2.2 (Waldstreicher, Santoro et al. 1988). 3

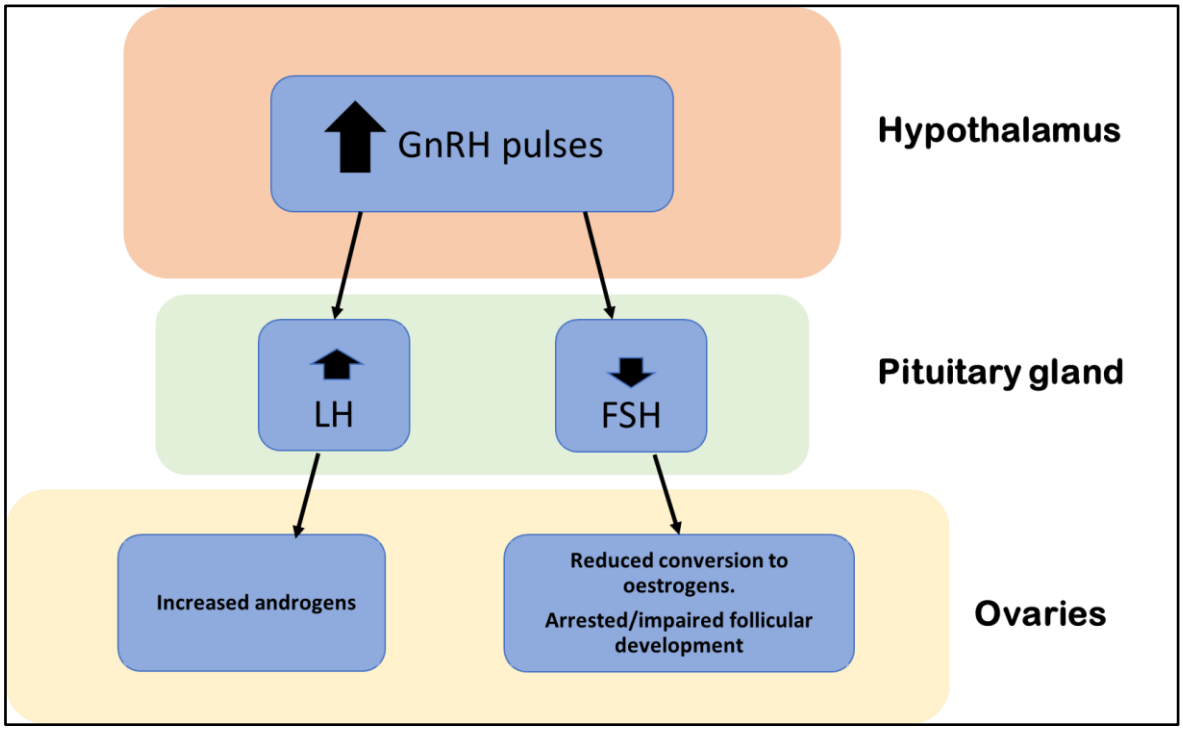

Figure 2.2- The hypothalamic-pituitary axis that leads to the hormone imbalance between LH and FSH in women with PCOS.
GnRH, gonadotrophin releasing hormone neurons; LH, luteinising hormone; FSH, follicle stimulating hormone.

Excess anti-müllerian hormone (AMH) can be indicative of ovarian dysfunction. While AMH is vital for normal follicular maturation and recruitment, excessive AMH has been reported to be contribute to anovulation and the polycystic ovary morphology (Cassar, Teede et al. 2014, Dewailly, Andersen et al. 2014, Dumont, Robin et al. 2015). AMH is elevated in women with PCOS and more highly expressed in the granulosa cells compared to women without PCOS. Follicular genesis is disrupted by AMH by reducing the follicular sensitivity to FSH, causing follicular arrest of the pre-antral and antral follicles in the ovary (Dewailly, Andersen et al. 2014). The mechanisms resulting in increased AMH in PCOS are poorly understood but have been attributed to obesity, insulin resistance, hyperandrogenism, gonadotrophins and their complex interactions.

Inflammation in PCOS is a contentious issue with a plethora of studies highlighting that obesity and inflammation go hand in hand. However, inflammation has been shown to be independent of obesity and associated with elevated levels of common inflammatory markers (IL-6, TNF-α and hsCRP) in women with PCOS who are considered lean. It has been postulated that the chronic low-grade inflammation is driven by hyperandrogenism and enhances androgen production in the ovaries (Ebejer and Calleja-Agius 2013, Shorakae, Teede et al. 2015). Further, follicular development has been associated with systemic and local inflammation, with evidence showing that inflammation can suppress ovarian function (Clancy, Baerwald et al. 2013, Clancy, Klein et al. 2013, Clancy, Baerwald et al. 2016). This indicates that the immune system and its associated cytokines may be involved in the pathogenesis of PCOS.

Inflammation may also act as a link between insulin resistance and hyperandrogenism in PCOS and is associated with both (Gonzalez 2012, Shorakae, Teede et al. 2015). Increased

nutrient load (hyperglycaemia) stimulates ROS generation in the immune cells. ROS can then stimulate the release of cytokines (specifically TNF-α) driving the inflammatory state. Further, evidence has shown the hyper-androgenic milieu promotes inflammation by activating and sensitising immune cells to glucose (Gonzalez, Rote et al. 2006, Gonzalez 2012, Gonzalez, Sia et al. 2014). Several lines of evidence now suggest that an overabundance of nutrients can influence the phenotype of resident or infiltrating immune cells and could thus be central to the dramatic changes affecting the immune system in metabolic diseases (Sell, Habich et al. 2012, Raghuraman, Donkin et al. 2016).

2.3.2 Psychological Impacts

Many chronic illnesses have mental health impacts (Lustman, Anderson et al. 2000, DeJean, Giacomini et al. 2013, Panagioti, Scott et al. 2014). Women with PCOS have reduced mental health and lower quality of life when compared to women without PCOS (Deeks, Gibson-Helm et al. 2011, Banting, Gibson-Helm et al. 2014). Women with PCOS are four times more likely to suffer from moderate to severe symptoms of depression and six times more likely to suffer from severe symptoms of anxiety (Cooney, Lee et al. 2017). Studies have found that the longer it takes for confirmation of diagnosis, a common issue in PCOS (Gibson-Helm, Lucas et al. 2014), the more likely women are to have symptoms of anxiety or depression (Deeks, Gibson-Helm et al. 2011). Obesity is recognised as a risk factor for increased symptoms of depression, however in women with PCOS the increased symptoms of depression have been found to be independent of BMI (Cooney, Lee et al. 2017). There is increasing evidence that psychological and the physiological manifestations are inter-related in PCOS however more research is required (Farrell and Antoni 2010).

2.3.3 Metabolic dysfunction

Women with PCOS are two to four times more likely to experience metabolic dysfunction than women without PCOS (Moran, Misso et al. 2010). Independent of weight, women with PCOS have higher prevalence of impaired glucose tolerance (IGT) and Type 2 Diabetes Mellitus (T2DM) (Moran, Misso et al. 2010, Hart and Doherty 2015, Moran, Norman et al. 2015). In addition, women with PCOS have a more rapid progression from normal glucose function to impaired glucose tolerance and T2DM compared with women without PCOS (Norman, Masters et al. 2001, Celik, Tasdemir et al. 2014). This is not surprising since women with PCOS regardless of BMI, diagnostic criteria or ethnicity have 27% lower insulin sensitivity than women without PCOS, established in a meta-analysis of hypersulinaemic-euglycaemic studies (Cassar, Misso et al. 2016). In an Australian cohort (n=79) it has been found that 75% of lean (BMI<27kg/m2) and 95% of overweight (BMI>27kg/m^2) women with PCOS were insulin resistant (Stepto, Cassar et al. 2013) using the gold standard euglycaemic-hyperinsulinaemic clamp technique. In a clinical setting oral glucose tolerance test (OGTT) are routinely performed and surrogate indices such as homeostatic model assessment (HOMA) (Meyer, McGrath et al. 2007) are used to test for insulin resistance. Studies using OGTT testing also show women with PCOS are more insulin resistant and have higher rates of IGT compared with women without PCOS (Legro, Kunselman et al. 1999). Women with PCOS have a higher prevalence of overweight, obesity and have greater central adiposity than women without PCOS (Lim, Davies et al. 2012, Teede, Joham et al. 2013). Studies have shown that overweight and obese women with PCOS are 2 to 10 times more likely to develop T2DM compared to healthy-weight women with PCOS (Norman, Masters et al. 2001, Celik, Tasdemir et al. 2014, Rubin, Glintborg et al. 2017).

2.3.3.1 Insulin resistance and hyperinsulinemia

The biological origins and pathophysiology of PCOS are poorly understood, partially due to complex relationship between the multiple biological systems impacted by PCOS. Insulin resistance has been postulated to play a central aetiological role in PCOS (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Stepto, Cassar et al. 2013, Moran, Norman et al. 2015, Cassar, Misso et al. 2016). Insulin resistance contributes to both the metabolic and reproductive features of PCOS (Figure 2.1). It is thought that the intrinsic insulin resistance or obesity-independent insulin resistance to be a potential mechanism in the multifactorial aetiology of PCOS (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Stepto, Cassar et al. 2013, Moran, Norman et al. 2015, Cassar, Misso et al. 2016).

Insulin resistance and the associated compensatory hyperinsulinemia is considered to strongly correlate with hyperandrogenism and clinical features of PCOS (Burghen GA 1980). Insulin is considered not only a metabolic hormone but also a reproductive hormone that directly regulates steroidogenesis (Diamanti-Kandarakis and Dunaif 2012). Early seminal studies provided the first evidence of significant positive correlations between insulin and testosterone levels (Plymate, Jones et al. 1988, Nestler, Powers et al. 1991). Insulin is a negative regulator of SHBG by supressing production in hepatic cells this increases the concentration of free testosterone circulating in the blood (Plymate, Jones et al. 1988, Nestler, Powers et al. 1991). It was also discovered in ovarian theca cells and granulosa cells, insulin bind its insulin receptor and activates the release of LH and stimulating excessive androgen production (Willis and Franks 1995, Nestler, Jakubowicz et al. 1998, Tosi, Negri et al. 2012). Initially it was thought that insulin would bind the IGF-1 receptor in ovarian tissue due to its similarity in structure. However early studies demonstrated that insulin binds its own receptor rather than the IGF-1 receptor (Willis and Franks 1995). Due to the insulin resistance, the β-

cells compensate and increase insulin secretion resulting in hyperinsulinemia (Diamanti-Kandarakis and Dunaif 2012). Insulin can act as an extra-ovarian modulator and override normal mechanisms that control ovarian androgen production (Rosenfield and Ehrmann 2016). There is increased release of LH from the pituitary gland due to the persistently increased GnRH pulse frequency (Burt Solorzano, Beller et al. 2012, Patel and Shah 2018). In combination with the augmentation of LH, insulin sensitises ovarian tissue by up-regulating LH binding sites and therefore this enhances androgen production and disrupts the normal cycling of LH and FSH required for normal follicular genesis (Rosenfield and Ehrmann 2016).

This apparent paradox occurs simultaneously with the peripheral insulin resistance in the adipose tissue and skeletal muscle which manifests as metabolic features of PCOS. Skeletal muscle accounts for 70-80% of insulin stimulated glucose uptake (DeFronzo 1988, Lundsgaard and Kiens 2014) and therefore any major defect in this tissue may have profound effects on whole body insulin sensitivity. The underlying mechanisms of insulin resistance that is unique in PCOS remain ill-defined (Teede, Misso et al. 2011, Diamanti-Kandarakis and Dunaif 2012), contributing to controversy over exclusion of insulin resistance in the diagnostic criteria, and a lack of optimal therapies that warrant the need for further research. Currently, there is a hypothesis that PCOS-specific insulin resistance (obesity independent) in peripheral tissues is due to a PCOS-specific inhibitory serine kinase that is phosphorylating, external to the canonical insulin signalling pathway, that targets the proximal proteins, including the insulin receptor and its immediate substrate the insulin receptor substrate 1 and 2 (IRS1/2) protein complex (Diamanti-Kandarakis and Dunaif 2012). However, in-vivo evidence across insulin sensitive tissue of excessive serine phosphorylation of either insulin receptor or its immediate substrate proteins IRS1/2 in skeletal muscle and its impact of insulin

stimulated glucose uptake in women with PCOS remains equivocal (Cusi, Maezono et al. 2000, Corbould, Kim et al. 2005, Corbould, Kim et al. 2005, Copps and White 2012).

2.3.3.2 An alternate pathway for insulin resistance

Recently Raja-Khan et al. (Raja-Khan, Urbanek et al. 2014) proposed an alternative hypothesis that dysfunctional transforming growth factor-beta (TGFβ) superfamily ligands may contribute to intrinsic insulin resistance associated with PCOS. Based on candidate gene studies TGFβ1 gene polymorphisms have been associated with susceptibility to PCOS (Yang, Zhong et al. 2015, Roh, Yoon et al. 2017). Further, TGFβ1 is elevated in the serum of women with PCOS and was positively associated with metabolic and reproductive variables measured in PCOS (FAI and HOMA-IR) (Tal, Seifer et al. 2013).The TGFβ superfamily are a group of structurally related regulatory proteins with diverse biological functions including reproduction, cancer progression, extracellular matrix formation, inflammation, metabolism, and development of bone, skeletal muscle, and fat (Diamanti-Kandarakis and Dunaif 2012). The TGFβ superfamily of ligands includes inhibins, activins, AMH, growth and differentiation factors, bone morphogenetic proteins (BMPs), and finally the TGFβ family which consists of 3 primary isoforms: TGFβ1, TGFβ2, and TGFβ3 (Raja-Khan, Urbanek et al. 2014). The synthesis of TGFβ ligands is not restricted to a particular tissue with most cells in the body expressing TGF-β receptors and can respond to a variety TGF-β ligands (Raja-Khan, Kunselman et al. 2010, Cassar, Teede et al. 2014). Each TGFβ ligand binds a specific set of TGFβ receptors that dimerise, and phosphorylates a specific protein from the Smad family that translocate to the nucleus activating a variety of target genes (Massague 2000, Lin, Lee et al. 2009). Of interest in PCOS is the TGF-β/Smad 3 signalling pathway as it regulates glucose metabolism and energy homeostasis. Specifically elevated TGFβ/Smad3 signalling

down-regulates insulin gene transcription, supresses the secretion of insulin and interferes with β-cell function. Further, it can repress expression of genes that promote glucose sensing, glucose metabolism and glucose-stimulated insulin secretion and the endocrine function of adipose tissue (Yadav and Rane 2012) all of which are important for insulin signalling. Therefore, it is plausible that dysfunctional TGFβ network signalling may play a direct signalling role in intrinsic insulin resistance in PCOS however further research is warranted.

Fibrillin isoforms 1, 2 and 3 are extracellular matrix glycoproteins. They form the major component of microfibrils that ensures structural integrity and support elastin in connective tissues (Piha-Gossack, Sossin et al. 2012, Raja-Khan, Urbanek et al. 2014). Fibrillin's regulate the TGFβ signalling pathway of the extracellular matrix (Raja-Khan, Urbanek et al. 2014, Bastian, Bayne et al. 2016) which surround tissue, including myofibrils in muscle. Defects in the fibrillin gene lead to dysfunctional TGFβ ligand signalling in cardiovascular disease, Marfan syndrome and fibrotic conditions (Akhurst and Hata 2012, Raja-Khan, Urbanek et al. 2014). A recently discovered gene variant fibrillin 3 appears to be unique to women with PCOS and has been associated with dysfunctional TGF-β signalling and increased fibrosis in ovarian tissue (Raja-Khan, Urbanek et al. 2014, Bastian, Bayne et al. 2016). AMH (Cassar, Teede et al. 2014) and TGFβ1 (Tal, Seifer et al. 2013, Liu, Gao et al. 2015) are also elevated in women with PCOS. These ligands likely act via their respective receptors to activate the Smad signalling proteins that are not only negative regulators of Akt (Chen, Colgan et al. 2016, Parker, Shaw et al. 2017) but are key signals for extracellular matrix deposition. The role of this aberrant extracellular matrix remodelling in the aetiology of PCOS is underexplored but may provide novel mechanisms linking abnormalities in whole body metabolism, ovarian function, insulin signalling in skeletal muscle and adaptations to exercise (Böhm, Hoffmann et al. 2016).

2.4 Management of PCOS

Lifestyle management (exercise and diet) is one of the first line treatment for obese women with PCOS (Teede, Misso et al. 2011). It is known that weight loss, as little as 5-10%, through diet and exercise improves the clinical features of PCOS (Clark, Thornley et al. 1998, Huber-Buchholz, Carey et al. 1999, Teede, Misso et al. 2011). Metabolic factors such as insulin resistance are improved by exercise independent of changes in BMI (obesity) (Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012). Moderate intensity aerobic exercise improves metabolic features (insulin resistance, dyslipidaemia, and cardio-vascular disease risk factors), body composition (decreases visceral fat), reproductive features (anti-müllerian hormone [AMH]) and psychological well-being in overweight women with PCOS, and in the general population (Harrison, Lombard et al. 2011, Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012). Greater improvements in metabolic health and cardio-respiratory fitness have been reported with high intensity exercise when compared to recommended lower intensity exercise (Kessler, Sisson et al. 2012, Greenwood, Noel et al. 2016). High intensity interval training (HIIT) consists of repeated, short bouts of vigorous exercise interspersed with rest periods. No studies to date have examined the health benefits of HIIT training alone in women with PCOS compared to moderate intensity exercise. However, a mixed protocol alternating between moderate intensity and high intensity has reported positive clinical outcomes in insulin resistance, AMH and visceral fat (Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012). Whether moderate or high intensity exercise is more effective in promoting optimal metabolic, reproductive and mental health outcomes in overweight women with polycystic ovary syndrome is not known.

2.5 Biological origins- genetics and epigenetics

Insulin resistance is thought to underpin PCOS and to increase hyperandrogenism that drives inherent metabolic and reproductive features of the condition. While instrumental in the pathophysiology we know that insulin resistance does not explain the whole molecular background in the syndrome. In parallel with exploring insulin resistance mechanisms, we therefore explored biological origins (genetics and epigenetics) which are also thought to play a role in driving the metabolic and reproductive features of PCOS.

2.5.1 Genetic predisposition

PCOS appears to have a polygenic predisposition that is exacerbated by environmental and lifetysle factors. Familial clustering of PCOS symptoms is well documented, providing evidence for a genetic contribution to the condition (Legro, Driscoll et al. 1998). Monozygotic twin studies have demonstrated the heritability of PCOS to be approximately 70% (Vink, Sadrzadeh et al. 2006). Genetic studies have been conducted to elucidate genes and pathways that may be involved in the aetiology of PCOS. Single nucleotide polymorphisms (SNPs) are mutations or variations in a base-pair of a gene that can lead to altered gene expression and protein function causing phenotypic changes. Gene association studies have generally investigated SNPs in genes that are associated with the known pathophysiology of the syndrome including; insulin signalling, steroid production and action, gonadotrophin synthesis and action, follicle development, low-grade inflammation and regulation of metabolism (Mykhalchenko, Lizneva et al. 2017). Investigation of these SNPs has resulted in mixed outcomes from single genetic association studies (Day, Hinds et al. 2015, Mykhalchenko, Lizneva et al. 2017).

Genome-wide association studies (GWAS), have gained validity and are an important tool able to screen the entire human genome to identify the risk loci or regions of interest that could be associated with complex diseases such as PCOS (Hirschhorn and Daly 2005). In PCOS, several GWAS have been conducted in Chinese and European cohorts and have identified fifteen genome-wide significant PCOS risk SNPs from 11 gene loci. Many of the risk loci identified are located near neuroendocrine, hormones, insulin signalling and organ growth genes (Day, Hinds et al. 2015, Mykhalchenko, Lizneva et al. 2017). GWAS do not identify candidate genes but regions of interest and therefore candidate gene studies and functional analysis is required to validate and decipher the functional impact of gene variants to establish the clinical relevance of GWAS findings (Wilkening, Chen et al. 2009, Vlahovich, Hughes et al. 2017, Williams, Williams et al. 2017). Only a limited number of studies have looked at the functional implications of the risk loci established by GWAS including: luteinizing hormone/choriogonadotropin receptor (LHCGR), insulin receptor (INSR) and DENN domain-containing protein 1A (DENND1A). The LHCGR gene has been found to be over-expressed in adipose tissue, theca, and granulosa cells (Jones, Brower et al. 2015). In obese women with PCOS, INSR has shown to be down-regulated in skeletal muscle and adipose tissue but up-regulated in the ovaries consistent with what is known in the current literature about the impact of hyperinsulinemia and insulin signalling in women with PCOS (Jones, Brower et al. 2015). DENND1A splice variant DENND1A.V2 has been found to be up-regulated in the theca tissue of women with PCOS (McAllister, Modi et al. 2014). Further functional studies are required in different tissue types to establish the biological role of the identified risk loci.

With the gaining interest in the genetic basis of PCOS there is an extensive amount of literature in this area. But the following key questions remain:

- Does this existing literature provide a broader picture of genetics role in PCOS?
- Is there comparability between studies?
- Are GWAS locating risk loci in genes that have functional implications in PCOS?

A systematic approach is required to gain a better understanding of the role of genetic variation in PCOS.

2.5.2 Gene-environmental interactions: epigenetics

Exposure to environmental factors such as stress, nutrition, placental insufficiency, endocrine imbalances and pollution can alter the epigenome especially in early life. This can lead to permanent phenotypic changes that can influence the health of an individual and increase the risk of metabolic disease later in life, this is known as the developmental origins of health and disease hypothesis (Zheng, Xiao et al. 2014, Lillycrop and Burdge 2015). The prenatal environment, in particular exposure to androgen excess and hyperinsulinemia, has been hypothesised to play a role in the metabolic and reproductive pathology of PCOS (Zhu, Zhu et al. 2010, Xu, Kwon et al. 2011, Xu, Chua et al. 2014, Lambertini, Saul et al. 2017). While the underlying mechanism of intrauterine programming have not been fully elucidated, animal models suggest that perturbations to the epigenome through DNA methylation may link the in-utero environment and the development of PCOS in later life (Zhu, Zhu et al. 2010, Xu, Kwon et al. 2011, Xu, Chua et al. 2014, Zhang, Cong et al. 2014). A recent study compared umbilical cord blood between women with and without PCOS and found differences in epigenetic patterns predominantly in pathways that are involved in lipid, and carbohydrate metabolism, inflammation and the immune system (Lambertini, Saul et al. 2017). Adding further evidence that the maternal intra-uterine environment does affect epigenetic programming in a developing embryo.

With genetic studies only being able to account for approximately 10% of the heritability of PCOS this indicates other factors must affect heritability (Chen, Zhao et al. 2011, Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015). Epigenetics is defined as changes in gene expression due to modifications of DNA without the DNA sequence being altered (Qiu 2006, Barrès, Yan et al. 2012). There are multiple types of epigenetic modifications including: histone modification, chromatin re-modelling, microRNAs, lncRNAs and DNA methylation (Tammen, Friso et al. 2013). In this thesis we focused on one type of epigenetic modification, DNA methylation. DNA methylation can alter protein-protein and protein-DNA interactions that can affect chromatin structure and modify transcription of the DNA in two different ways. Firstly, by modulating the access of the transcription machinery to the chromatin by binding a methyl unit to the 5' position of a cytosine base. Secondly, by recruiting methyl-specific binding proteins that recruit enzymatic machinery capable of locally altering histone modification.(Qiu 2006, Barres and Zierath 2011, Tammen, Friso et al. 2013). Inappropriate epigenetic programming is evident in the aetiology of common metabolic diseases (Barres and Zierath 2011) and in PCOS (Shen, Qiu et al. 2013, Wang, Wei et al. 2014) indicating an interaction of genetics and lifestyle.

2.5.2.1 Techniques for measuring DNA methylation

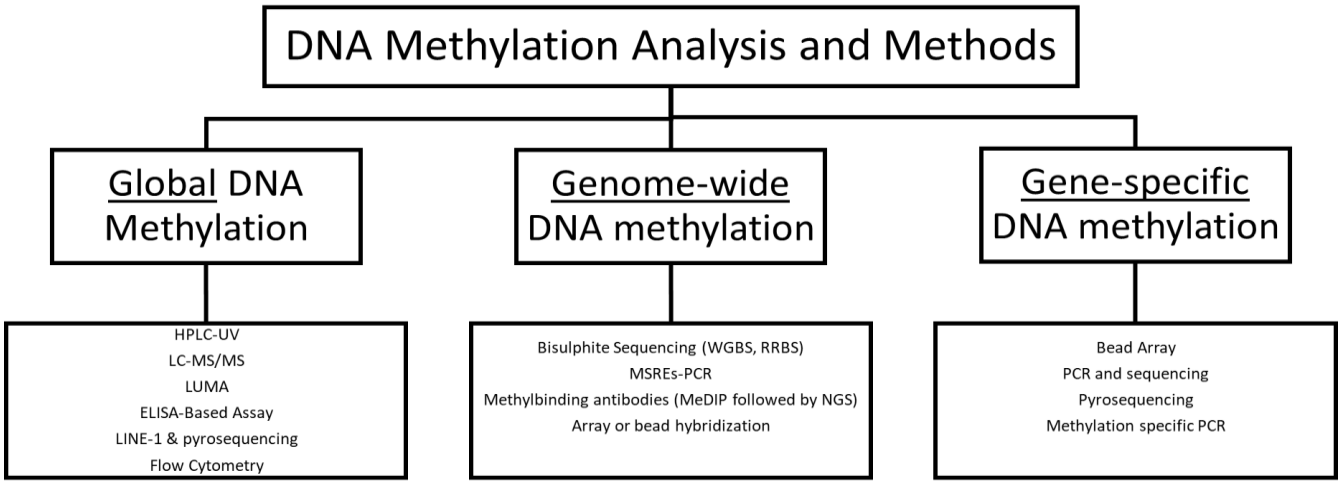

Figure 2.3: Overview of DNA methylation methods (Kurdyukov and Bullock 2016). HPLC-UV, High Performance Liquid Chromatography-Ultraviolet; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; LUMA, Luminometric Methylation Assay; ELISA, Enzyme-Linked Immunosorbent Assay; LINE-1, Long Interspersed Nuclear Elements; WGBS, Whole Genome Bisulphite Sequencing; RRBS, Reduced Representation Bisulphite Sequencing; MSREs, Methylation Sensitive Restriction Enzymes; PCR, Polymerase Chain Reaction; MeDIP, Methylated DNA Immunoprecipitation.

Multiple techniques have been developed to measure DNA methylation from global methylation of a tissue to screening methylation across genome-wide loci to more specific regions of interest and single gene methylation (see Figure 2.3 for overview). While there are numerous techniques to measure methylation of the DNA they all follow one of three basic principles: conversion of methylated or unmethylated cytosine's, methylation sensitive restriction enzymes or an antibody that can interact with methylcytosine (5MeC) (Ammerpohl, Martin-Subero et al. 2009). Global methylation refers to non-sequence dependent measurement of methyl-cytosine content. As it is non-sequence dependent it does not establish what loci or gene may be affected. However, global methylation status can reflect environmental factors or be used as a biomarker of a disease. Global methylation status

in peripheral blood has been used as a biomarker for many chronic conditions such as stroke (Baccarelli, Wright et al. 2010), cancer (Choi, James et al. 2009) and T2DM (Martin-Nunez, Rubio-Martin et al. 2014). Genome-wide DNA methylation establishes differentially methylated loci or genes across the whole genome and can elucidate genes and enriched molecular pathways that are affected by a condition. This is particularly useful in finding novel molecular pathways that are associated with or causal of a disease. While genome-wide DNA methylation analysis is a methylation status screen of all loci it is does not give an indication of the precise location of the CpG methylation nor which nucleotide in the loci is methylated or unmethylated. Gene-specific DNA methylation establishes the methylation of individual CpGs at a locus of interest. It is of particular interest to determine the location of the methylation (i.e.- transcription start site, gene body, CpG islands, shelves and shore regions in a gene, enhancer or promoter) as this is important when evaluating the impact to gene expression and phenotype (Crider, Yang et al. 2012).

2.5.2.2 Immune system and epigenetics

The immune system plays a leading role in fighting pathogens, by regulating the innate and adaptive arms of immunity. There is a growing body of evidence that associates the immune system with metabolism and endocrine regulation (Pate, Toyokawa et al. 2010, Raghuraman, Donkin et al. 2016, Nilsson, Benrick et al. 2018). The immune system is epigenetically regulated and it has been shown that in related morbidities of PCOS like obesity and T2DM, have a distinct DNA methylome compared to apparently healthy individuals (Wang, Zhu et al. 2010, Lawson, Eleftheriadis et al. 2012, Simar, Versteyhe et al. 2014, Raghuraman, Donkin et al. 2016). The order of events in systemic inflammation and metabolic, and reproductive dysfunction remain incompletely understood (Chen, Ge et al. 2016). Immune cells may infiltrate other tissues (skeletal muscle, ovaries, adipose tissue) integrating any

physiological and pathophysiological changes that are occurring in these tissues (Bukulmez and Arici 2000, Pate, Toyokawa et al. 2010). Conversely other evidence indicates that the epigenome of immune cells can adapt their environmental milieu (cytokines, reactive oxidative species, diet, hormones) and then influence the epigenome of surrounding tissues (Paparo, di Costanzo et al. 2014, Obata, Furusawa et al. 2015). Indicating that the immune system may reprogram metabolically active tissue through epigenetic modification, and that these modifications may result in the development of metabolic disease (Kintscher, Hartge et al. 2008, Wang, Zhu et al. 2010, Barres and Zierath 2011, Raghuraman, Donkin et al. 2016) however further research is required to establish causality.

As established earlier in this literature review both metabolic and endocrine dysfunction are involved in the pathophysiology of PCOS (Teede H, Deeks A et al. 2010). In both animal models and human ovarian tissue altered immune responses and inflammatory markers have been found in the tissues of the ovaries, suggesting a role in the pathophysiology of the reproductive features of PCOS (Pate, Toyokawa et al. 2010, Figueroa, Davicino et al. 2012, Schmidt, Weijdegard et al. 2014). With implications in regulating immune function, differential patterns of genome-wide DNA methylation have been found in whole blood in women with PCOS (Shen, Qiu et al. 2013, Li, Zhu et al. 2016). Further, differential genome-wide DNA methylation patterns have been found in a variety of other tissues in women with PCOS including adipose tissue (Kokosar, Benrick et al. 2016), ovaries (Wang, Wei et al. 2014, Yu, Sun et al. 2015) and the granulosa cell (Xu, Bao et al. 2016). With changes in DNA methylation corresponding with genes associated with the inflammatory response, metabolism of sex steroids and metabolism. Additionally a recent study in skeletal muscle also found the most significantly enriched pathways were involved in immune function or immune diseases (Nilsson, Benrick et al. 2018). Altogether this provides further evidence that

alterations in DNA methylation are associated with impaired immune function in various tissues in women with PCOS (Nilsson, Benrick et al. 2018) and may be associated with the pathophysiology of PCOS. However, at this stage it is unknown what the order of events are: does immune dysfunction come first or, is it metabolic, and reproductive dysfunction?

Peripheral whole blood is a heterogeneous tissue that consists of platelets, red blood cells, and white blood cells. White blood cells are made up of two fractions: polymorphonuclear cells (PNCs) and peripheral blood mononuclear cells (PBMC). Eosinophils, basophils and neutrophils make up the PNC fraction, while lymphocytes (T cells, B cells and natural killer cells [NK]) and monocytes make up the PBMC fraction. Cellular heterogeneity is a confounding factor in DNA methylation analysis in both global (Simar, Versteyhe et al. 2014) and genome-wide DNA methylation analysis (Glossop, Nixon et al. 2013, Mamrut, Avidan et al. 2015). This was highlighted by a study on global DNA methylation in T2DM. Differences in global DNA methylation in whole PBMC fractions were not observed. However, when specific cell populations in blood were isolated and analysed separately there was increased methylation in B cells and NK cells in T2DM (Simar, Versteyhe et al. 2014). In PCOS, one study has investigated global DNA methylation and there were no unique differences in non-specific analysis of PBMC between women with and without PCOS (Xu, Azziz et al. 2010). Multiple studies have detected differences in genome-wide DNA methylation patterns in a cell-specific manner in apparently healthy women (Glossop, Nixon et al. 2013, Mamrut, Avidan et al. 2015). Glossop et al. (2013) found unique genome-wide DNA methylation patterns in T and B cells. This finding was also confirmed by (Mamrut, Avidan et al. 2015), who also identified unique methylation patterns in additional immune cell populations (B cells and monocytes). Alterations in the DNA methylome of immune cells could have consequences in their functioning and contribute to the low-grade inflammation

in PCOS. Cause and effect have yet to be determined however immune cells could infiltrate many tissues (skeletal muscle, ovaries, adipose tissue) and integrate any physiological and pathophysiological changes that are occurring in these tissues (Bukulmez and Arici 2000, Pate, Toyokawa et al. 2010). Or the DNA methylome of immune cells can adapt to their environmental milieu and then influence the epigenome of surrounding tissues (Paparo, di Costanzo et al. 2014, Obata, Furusawa et al. 2015). Non-specific analysis of PBMCs could potentially be hiding cell-specific and disease-specific changes that may play a significant role in the molecular mechanisms of PCOS requiring further investigation (Adalsteinsson, Gudnason et al. 2012, Glossop, Nixon et al. 2013, Simar, Versteyhe et al. 2014).

2.5.3 Exercise and DNA methylation

Exercise elicits a positive clinical outcome for women with PCOS however, there are large gaps of knowledge as to the molecular mechanism that facilitate this improvement. Lifestyle factors such as diet and exercise can remodel DNA methylation (Rönn, Volkov et al. 2013, Ling and Ronn 2014). Alterations to DNA methylation patterns after an intervention, could be a key factor contributing to improvements in metabolic status. Epigenetics may explain how exercise can improve health status in PCOS (Ling and Ronn 2014) or potentially even its limited impact. Previous studies have shown skeletal muscle DNA methylation is remodelled by exercise and has a unique intensity-dependent exercise effects on DNA methylation (Barrès, Yan et al. 2012, Nitert, Dayeh et al. 2012, Rönn, Volkov et al. 2013, Rasmussen, Zierath et al. 2014, Barres and Zierath 2016). Whether an exercise intervention in women with PCOS can alter DNA methylation patterns in skeletal muscle, peripheral blood or adipose tissue has not been investigated. Gaining a better understanding of whether methylation is modifiable by lifestyle interventions such as exercise, may improve future

643 management of PCOS through the identification of new therapeutic targets and/or effective
644 lifestyle interventions.

2.6 Aims:

Considering the gaps identified in this review of literature, the aims of this thesis are;

1. To conduct an overview of systematic reviews to synthesise the current evidence and the quality of evidence for the relationship between genetic variation and PCOS. (chapter 3)
2. To investigate global DNA methylation profile in specific immune cell population and its associations with key clinical features including BMI, metabolic and reproductive function in women with and without PCOS. (chapter 4)
3. To investigate the transcriptome and methylome in T helper cells of women with and without PCOS to further elucidate the molecular pathways that may be affected. (chapter 5)
4. Explore the proteins in the insulin signalling pathways that may be altered by the intrinsic and extrinsic insulin resistance in polycystic ovary syndrome. (chapter 6)
5. Design of a randomised control trial using an exercise intervention to examine the effectiveness of different exercise intensities on insulin sensitivity, reproductive hormone profiles, psychosocial health and epigenetic reprogramming (chapter 7).

CHAPTER 3. THE GENETICS OF PCOS: AN OVERVIEW OF SYSTEMATIC REVIEWS

3.1 General background

PCOS is a complex endocrine condition with apparent heritability as demonstrated in genetic association studies and familial clustering by twin and family studies (Legro, Driscoll et al. 1998, Legro, Bentley-Lewis et al. 2002, Kaminsky, Tang et al. 2009). Many genetic association studies have been undertaken from candidate gene studies, twin studies to the large genome-wide association studies (GWAS). These studies seek to identify single nucleotide polymorphisms (SNPs) that are associated with PCOS and its clinical features including insulin signalling, steroid production and action, gonadotrophin synthesis and action, follicle development, low-grade inflammation and regulation of metabolism. Several GWAS have been conducted in Chinese and European cohorts and have identified fifteen genome-wide significant PCOS risk SNPs from 11 gene loci. Many of the risk loci identified are located near neuroendocrine, hormones, insulin signalling and organ growth genes (Day, Hinds et al. 2015, Mykhalchenko, Lizneva et al. 2017). GWAS while informative, only identify regions of interest but not specific genes. Candidate gene studies and functional analysis are required to validate and decipher the functional gene variants and the clinical relevance of GWAS findings (Wilkening, Chen et al. 2009, Vlahovich, Hughes et al. 2017, Williams, Williams et al. 2017).

In this chapter I conducted an overview of systematic reviews using gold standard methods of PRISMA supplementary Supplementary table 3.7 (Moher, Liberati et al. 2009) complimented with the AMSTAR quality assessment tool (Shea, Grimshaw et al. 2007). I systematically explored the literature and synthesis of evidence using a novel approach (systematically reviewing systematic reviews) to understand the potential contributions of *a-priori* selected single candidate gene variants and their associations with PCOS. This original research methodology has recently been introduced due to a substantial increase in publications of

systematic reviews in biomedical and clinical sciences. This approach affords us the opportunity to assess the methodological quality of systematic reviews on a given topic and the consistency of evidence contained in them. This overview highlighted methodological flaws that should be addressed in future systematic reviews and primary genetic studies to improve the comparability of results when examining genetics in PCOS. This is of importance, particularly in genetics with the introduction of GWAS, where candidate gene studies will play an imperative role in validating and deciphering the functional gene variants and is vital for determining the clinical relevance and application of GWAS findings (Wilkening, Chen et al. 2009, Vlahovich, Hughes et al. 2017, Williams, Williams et al. 2017). These recommendations will improve the quality of not only systematic reviews but also candidate gene studies and will allow the field of genetics in PCOS to progress.

This chapter consolidates the existing literature around the contribution of SNPs in genetic basis of PCOS, and is currently under review in the Journal of Metabolism (Clinical and Experimental). The work contained in this chapter was led by myself and is an international collaboration where all listed authors have consented to this work being submitted in this thesis. The following people contributed to the work; Alba Moreno-Asso[1], Helena Teede[2], Joop Laven[3], Nigel K Stepto[1], Lisa J. Moran[2], Melanie Gibson-Helm[2].

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3.2 Abstract

Polycystic Ovary Syndrome (PCOS) is a complex condition with mechanisms likely to involve the interaction between genetics and lifestyle. While heritability is clearly involved, genetic studies have resulted in mixed outcomes. Therefore, there is a growing number of systematic reviews (SRs) regarding genetic studies in PCOS. This creates a need to synthesise and summarise these publications in an overview that generates bigger picture insights into the genetics of PCOS. Databases (MEDLINE, EMBASE, CINAHL PLUS and PROSPERO) were searched to identify systematic reviews with or without meta-analyses. Review quality was assessed with the Assessing the Methodological Quality of Systematic Reviews (AMSTAR) tool. The protocol was registered in the international prospective register of systematic reviews PROSPERO (CRD42016052649). Sixteen systematic reviews were included in this overview. None met all 11 AMSTAR criteria and the median overall quality score was 7 (interquartile range: 6 to 7). Multiple genetic polymorphisms were found to be associated with PCOS; however, poor consistency and limitations did not allow definitive conclusions to be reached. Further high quality genetic association systematic reviews and primary studies are required to fully elucidate the role of genetic polymorphisms in PCOS as they cannot be concluded from this overview. While only candidate gene studies were systematically reviewed in this manuscript the recommendations made are applicable to genome-wide association studies, which are becoming increasingly common in complex diseases. This overview highlights the need for standardisation in systematic review design and their analyses to improve reproducibility and impact, and to elucidate the role of genetics in PCOS.

3.3 Introduction

Polycystic ovary syndrome (PCOS) is a major public health concern affecting 6-10% of reproductive aged women (Bozdag, Mumusoglu et al. 2016). PCOS is exacerbated by obesity and has significant metabolic, reproductive and psychological features, including an increased risk of Type 2 Diabetes with an earlier age of onset, sub-fertility, and increased risk of depression and anxiety symptoms (Moran, Misso et al. 2010, Teede, Misso et al. 2011, Cooney, Lee et al. 2017). At present the internationally accepted criteria for diagnosis of PCOS is the revised Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group 2004) which requires exclusion of other causes of adrenal or pituitary dysfunction and presence of two of the following three characteristics: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasound. The Rotterdam criteria yield four phenotypes of PCOS and there is evidence that the different PCOS phenotypes have varying degrees of adiposity, and may differ in metabolic and reproductive profiles (Moran, Norman et al. 2015). The proposed pathophysiology of PCOS is a synergistic relationship between perturbed GnRH pulsatility, hyperandrogenism accompanied by insulin resistance and inflammation, however the nuances of these relationships are yet to be fully elucidated, see Figure 3.1 (Nestler and Jakubowicz 1996, Shorakae, Teede et al. 2015, Cassar, Misso et al. 2016). PCOS appears to have a polygenic predisposition that is exacerbated by lifestyle factors such as overweight and obesity: therefore lifestyle management is the recommended first-line treatment (Teede, Misso et al. 2011).

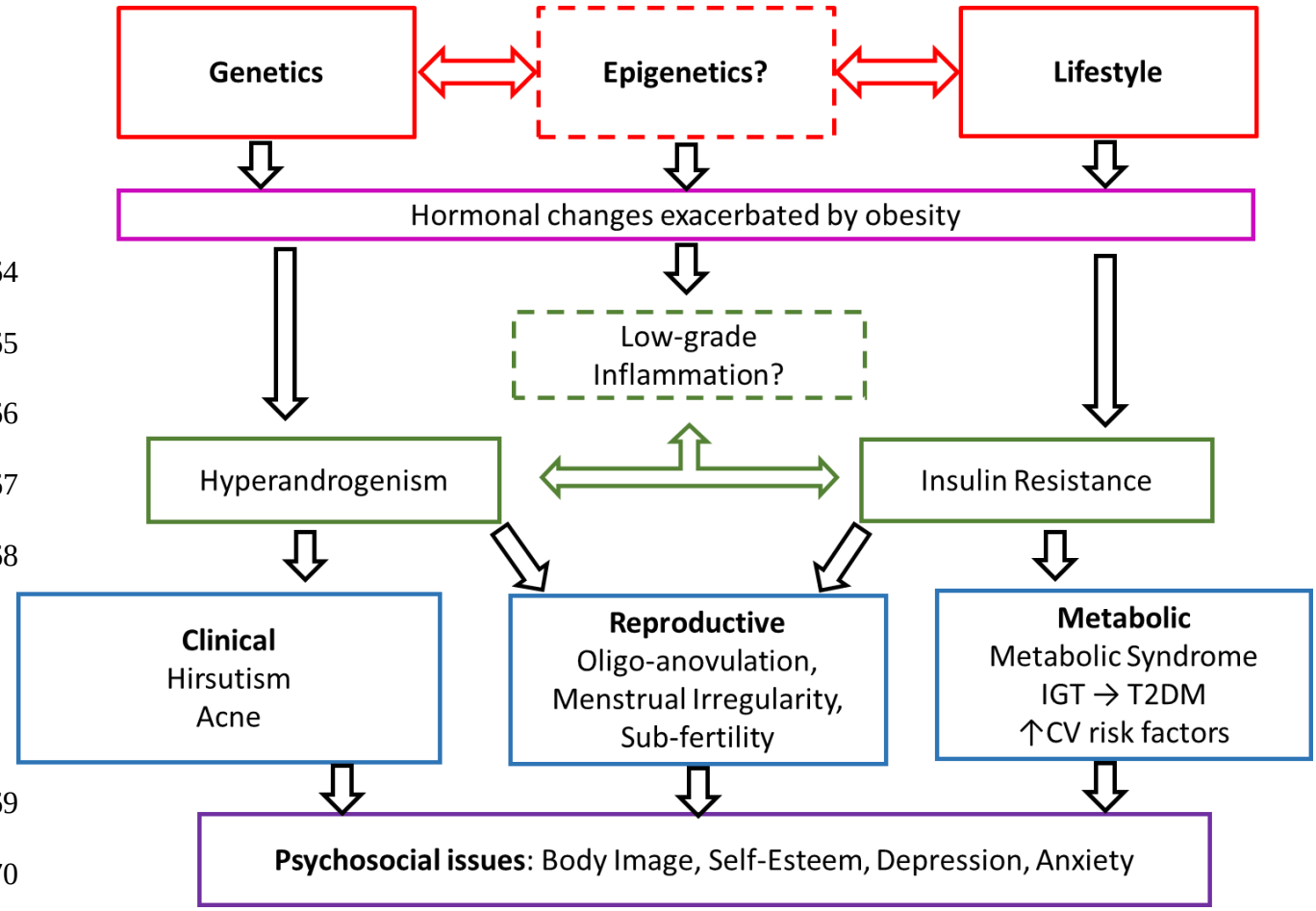

Figure 3.1: Proposed pathophysiology and features of Polycystic Ovary Syndrome. Adapted and reproduced with permission (Teede, Misso et al. 2011). CV, cardiovascular; IGT, impaired glucose tolerance; TD2M, type 2 diabetes.

Familial clustering of PCOS symptoms is well documented, providing evidence for a genetic contribution to the condition (Legro, Driscoll et al. 1998). Monozygotic twin studies have demonstrated the heritability of PCOS to be approximately 70% (Vink, Sadrzadeh et al. 2006). Genome Wide Association Studies (GWAS) conducted in Chinese and European cohorts have identified fifteen genome-wide significant PCOS risk loci, however these account for only a small portion of the heritability (Chen, Zhao et al. 2011, Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015). Investigations to identify the single nucleotide polymorphisms (SNPs) that may be involved in the genetic basis of PCOS have resulted in

783 mixed outcomes (Day, Hinds et al. 2015). Therefore, systematic reviews and meta-analyses are
784 important to elucidate the overall impact of SNPs on predisposition to PCOS.
785
786 High quality systematic reviews (SRs) of genetic association studies establish the consistency
787 and the significance of the individual gene-disease associations (Sagoo, Little et al. 2009).
788 They can help overcome many common limitations of a single genetic association study such
789 as sample size and selection bias from confounding variables, such as ethnicity, diagnostic
790 criteria, BMI, and source of participants, which can result in disparate results (Lewis 2002,
791 Sagoo, Little et al. 2009). While systematic reviews are vital to help synthesise the myriad of
792 articles published each year, they must be conducted according to best practice and be reliable
793 bodies of evidence to progress this field of study. Individual systematic reviews often focus on
794 single gene associations and therefore fail to provide a broader picture of genetics in PCOS.
795 This creates a need to synthesise and summarise existing systematic reviews to appropriately
796 inform clinicians, researchers, and consumers and to guide further research. An overview of
797 systematic reviews aims to assess the methodological quality of systematic reviews on a given
798 topic and the consistency of evidence contained in them (Silva, Grande et al. 2012). The aim
799 of this overview of systematic reviews was to synthesise the current evidence and the quality
800 of evidence for the relationship between genetic variation and PCOS.
801

802 ## 3.4 Methods

803 Inclusion criteria

804 The Participant, Intervention, Comparison, Outcomes, and Studies (PICOs) framework was
805 used for this overview of systematic reviews (Supplementary table 3.5). The population was
806 any systematic review with or without a meta-analysis where the genetics of PCOS was the

primary focus, either as interventions in PCOS or a comparison of women with and without PCOS for a specific outcome. Systematic reviews not on the genetics of PCOS (e.g. focusing on assessment or treatment) were excluded and are the focus of separate overview of systematic reviews. The intervention was any systematic reviews with or without a meta-analysis. The specific inclusion criteria for systematic reviews were a publication date from 2009 onwards, description of a search strategy containing at least key words or terms, inclusion of the number of identified and included articles, and quality appraisal of the articles. The comparison term was not applicable in this context. The outcomes included the methodology, results, and quality of each systematic review. The study type was systematic reviews with or without meta-analyses. Only articles published in English were included. The protocol was registered in the international prospective register of systematic reviews PROSPERO (CRD42016052649).

Article selection

A comprehensive database search was conducted on the 17th of October 2016. The following electronic databases were used to identify relevant systematic reviews: Medline in-process and other non-indexed citations (Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) 1946 to Present), EMBASE (EBM Reviews - Cochrane Database of Systematic Reviews 2005 to October 12, 2016, EBM Reviews - ACP Journal Club 1991 to September 2016, EBM Reviews - Database of Abstracts of Reviews of Effects 1st Quarter 2015, EBM Reviews - Cochrane Central Register of Controlled Trials September 2016, EBM Reviews - Cochrane Methodology Register 3rd Quarter 2012, EBM Reviews - Health Technology Assessment 3rd Quarter 2016, EBM Reviews - NHS Economic Evaluation Database 1st Quarter 2015), and CINAHL PLUS. The search strategy for MEDLINE is documented in Supplemental table 3.6. This search was modified for EMBASE and CINAHL using their subject headings instead of the MeSH subject headings.

The international prospective register of systematic reviews PROSPERO (http://www.crd.york.ac.uk/PROSPERO/) was additionally searched on the 1st December 2016 using key words "PCOS" or "polycystic ovary syndrome". Two independent reviewers (L.J.M. and D.H), who were not blinded to the names of investigators or sources of publication, identified, and selected the systematic reviews that met the inclusion criteria. At all stages of eligibility assessment of articles, disagreements between the two reviewers were discussed and resolved by consensus, or arbitration (M.G.H).

Data extraction

All eligible systematic reviews were examined and extracted independently by two reviewers (D.H and A.M.A). The data extracted included information on authors, country, publication date, inclusion criteria, systematic review methodology, systematic review outcomes, number of studies identified, number of participants in the systematic review, whether a meta-analysis was conducted, and quality of identified articles in each systematic review. When several versions of Cochrane reviews were identified, only the most recent was included. Methodological variables specific to genetic association studies were extracted: source of participants, whether the control group were in Hardy-Weinberg Equilibrium (HWE), and the method by which the control group was dealt with in the systematic review if it departed from HWE. The Hardy-Weinberg principle states that if control groups are healthy and therefore "disease-free" they should be in equilibrium and a population in which genetic variation remains constant (Hardy 1908). Departures from HWE can indicate a number of methodological issues including study design or genotyping errors. There is no consensus on which method is most appropriate to deal with deviations from HWE, but common procedures are excluding any studies that have a significant deviation from HWE before conducting meta-analysis, conducting sensitivity analysis to examine whether meta-analysis results are altered

when studies containing control groups not in HWE are excluded, or correcting the pooled odds ratio (Zintzaras and Lau 2008). For each systematic review the diagnostic criteria for PCOS and the criteria for defining the control group were extracted.

Quality assessment of systematic reviews

All included systematic reviews were evaluated by two independent reviewers (D.H and A.M.A) using the Assessing the Methodological Quality of Systematic Reviews (AMSTAR) tool (Shea, Grimshaw et al. 2007), which contains 11 items to appraise the methodological aspects of systematic reviews. This includes *a priori* design, duplicate study selection and data extraction, comprehensive literature search, status of publication used as an inclusion criterion, included and excluded studies listed, characteristics of included studies described, assessment of scientific quality of the included studies, use of scientific quality of studies in formulating conclusions, appropriate methods for combining study findings, assessment of publication bias, and documenting conflicts of interest. Each item was scored as yes=1 or no/unclear=0, with a total score ranging from 0 to 11. AMSTAR does not provide guidelines on how to integrate the score into an overall judgement of the methodological quality, so to determine the risk of bias, and therefore methodological quality, an overall summarised risk of bias was calculated as described previously (EMSurg Collaborators 2017). This was based on four items from the AMSTAR tool: a comprehensive literature search (minimum of two electronic databases searched), assessment of the scientific quality of the included studies, appropriate use of quality assessments in formulating review conclusions, and appropriate use of methods to combine findings. Systematic reviews with low risk of bias must have addressed all four key criteria. At all stages of data extraction and quality assessments disagreements between the two reviewers (D.H and A.M.A) were discussed and resolved by consensus, or arbitration (M.G.H).

3.4.1 Results

Eligibility assessment

The search yielded 856 citations and 733 citations remained after removal of duplicates. A further 32 citations were identified from PROSPERO. Based on *a priori* selection criteria, screening for title or abstract identified 228 studies for assessment of full text. Of these, 117 articles were excluded due to not conducting quality assessment, not being in English or no search terms, or search strategy identified. Detailed characteristics of excluded studies have been reported in Supplementary table 3.7. Of the remaining 111 full-text systematic reviews, 16 were related to genetics and included in this overview of systematic reviews (Figure 3.2).

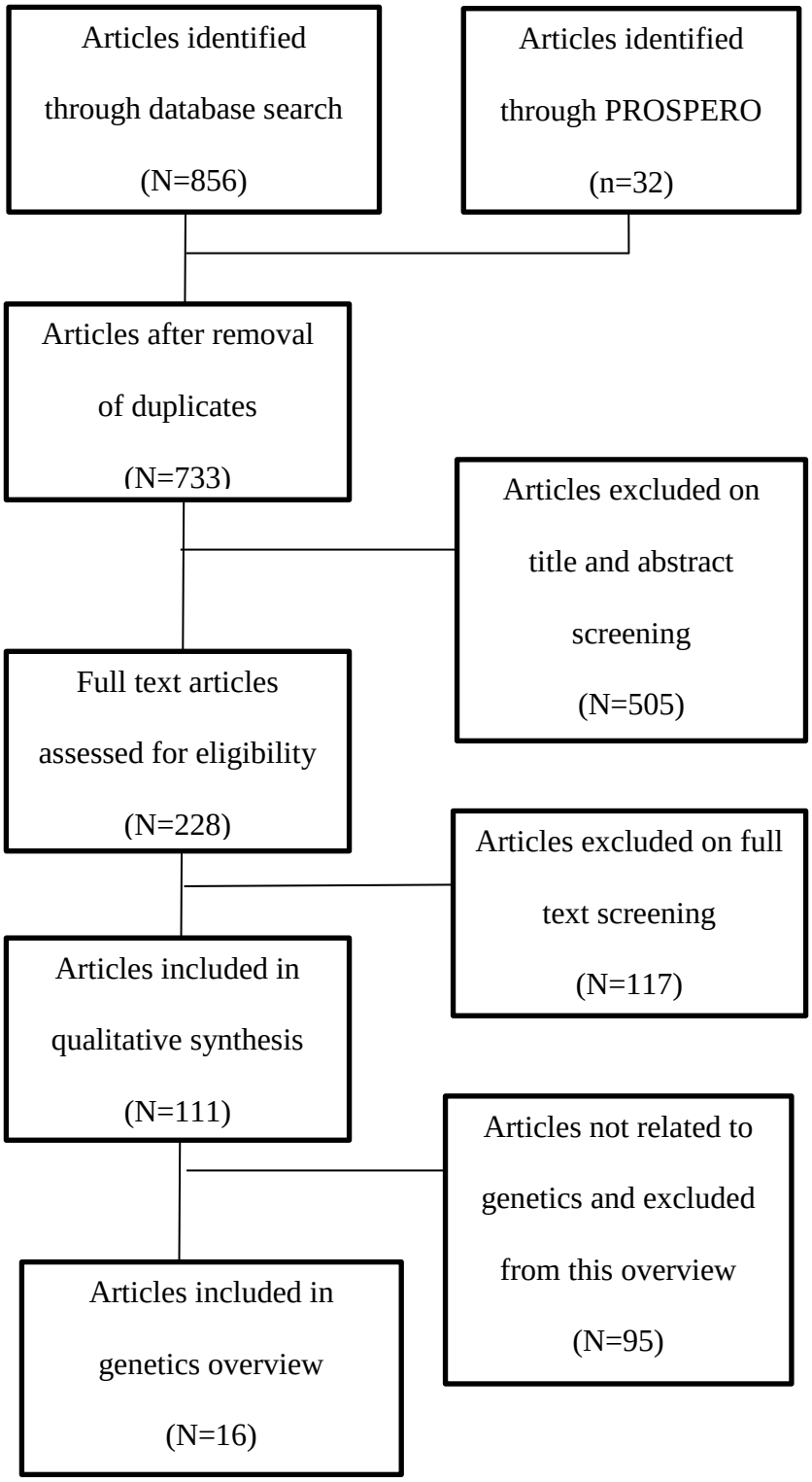

Figure 3.2: Identification and selection of systematic reviews of genetics and polycystic ovary syndrome

Table 3.1: Characteristics of included systematic reviews assessing the genetics of PCOS.

Author	Publication date	Countries the primary studies were conducted in	Languages included in systematic review	Sample size of SR (n= participants)	Types of primary studies included	Meta-analysis performed?	systematic review methodology	QA methodology and authors judgement of quality (if available) of primary studies
(Feng, Lv et al. 2015)	2015	UK, USA, China, Korea, India, Turkey, Romania, Brazil, Iran, Japan, Croatia and Netherlands	No restriction	PCOS 17 460; Controls 23 845	Case-control or cohort	Yes	Yes- PRISMA	NOS 6.75/9 (did not specify if considered high or low quality)
(Guo, Zheng et al. 2015)	2015	UK, USA, China, Korea, India, Turkey, Romania, Brazil, Iran, Japan, Croatia and Netherlands	No restriction	PCOS 1 665; Controls 1 687	Case-control	Yes	Yes- PRISMA	NOS High quality
(Jia, Yu et al. 2012)	2012	China, Korea, Japan, Iran, Spain, Greece, Finland, Turkey, Germany	English	T45G SNP PCOS 1 104; Controls 1 717 G276T PCOS 823; Controls 982	Case-control	Yes	No	Modified scoring system Low quality

(Li, Liu et al. 2012)	2012	Poland, America, China, Korea, Chile, Turkey, India	No restriction	PCOS 1321; Control 1017	Case-control	Yes	No	QA methodology not specified. Quality of primary studies was satisfactory[a]
(Qiu, Liu et al. 2015)	2015	Japan, China, UK, Turkey, Netherlands, Italy, Korea	English and Chinese	PCOS 2670; Control 7752	Case-control	Yes	No	CASP & STREGA Medium to high quality
(Ramos, Fabris et al. 2015)	2015	UK, Ireland, Greece, Korea, China, Czech Republic, Brazil, Tunisia	No restrictions	PCOS 1892; Control 2695	Case-control and cross-sectional	Yes	Yes- PRISMA	NOS Range 5 to 7
(Ruan, Ma et al. 2012)	2012	France, Chile, USA, Turkey, Spain, Germany, Taiwan, Japan, Greece, Slovak, Italy	English	PCOS 1877; Control 2 444	Case-control and cross-sectional	Yes	No	QA methodology not specified [a]
(Shen, Li et al. 2013)	2013	Spain, UK, Germany, China, Chile, Korea, Turkey, India	No Restriction	PCOS 2123; Control 3612	Case-control	Yes	No	NOS Good quality
(Shen, Li et al. 2013)	2013	India, Turkey, China	No Restriction	PCOS 521; Control 515	Case-control	Yes	No	STROBE Moderate to high quality

(Shen, Li et al. 2014)	2014	India, Turkey, China, Greece, Spain, USA, UK	No Restriction	PCOS 1571; Control 1918	Case-control	Yes	No	NOS Poor to good Quality
(Shen, Li et al. 2014)	2014	Brazil, Tunisia, Czech Republic, Korea, China, Greece, UK	No Restriction	PCOS 2458; Control 5109	Clinical cohort and case-control	Yes	No	NOS Poor to good quality
(Shi, Xie et al. 2016)	2016	UK, China, South Korea, Turkey, India, Iran, Japan, Croatia, Iraq, France, Chile, Germany, USA, Spain, Taiwan Italy, Greece, Slovakia	English or Chinese	PCOS 2975; Control 3011	Case-control	Yes	No	NOS Low to high Quality
(Wang, Tong et al. 2015)	2015	Turkey, India, Austria	English or Chinese	PCOS 351; Control 464	Case-control	Yes	No	10 point scoring system Fair quality
(Wu, Yu et al. 2015)	2015	Australia, China, Turkey, India, Finland, Austria, Korea	No restriction	TNFα 308G > A: 582 PCOS, 563 control TNFα -805C > T: 87 PCOS, 115 control; TNFα -1031 T > C:	Case-control	Yes	Yes- PRISMA	NOS Low to high quality

				500 PCOS, 450 control; IL-1A -889C > T 310 PCOS, 279 controls IL-1B -511C > T 482 PCOS, 421 control IL-1B +3953C > T 223 PCOS, 188 control IL-6 -174G > C 416 PCOS, 569 control IL-10 -819C > T 91 PCOS, 75 control IL-10 -1082A > G 188 PCOS, 170 control IL-18 -607C > A 118 PCOS, 79 control; IL-18 -137G > C 244 PCOS, 192 controls				

(Yan, Liang et al. 2014)	2014	UK, Czech republic, Finland, Estonia, Slovenia, China, Korea, Croatia	English and Chinese	PCOS 1075; Control 2878	Case-control	Yes	Yes- PRISMA	NOS Low to high Quality
(Zhang, Liang et al. 2013)	2013	Singapore, Australia, Finland, USA, Korea, Slovenia, China, Estonia, Croatia, Germany	No restriction	PCOS 1144; Control 1409	Not stated	Yes	Yes- MOOSE	QA methodology not specified [a]

CASP, Critical Appraisal Skills Programme; IL, Interleukin; MOOSE, Meta-analysis Of Observational Studies in Epidemiology; NOS, Newcastle Ottawa Scale; PCOS: Polycystic ovary syndrome; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; QA, Quality Appraisal; SNP, Single Nucleotide Polymorphisms; STREGA, Strengthening The Reporting of Genetic Association studies; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; TNF-α, Tumor Necrosis Factor-Alpha.

[a] Li et al. (2012), Ruan et al. (2012), and Zhang et al. (2013) completed quality appraisal and therefore fulfilled the PICO inclusion criteria however did not specify by which method they assessed quality or indicate the quality of the primary studies.

9 **Table 3.2: Outcomes, main findings, sub-analyses and conclusions reported by the included systematic reviews.**

Author	Outcome assessed	Main Findings	Sub-analysis	Conclusion
Metabolic Dysfunction				
(Feng, Lv et al. 2015)	Association between polymorphisms in INSR gene and PCOS.	Three SNPs in the INSR gene investigated – rs1799817 [His1057 C/T] no significant association – rs2059806- no significant association – rs2059807- 3/4 primary studies found significant associations however pooled OR was not calculated.	– rs1799817- ethnicity, BMI and diagnostic criteria - no significant associations.	No significant association between SNPs (rs1799817 or rs2059806) and PCOS even when BMI and ethnicity were taken into account. Further investigation into the SNP rs2029807 is warranted. Further sub-group analysis according to BMI or IR status could help with systematic review power.
(Jia, Yu et al. 2012)	Association between polymorphisms in Adiponectin gene polymorphisms (T45G & G276T) and PCOS.	– G276T- T allele was negatively associated with PCOS in the allelic genetic model. – T45G- no significant association.	– G276T-ethnicity– significant negative association in East Asian populations. – T45G- ethnicity - no significant associations	There was a significant negative association of G276T polymorphism in the adiponectin gene and PCOS in the East Asian ethnicity but not Caucasians. Adiponectin might play a role in the aetiology of PCOS. Further investigation of the effect of this SNP, as well as gene-gene and gene-environment interactions is required.
(Ramos, Fabris et al. 2015)	Association between polymorphisms in the TCF7L2 gene and PCOS.	– No significant associations between TCF7L2 SNPs [C/G] or	– Ethnicity – no significant associations	No significant association in polymorphisms rs7903146 or

		[G/T] irrespective of the genetic models used.		rs23366372 in the TCF7L2 gene and PCOS.
(Ruan, Ma et al. 2012)	Association between polymorphisms in the IRS-1 gene and IRS-2 gene and PCOS.	– IRS-1 [Gly972Arg] Arg allele is positively associated with PCOS in the dominant, heterozygote and the allele contrast model. – No significant associations in the IRS2 [Gly1057Asp].		There was a significant positive association between the A allele of the IRS-1 Gly972Arg polymorphism and PCOS. However, no significant association was detected between IRS-2 Gly1057Asp polymorphism and PCOS. Further investigation of protein levels of the gene, the effect of gene-gene and gene-environment interactions is required.
(Shen, Li et al. 2013)	Association between polymorphisms in the Calpain-10 gene and PCOS.	– UCSNP-19 [del/ins] significant positive association with del variant and PCOS – UCSNP-44 [T/C] significant positive association with C variant and PCOS – UCSNP-63 [C/T] significant positive association with C variant and PCOS – No significant associations in USCN-22, USCN- 43, USCN-45, USCN-56, USCN-58 or USCN- 110.	– Ethnicity: (UCSNP-19, UCSNP-63) significant positive associations in Asian populations. – Population-based recruitment: (UCSNP-19, UCSNP-44, UCSNP-63) significant positive association. – SNP genotype method: (UCSNP-19, UCSNP-44, UCSNP-63) - significant positive association.	Three SNPs UCSNP-19, UCSNP-63 and UCSNP-44 in the Calpain-10 gene may be positively associated with PCOS, especially among Asian populations and may be useful biomarkers for predicting susceptibility to PCOS. Further investigation of the effect of gene-environment interactions is required.
(Shen, Li et al. 2013)	The associations between	– CYP1A1 [T/C] C Variant is positively associated with PCOS.	– Country: significant positive associations in the Turkish and Indian subgroups.	The CYP1A1 (T/C) polymorphism may be positively associated with

	polymorphisms in the CYP1A1 gene and PCOS.	– CYP1A1 [A/G]- No significant associations	– Significant positive association in population based recruitment of control groups and SNP genotyping method (PCR-RFLP) with PCOS.	PCOS, especially among Turkish and Indian populations. Further investigation of the CYP1A1 [A/G] SNP is required and the effect of gene-gene interactions is required.
(Shen, Li et al. 2014)	The associations between polymorphism in the of CYP1A1 gene and microsatellite repeat polymorphisms [TTTA]n in the CYP11A1 gene and PCOS.	– CYP1A1 [T/C]- C variant is positively associated with PCOS. – CYP1A1 [A/G]- No significant associations. – CYP11A1[TTTA]n- polymorphism significant positive association with PCOS.	CYP1A1 – Significant positive association in Caucasian populations, population based recruitment of control groups and SNP genotyping method (PCR-RFLP). – CYP11A1 [TTTA]n Significant positive association in Caucasian populations, population based recruitment of control groups and SNP genotyping method (PCR-RFLP).	Polymorphisms in the CYP1A1 [T/C] and CYP11A1 microsatellite [TTTA]n repeat polymorphisms may contribute to increasing susceptibility to PCOS among Caucasian populations. These polymorphisms could serve as potential biomarkers for diagnosis of PCOS however further investigation is required.
(Shen, Li et al. 2014)	Association between polymorphisms in the TCF7L2 gene and PCOS	– TCF7L2 polymorphism (C/T) C variant is positively associated with PCOS – TCF7L2 polymorphism (G/T) No significant association with PCOS.	– Ethnicity- significant positive association in Caucasian and Asian populations. – SNP genotyping method and sample size- significant positive association	TCF7L2 polymorphisms may contribute to increased susceptibility to PCOS, especially for the C/T polymorphism among Caucasians and Asians.

				This polymorphism could serve as potential biomarkers for diagnosis of PCOS however further investigation is required.
(Shi, Xie et al. 2016)	Association between polymorphisms in the INSR, IRS-1and IRS-2 genes and PCOS.	– INSR His1058 C/T - No significant associations with PCOS. – IRS-1 [Gly972Arg]- G allele is negatively associated with PCOS. – IRS-2 Gly1057Asp- G allele is negatively associated with PCOS.	– INSR His1058 C/T- Ethnicity no significant associations – IRS-1 Gly972Arg- Ethnicity- G allele is negatively associated with PCOS in Caucasian populations. – IRS-2 Gly1057Asp- Ethnicity- G allele is negatively associated with PCOS in Asian populations.	IRS-1 Gly972Arg is associated with PCOS in Caucasian population. IRS-2 Gly1057Arg is associated with PCOS in Asian population. INSR His1058 C/T is not implicated in PCOS. Further investigation with larger samples sizes is required.
(Yan, Liang et al. 2014)	Association between Insulin gene variable number of tandem repeats (INS INVTR) and PCOS	– INS VNTR (III/I) III allele is positively associated with PCOS.	– Population based recruitment of controls- significant positive association. – Hospital based recruitment of controls- no significant association.	There was evidence of significant positive association between the III allele in INS VNTR and PCOS.
Imbalances in androgens and gonadotrophins				
(Li, Liu et al. 2012)	Association between polymorphisms in the CYP17 and PCOS	– No significant association.	– Ethnicity- No significant association. – Limiting analysis to the primary studies whose control group is in HWE resulted in a significant positive association with PCOS.	CYP17 T/C polymorphism may be not associated with PCOS risk. The significant differences that were observed may be due to small-study bias.

				Further investigation of the effect of gene-gene and gene-environment interactions may provide further information between understanding the association between CYP17 T/C SNP and risk of PCOS.
(Qiu, Liu et al. 2015)	Association between polymorphisms in the FSHR gene and PCOS.	– Thr307Ala- No significant association. – Asn680Ser- Asn allele is negatively associated with PCOS.	Asn680Ser- Ethnicity- significant negative association with PCOS in the Caucasian population.	Overall, there was no evidence for significant association of Thr307Ala polymorphism with PCOS susceptibility. There might be a significant association between Asn680Ser polymorphism and reduced susceptibility to PCOS. Further investigation with larger sample sizes and of the effect of gene-gene and gene-environment interactions is required.
(Zhang, Liang et al. 2013)	Association between the CAG length in the Androgen Receptor (AR) gene and PCOS risk and Testosterone (T) levels.	– CAG length and PCOS- No significant association in the biallelic mean length between women with and without PCOS. No significant associations with PCOS of individuals with the biallelic mean less than median, with the short CAG allele, or with the X-weighted biallelic mean.	– CAG length and Testosterone levels- No significant association between CAG repeat length and T levels in PCOS populations. The biallelic mean was not significantly different between PCOS populations with high T and those with low T.	There was no association between the CAG length variations in AR gene and PCOS risk. The CAG length may be positively associated with T levels in PCOS. Further investigation with larger sample sizes and the effect of gene-environment interactions is required.

			– The summary correlation r of 3/11 primary studies indicated that the CAG biallelic mean was positively associated with T levels in PCOS.	
Inflammation				
(Guo, Zheng et al. 2015)	Association between polymorphisms in TNF-alpha, IL-6 and IL-beta genes and PCOS.	– TNF-alpha (-308 G/A) - no significant association. – IL-beta (-511 C/T) - No significant association. – IL-6 (-174 G/C) - C allele negatively associated with PCOS in the allelic and homozygote comparison with PCOS.	– TNF-alpha (-308 G/A) HWE in controls, BMI, sample size, SNP analysis method, diagnostic criteria and ethnicity - no significant association. – IL-beta (-511 C/T) - HWE in controls, sample size, SNP analysis method, and ethnicity- no significant association. – IL-6 (-174 G/C) – Sample size (>200), SNP genotyping (PCR-RFLP)- significant negative association. HWE in controls, sample size ≤ 200 and genotyping method (pyrosequencing) - no significant association.	Polymorphisms in TNF-alpha (−308 G/A), IL-6 (−174 G/C), and IL-1beta (−511 C/T) genes might not be genetic risk factors for PCOS. Further investigation with larger sample sizes and of the effect of gene-gene and gene-environment interactions is required.
(Wang, Tong et al. 2015)	Association between polymorphisms in the IL-6 gene and PCOS.	– IL-6 (-174 G/C)- C allele was negatively associated with PCOS under the allele model only. No further significant results found.	– HWE in control group- no significant association.	IL-6 –174 G/C polymorphism may be not related to susceptibility of PCOS.

				Further investigation with larger sample sizes and of the effect of gene-gene and gene-environment interactions is required.
(Wu, Yu et al. 2015)	Associations between polymorphisms in the TNF-α and IL genes and PCOS.	– TNFα (-308G/A) & (-805C/T) no significant association. – TNFα (-1031 T/C) C allele was positively associated with PCOS. – IL-6 (-174G/C) C allele was negatively associated with PCOS. – No associations were found in IL-1A -889C > T, IL-1B -511C > T, IL-1B +3953 T > C, IL-10 -819C > T, IL-10 -1082A > G, IL-18 -607C > A, and IL-18 -137G > C.		Significant associations between the TNF-α -1031 T > C and IL-6 -174G > C polymorphisms and PCOS. No associations are found between PCOS risk and the TNF-α -308G > A, TNF-α -805C > T, IL-1A -889C > T, IL-1B -511C > T, IL-1B +3953C > T, IL-10 -819C > T, IL-10 -1082 A > G, IL-18 -607C > A, and IL-18 -137G > C polymorphisms. Further investigation with larger samples sizes, in multiple ethnicities, using homogenous populations of cases and well-matched controls are required.

AR, Androgen Receptor; BMI, Body Mass Index; CYP1A1, Cytochrome P450 Family 1 Subfamily A Member 1; CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; CYP17, Cytochrome P450 Family 17 Subfamily A Member 1; FSHR, Follicle Stimulating Hormone Receptor; HWE, Hardy-Weinberg Equilibrium; IL, Interleukin; INSR, Insulin Receptor; INS INVTR, Insulin gene variable number of tandem repeats; IRS-1, Insulin Receptor Substrate-1; IRS-2, Insulin Receptor Substrate-2; OR, Odds Ratio; PCOS, Polycystic Ovary Syndrome; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; SNPs, Single Nucleotide Polymorphisms; TCF7L2, Transcription Factor 7-Like 2; T, Testosterone; TNF-α, Tumor Necrosis Factor-Alpha.

17 **Table 3.3: Systematic review methodology characteristics specifically relevant to PCOS.**

Author	What PCOS diagnostic criteria did the systematic review accept?	Did the systematic review note the source of participants for their included studies?	Did the systematic review have clearly defined control inclusion criteria?	Were controls not in HWE included in meta-analysis? Was sensitivity analysis performed?
Metabolic dysfunction				
(Feng, Lv et al. 2015)	– Primary studies where diagnostic criteria was not defined – Rotterdam – NIH – JSOG	Not described	Not described	Yes HWE was tested. No Sensitivity analysis was performed
(Jia, Yu et al. 2012)	– Rotterdam – NIH	Not described	Healthy women without PCOS	Yes HWE was tested. Sensitivity analysis was conducted.
(Ramos, Fabris et al. 2015)	– Not described however all primary studies fulfilled Rotterdam criteria	Not described	Healthy women	No primary studies were excluded if the control group deviated from HWE.
(Ruan, Ma et al. 2012)	– Not described	Not described	Unrelated healthy women	Yes HWE was tested. Sensitivity analysis was conducted.
(Shen, Li et al. 2013)	– Rotterdam – NIH	Source of controls: Hospital-based and population-based.	Healthy women with normal menstrual cycles, ovarian morphology, and without history of subfertility treatment	No primary studies were excluded if the control group deviated from HWE.

(Shen, Li et al. 2013)	– Not described	Matched recruitment of cases and controls (Hospital-based and population-based).	Healthy women	No primary studies were excluded if the control group deviated from HWE.
(Shen, Li et al. 2014)	– Rotterdam – NIH	Source of controls: Hospital-based and population-based.	Healthy women with normal menstrual cycles, ovarian morphology, and without history of subfertility treatment	No primary studies were excluded if the control group deviated from HWE.
(Shen, Li et al. 2014)	– Rotterdam – NIH	Not described	Not described	No primary studies were excluded if the control group deviated from HWE.
(Shi, Xie et al. 2016)	– Not described	Not described	No criteria however stated they included women who were infertile or had other gynaecological conditions in their control group.	Yes HWE was tested. Sensitivity analysis was conducted.
(Yan, Liang et al. 2014)	– Not described	Source of controls- Hospital and population based.	Not described	Yes HWE was tested. Sensitivity analysis was conducted.
Imbalances in Androgens and Gonadotrophins				
(Li, Liu et al. 2012)	– Not described	Not described	Not described	Yes HWE was tested. Sensitivity analysis was conducted.
(Qiu, Liu et al. 2015)	– Rotterdam – Only specific phenotypes accepted [Hyperandrogenism + irregular cycles + oligo or anovulation;	Not described	Age and ethnicity matched healthy women	All control groups were in HWE.

	Hyperandrogenism + Polycystic Ovaries + irregular cycles; Polycystic ovaries + oligo or anovulation + irregular cycles]			
(Zhang, Liang et al. 2013)	– Rotterdam – NIH – Androgen Excess & PCOS Society	Source of controls- Hospital and population based.	Healthy women with proven fertility	Not tested
Inflammation				
(Guo, Zheng et al. 2015)	– Rotterdam – NIH	Not described	Healthy women	Yes HWE was tested. Sensitivity analysis was conducted.
(Wang, Tong et al. 2015)	– Not described	Not described	Alive and free from diagnosed PCOS women	Yes HWE was tested. Sensitivity analysis was conducted.
(Wu, Yu et al. 2015)	– Rotterdam – NIH	Not described	Healthy women	Yes HWE was tested. Sensitivity analysis was conducted.

18 HWE, Hardy-Weinberg Equilibrium; JSOG, Japanese Society of Obstetrics and Gynaecology; NIH, National Institute of Health; PCOS, Polycystic
19 Ovary Syndrome; SR, Systematic Review.
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22 **Table 3.4: AMSTAR appraisal of included systematic reviews (EMSurg Collaborators 2017).**

Author	A *Priori* design	Duplicate study selection and data extraction	Comprehensive literature search*	Status of publication used as an inclusion criterion	List of studies (included and excluded) provided	Characteristics of the included studies described	Scientific quality assessed*	Conclusions based on quality of studies*	Appropriate methods used to combine the findings*	Publication bias assessed	Conflict of interest stated	No. of AMSTAR criteria met by this SR (of 11)[#]	Summarised risk of bias of this SR*
Metabolic dysfunction													
(Feng, Lv et al. 2015)	Yes	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	8	Low risk of bias
(Jia, Yu et al. 2012)	No	Yes	No	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	6	High risk of bias
(Ramos, Fabris et al. 2015)	Yes	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	8	Low risk of bias
(Ruan, Ma et al. 2012)	No	Yes	Yes	Unclear	No	Yes	No	No	Yes	Yes	No	5	High risk of bias
(Shen, Li et al. 2013)	No	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
(Shen, Li et al. 2013)	No	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
(Shen, Li et al. 2014)	No	No	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
(Shen, Li et al. 2014)	No	No	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	6	Low risk of bias
(Shi, Xie et al. 2016)	No	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias

(Yan, Liang et al. 2014)	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Unclear	8	Low risk of bias
Median [Interquartile Range] AMSTAR score													7 [6, 8]
Imbalances in androgens and gonadotrophins													
(Li, Liu et al. 2012)	No	Yes	Yes	Unclear	No	Yes	Unclear	No	Yes	Yes	No	5	High risk of bias
(Qiu, Liu et al. 2015)	No	No	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
(Zhang, Liang et al. 2013)	No	Yes	No	Unclear	Yes	Yes	Unclear	No	Yes	Yes	No	5	High risk of bias
Median [Interquartile Range] AMSTAR score													5 [5]
Inflammation													
(Guo, Zheng et al. 2015)	No	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
(Wang, Tong et al. 2015)	No	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	No	Unclear	6	Low risk of bias
(Wu, Yu et al. 2015)	No	Yes	Yes	Unclear	No	Yes	Yes	Yes	Yes	Yes	No	7	Low risk of bias
Median [Interquartile Range] AMSTAR score													7 [7]
OVERALL MEDIAN [Interquartile Range] AMSTAR SCORE													7 [6,7]

23 AMSTAR, Assessing the Methodological Quality of Systematic Reviews; SR, Systematic Review.

3.4.2 General characteristics of included systematic reviews

All sixteen systematic reviews included a meta-analysis and were based mainly on case-control or cross-sectional primary studies (Table 3.1). Ten systematic reviews did not restrict their search by language, four only included Chinese or English articles, and two systematic reviews only included articles in English. The majority of the systematic reviews (9/16) used the Newcastle Ottawa Scale (NOS) as their quality appraisal tool. Only six systematic reviews (37%) reported using a systematic review and meta-analysis guideline.

3.4.3 Outcomes investigated and conclusions made by the included systematic reviews

The genes investigated by each systematic review and the detailed findings are described in Table 3.2. Fourteen systematic reviews investigated SNPs (Jia, Yu et al. 2012, Li, Liu et al. 2012, Ruan, Ma et al. 2012, Shen, Li et al. 2013, Shen, Li et al. 2013, Shen, Li et al. 2014, Shen, Li et al. 2014, Feng, Lv et al. 2015, Guo, Zheng et al. 2015, Qiu, Liu et al. 2015, Ramos, Fabris et al. 2015, Wang, Tong et al. 2015, Wu, Yu et al. 2015, Shi, Xie et al. 2016). The remaining two systematic reviews investigated CAG length or tandem repeats respectively (Zhang, Liang et al. 2013, Yan, Liang et al. 2014). Candidate genes focused on three main aspects of PCOS pathophysiology: metabolic dysfunction,(Jia, Yu et al. 2012, Ruan, Ma et al. 2012, Shen, Li et al. 2013, Shen, Li et al. 2013, Shen, Li et al. 2014, Shen, Li et al. 2014, Yan, Liang et al. 2014, Feng, Lv et al. 2015, Ramos, Fabris et al. 2015, Shi, Xie et al. 2016) imbalances in androgen and gonadotrophins, (Li, Liu et al. 2012, Zhang, Liang et al. 2013, Qiu, Liu et al. 2015) and inflammation (Guo, Zheng et al. 2015, Wang, Tong et al. 2015, Wu, Yu et al. 2015).

The genes involved in metabolic function that were investigated were insulin receptor (INSR), adiponectin, transcription factor 7-like 2 (TCF7L2), insulin receptor substrate 1 (IRS-1),

insulin receptor substrate 2 (IRS-2), calpain-10, cytochrome P450, family 1, subfamily A, member 1 (CY1A1), cytochrome P450, family 11, subfamily A, member 1 (CYP11A1), and insulin gene (Table 3.2). Some polymorphisms in the IRS-1, calpain-10, insulin gene variable number of tandem repeats (INS VNTR), CYP1A1, and CYP11A1 genes were significantly positively associated with PCOS; however, associations were not consistent across all ethnicities, genotyping methods, or sources of recruitment. Only one adiponectin SNP was significantly negatively associated with PCOS and this was only in East Asian ethnicity but not in Caucasian ethnicity. Contrasting results were found between the two systematic reviews that investigated the IRS-2 and TCF7L2 genes. The SNPs within the INSR gene were not associated with PCOS. Overall due to heterogeneity, the data lacks clear association between metabolic genes and PCOS.

Three systematic reviews focused on androgens and gonadotrophins (Table 3.2) and investigated the cytochrome P450 17A1 (CYP17), follicle stimulating hormone receptor (FSHR), and the androgen receptor gene. CAG repeat length polymorphism in the androgen receptor gene was positively associated with serum testosterone but not with PCOS per se. There were no clear association between FSHR and PCOS, and SNPs in the CYP17 gene were not associated with PCOS. No clear associations emerged between hormonal genes and PCOS in this overview of systematic reviews.

Three systematic reviews focused on inflammation and investigated cytokine genes: Tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-β, IL-10, IL-18 (Table 3.2). Of the three SNPs within the TNF-α gene investigated, only one (TNF-α -1031 T/C) was positively associated with PCOS. All three systematic reviews concurred that the SNP (-174 G/C) in the IL-6 gene was not associated with PCOS. IL-β, IL-10, and IL-18 were not associated with

PCOS. Again, these findings provide no clear evidence of associations between inflammation genes and PCOS.

3.4.4 Methodological considerations specific to PCOS

Seven systematic reviews (44%) did not did not describe which PCOS diagnostic criteria they accepted in their inclusion criteria (Table 3.3) (Li, Liu et al. 2012, Ruan, Ma et al. 2012, Shen, Li et al. 2013, Yan, Liang et al. 2014, Ramos, Fabris et al. 2015, Wang, Tong et al. 2015, Shi, Xie et al. 2016). The remaining nine systematic reviews accepted diagnostic criteria consistent with the most inclusive Rotterdam diagnostic criteria, although they all included primary studies that selectively recruited specific phenotypes (such as the National Institute Health [NIH], or Androgen Excess PCOS [AE-PCOS] phenotypes) (Jia, Yu et al. 2012, Shen, Li et al. 2013, Zhang, Liang et al. 2013, Shen, Li et al. 2014, Shen, Li et al. 2014, Feng, Lv et al. 2015, Guo, Zheng et al. 2015, Qiu, Liu et al. 2015, Wu, Yu et al. 2015).

Only four systematic reviews (25%) described detailed meta-analysis inclusion criteria for the control groups: absence of irregular cycles, sub-fertility, polycystic ovarian morphology and signs of hyperandrogenism, or healthy with proven fertility Table 3.3. The remaining systematic reviews either did not describe any criteria for control group inclusion or described women in the control groups as healthy but without including any further detail.

3.4.5 Methodological considerations not specific to PCOS

Five systematic reviews excluded any individual studies where the control group did not conform to HWE (Bonasio, Tu et al. 2010, Shen, Li et al. 2013, Shen, Li et al. 2013, Shen, Li et al. 2014, Shen, Li et al. 2014, Ramos, Fabris et al. 2015). One systematic review did not test for HWE (Zhang, Liang et al. 2013). The remaining systematic reviews (8/17) all performed

sensitivity analysis of the individual studies whose control group did not conform to the HWE (Jia, Yu et al. 2012, Li, Liu et al. 2012, Ruan, Ma et al. 2012, Yan, Liang et al. 2014, Guo, Zheng et al. 2015, Qiu, Liu et al. 2015, Wang, Tong et al. 2015, Wu, Yu et al. 2015, Shi, Xie et al. 2016).

3.4.6 Assessment of systematic review quality using the AMSTAR tool

The median overall quality score was 7 [IQR 6, 7] but none of the systematic reviews met all 11 AMSTAR criteria Table 3.4 (Shea, Grimshaw et al. 2007). Almost all systematic reviews met the criteria of conducting a comprehensive literature search, assessing publication bias, assessing the quality of the individual studies, and made conclusions based on this. The majority of the systematic reviews failed to include *a priori* design, for example by referring to a published protocol. All systematic reviews also failed to adequately address the conflict of interest criterion, and all but one did not adequately list of the included and excluded primary studies. Of the sixteen systematic reviews 75% (12/16) were assessed as having low risk of bias (EMSurg Collaborators 2017): 8/10 systematic reviews relating to metabolic dysfunction, 1/3 relating to androgens and gonadotrophins, 3/3 relating to inflammation.

3.5 Discussion

This overview of systematic reviews summarises current systematic reviews about candidate gene polymorphisms that may be associated with PCOS. These fall into three broad categories: metabolic dysfunction, imbalances in androgens and gonadotrophins, and inflammation, with most systematic reviews relating to metabolic dysfunction.

3.5.1 Metabolic dysfunction

Metabolic dysfunction is involved in the aetiology of PCOS. Much research has been conducted in this area, supported by our finding that over half of the systematic reviews concerned metabolic dysfunction and the majority of these having a low risk of bias. More specifically, most systematic reviews examined SNPs within genes that may regulate insulin resistance, which is strongly implicated in the aetiology and reproductive and metabolic consequences of PCOS (Moran, Norman et al. 2015, Cassar, Misso et al. 2016). Significant positive associations of some genetic variants in the INS VTR and Calpain-10 genes with PCOS were identified, both of which are implicated in development of T2DM (Shen, Li et al. 2013, Yan, Liang et al. 2014). Adiponectin is a multifunctional adipocytokine that modulates insulin sensitivity and energy metabolism, and was identified to be negatively associated with PCOS (Jia, Yu et al. 2012). However, most of these associations were not consistent across ethnicities, genotyping methods or sources of recruitment. Consistency and reproducibility of findings regarding SNPs within the IRS-2 genes (involved in insulin signalling) was also problematic. The SNP INSR-His1058 C/T was not associated with PCOS, (Feng, Lv et al. 2015, Shi, Xie et al. 2016) further exploration of other SNPs within the INSR gene may be warranted (Feng, Lv et al. 2015). This overall lack of evidence is mirrored by recent GWAS studies which have not found any associations between PCOS and variants in obesity or T2DM genes (Hayes, Urbanek et al. 2015). At this stage it is unclear what role of metabolic gene variants play in the aetiology of PCOS.

3.5.2 Dysregulation of androgens and gonadotrophins

Excessive androgen production leads to follicular arrest and the subsequent menstrual dysfunction, and anovulation that is commonly observed in PCOS (Blank, McCartney et al.

2006). Factors including inflammation, insulin sensitivity, and neuroendocrine dysfunction are proposed to be associated with hyperandrogenism in PCOS (Shorakae, Teede et al. 2015).

Only one of the three systematic reviews concerning dysregulation of androgens and gonadotrophins was at low risk of bias. This systematic review concluded no significant association between PCOS and FSHR SNP Thr307Ala for all participants combined, but a negative association for women with Caucasian ethnicity in the SNP Asn680Ser in the FSHR gene (Qiu, Liu et al. 2015). This is in contrast to GWAS reporting associations between the FSHR and PCOS in a Chinese cohort using the Rotterdam diagnostic criteria (Shi, Zhao et al. 2012) and in a European cohort using the NIH diagnostic criteria (Hayes, Urbanek et al. 2015). Qui et al. (2015) used a multitude of diagnostic criteria including the Rotterdam criteria and non-specific case definitions which likely increased heterogeneity and reduced the ability to detect subtle associations (Zondervan and Cardon 2007). While Qui et al. (2015) performed subgroup analysis on ethnicity they did not stratify by diagnostic criteria. No systematic reviews focused on the follicle stimulating hormone (FSH) gene, which may be an important for future investigations as GWAS have consistently identified polymorphisms in the FSH Beta subunit gene (FSHB) in multiple ethnicities and using different diagnostic criteria (Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015). Additionally, these GWAS identified other neuroendocrine genes (i.e. the gonadotrophin Luteinising Hormone: [LH]) that are associated with PCOS. Taken together with the lack of high quality systematic reviews on the topic, there is scope for further high quality, adequately powered primary candidate gene studies and systematic reviews to follow up the associative gene variant findings of GWAS to establish causality.

3.5.3 Inflammation

Inflammation potentially acts as a link between insulin resistance and hyperandrogenism in PCOS and is associated with both (Gonzalez 2012, Shorakae, Teede et al. 2015). All three systematic reviews on the topic of inflammation were assessed to be at low risk of bias. Two focused on SNPs in the TNF-α which is a pro-inflammatory cytokine that has been associated with PCOS, ovarian function and ovulation and is a known mediator of insulin resistance (Gonzalez 2012, Guo, Zheng et al. 2015, Wu, Yu et al. 2015). Neither reported significant associations between the TNF-α (-308 G/A) polymorphism and PCOS (Guo, Zheng et al. 2015, Wu, Yu et al. 2015). One systematic review concluded that TNF-α-1031T/C was positively associated with PCOS suggesting this may be the functional polymorphism for susceptibility to PCOS (Wu, Yu et al. 2015). However, sample size and heterogeneity in the primary studies limit the conclusions that can be drawn. Whether low-grade inflammation is intrinsic to PCOS or a consequence of PCOS-related obesity is contentious. Although it is suggested that inflammation is independent of BMI in women with PCOS, the literature lacks consistency (Samy, Hashim et al. 2009, Duleba and Dokras 2012). Unfortunately, only one of the included systematic reviews investigated the confounding influence of BMI and none investigated body fat composition on inflammation gene variants (Guo, Zheng et al. 2015). Obesity is known to exacerbate many of the symptoms of PCOS and it is crucial for future systematic reviews to investigate both the intrinsic mechanisms in PCOS and extrinsic mechanisms such as BMI.

3.6 Future Considerations

We note a number of methodological aspects to address in future work to improve the comparability of systematic reviews examining genetics in PCOS. No systematic review explored associations between genetic polymorphisms and different PCOS phenotypes. This is

important as PCOS is known to have multiple phenotypes and some genetic polymorphisms may only apply to some phenotypes (Moran, Norman et al. 2015). This is supported by recent GWAS that found that LH/Choriogonadotropin Receptor and FSHR are associated with PCOS across all phenotypes as defined by the Rotterdam criteria, while FSHB is only associated with the NIH phenotype (Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015).

Criteria for control groups need to be clearly defined as most of the systematic reviews stated simply they included healthy women or did not define the relevant inclusion criteria. This may affect the strength of association, (Bloom, Schisterman et al. 2007) possibly reflected in two systematic reviews in this overview of systematic reviews that included the same primary studies but came to different conclusions (Shen, Li et al. 2014, Ramos, Fabris et al. 2015). While Ramos et al. (2015) excluded any controls from their meta-analysis that were not considered healthy, Shen et al. (2014) did not describe the inclusion criteria for the control group, therefore it is difficult to compare the meta-analyses.

Another contentious issue is whether to include or exclude individual studies whose control groups did not conform to Hardy-Weinberg Equilibrium (HWE) (Thakkinstian, McElduff et al. 2005, Zintzaras and Lau 2008). Departures from HWE can indicate problems with study design, genotyping error, population stratification, selection bias of controls, or small sample size (Thakkinstian, McElduff et al. 2005, Zintzaras and Lau 2008). In this overview of systematic reviews, a variety of methods were used to deal with the primary studies that departed from HWE and some systematic reviews did not consider this issue at all.

Almost all systematic reviews acknowledged they were limited by small sample size and this highlights the need for larger primary studies and systematic reviews to progress our

knowledge of the role of genetics in the aetiology and pathophysiology of PCOS. While most systematic reviews were at low risk of bias, there was a lack of consistent methodological rigour regarding clear definitions of cases and controls, and the differences in dealing with deviations from HWE. Therefore, very few conclusions can be made about the influence of genetic polymorphisms in PCOS.

With the introduction of GWAS, candidate gene studies will play an imperative role in validating and deciphering the functional gene variants and assist in determining the clinical relevance of GWAS findings (Wilkening, Chen et al. 2009, Vlahovich, Hughes et al. 2017, Williams, Williams et al. 2017). Hypothesis driven candidate gene studies requiring *a-priori* gene selection should utilise the GWAS data to provide stronger candidate gene selection to further validate a polymorphism (Wilkening, Chen et al. 2009, Cirulli and Goldstein 2010, Williams, Williams et al. 2017). While GWAS have progressed the field of genetics this methodology has limitations when studying less frequent gene variants (minor allele frequency <5%). Candidate gene studies allow a more targeted SNP analysis, including rare variants and those with known functions (Wilkening, Chen et al. 2009). Therefore, this overview of systematic reviews is a timely reminder of limitations and important methodological considerations needed to be considered for all future genetic studies, GWAS or candidate gene polymorphism studies, in PCOS and complex diseases more generally.

3.7 Conclusion

This overview of systematic reviews identified several candidate genes and their variants, but overall the data was underwhelming with no clear links between studied genes and biological origins and/or established pathophysiology of PCOS. However, this work identified the limitations and important methodological considerations that should inform and complement

243 future genetic studies. Specifically, data obtained from genome-wide association studies
244 (GWAS) still require validation by candidate gene association studies and functional studies in
245 PCOS. This overview of systematic reviews highlights the need for standardisation in
246 systematic review design and the analysis contained within them to improve reproducibility
247 and impact.

3.8 Supplementary Data

Supplementary table 3.5: Participant, Intervention, Comparison, outcomes and studies (PICOs) framework

	Participants	Interventions	Comparisons	Outcomes	Study design	Limits
Inclusion Criteria	-Any population where PCOS is PRIMARY FOCUS. -Can be interventions in PCOS, descriptions/characterisations of PCOS or comparisons of women with and without PCOS	Any systematic review with or without a meta-analysis -Review must include a search strategy containing at least key words or terms -Review must include the number of identified and included articles -Review must include some form of article quality appraisal	N/A	ANY systematic review with or without meta-analysis. -Following article screening and data extraction the reviews will be grouped by theme: genetics, assessment, fertility treatment, non-fertility treatment, lifestyle management, complementary or alternative medicines/therapies.	Any systematic review with or without a meta-analysis	-English Only -Humans -Published 2009 to current
Exclusion criteria	PCOS is a secondary outcome (not main focus of the systematic review)		N/A		RCTs Cohort studies Case-control Cross sectional Grey literature Longitudinal Qualitative Case study Editorial Narrative review	-Not written in English -Animal Models -Published prior to 2009

Supplemental table 3.6: Search strategy in MEDLINE

1. exp Polycystic Ovary Syndrome/
2. Polycystic Ovar$.tw
3. pco.tw or pcos.tw
4. (sclerocystic adj3 ovar$).tw
5. stein leventhal.tw
6. or/1–5
7. Meta-Analysis as Topic/
8. meta analy$.tw
9. metaanaly$.tw
10. Meta-Analysis/
11. (systematic adj (review$1 or overview$1)).tw.
12. exp Review Literature as Topic/
13. or/7-12
14. cochrane.ab.
15. embase.ab.
16. (psychlit or psyclit).ab.
17. (psychinfo or psycinfo).ab.
18. (cinahl or cinhal).ab.
19. science citation index.ab.
20. bids.ab.
21. cancerlit.ab.
22. or/14-21
23. reference list$.ab.
24. bibliograph$.ab.
25. hand-search$.ab.
26. relevant journals.ab.
27. manual search$.ab.
28. or/23-27
29. selection criteria.ab.
30. data extraction.ab.
31. 29 or 30
32. Review/
33. 31 and 32
34. Comment/
35. Letter/
36. Editorial/
37. animal/
38. human/
39. 37 not (37 and 38)
40. or/34-36,39
41. 13 or 22 or 28 or 33
42. 6 and 41
43. 42 not 40

Supplementary table 3.7: PRSIMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	45
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	48
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	49-51
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	51, 52
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	48, 52
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	52, 53
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	52, 53
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Supplementary table 3.5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	52, 53
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	53,54

Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	53, 54
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	54
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	53, 54
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Table 3.3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 3.2
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 3.1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table 3.4
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 3.2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A

DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Table 3.2, 77-81
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	Table 3.4
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	80-85
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	47

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

53 **Supplementary table 3.8: Articles excluded on full text screening.**

Author	Title	Notes
Abu Hashim 2016	Twenty years of ovulation induction with metformin for PCOS; what is the best available evidence?	Didn't include quality assessment
Al Khalifah 2015	The effectiveness and safety of treatments used for polycystic ovarian syndrome management in adolescents: a systematic review and network meta-analysis protocol	Protocol only
Atiomo 2009	Proteomic biomarkers for the diagnosis and risk stratification of polycystic ovary syndrome: a systematic review	Didn't include quality assessment
Bagos 2009	Plasminogen activator inhibitor-1 4G/5G and 5,10-methylene-tetrahydrofolate reductase C677T polymorphisms in polycystic ovary syndrome	Didn't include quality assessment or number of articles extracted on search
Bao 2016	Association of DENND1A Gene Polymorphisms with Polycystic Ovary Syndrome: A Meta-Analysis	Didn't include quality assessment
Baranova 2011	Systematic review: association of polycystic ovary syndrome with metabolic syndrome and non-alcoholic fatty liver disease	Didn't include number of articles extracted on search or quality assessment
Barba 2009	The effects of metformin on endogenous androgens and SHBG in women: a systematic review and meta-analysis	Not primarily focused on PCOS
Bayram 2010	Pulsatile gonadotrophin releasing hormone for ovulation induction in subfertility associated with polycystic ovary syndrome	Although Cochrane assessed content as up to date in 2010, search was conducted in 2003 so deemed this as meeting prior to 2009 exclusion criteria
Birch Petersen 2016	Mono-ovulation in women with polycystic ovary syndrome: a clinical review on ovulation induction	Didn't include search terms or number of articles extracted on search
Bouza-Alvarez	Safety and efficacy of metformin in improving clinical, hormonal and metabolic features of polycystic ovary syndrome. Systematic review and meta-analysis	? HTA, bulk of article in Spanish
Bronstein 2011	Age of onset of polycystic ovarian syndrome in girls may be earlier than previously thought	Didn't include quality assessment
Cahill 2009	PCOS	Didn't include search terms or number of articles extracted on search
Cahill 2015	Polycystic ovary syndrome (PCOS): metformin	Didn't include search terms or number of articles extracted on search
Cai 2014	Association between fat mass- and obesity-associated (FTO) gene polymorphism and polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Carlus 2016	Is MTHFR 677 C>T Polymorphism Clinically Important in Polycystic Ovarian Syndrome (PCOS)? A Case-Control Study, Meta-Analysis and Trial Sequential Analysis	Didn't include quality assessment

Chen 2014	Two follicle-stimulating hormone receptor polymorphisms and polycystic ovary syndrome risk: a meta-analysis	Didn't include quality assessment
Chittenden 2009	Polycystic ovary syndrome and the risk of gynaecological cancer: a systematic review	Didn't include quality assessment
Conte 2015	Mental Health and Physical Activity in Women with Polycystic Ovary Syndrome: A Brief Review	Didn't include quality assessment
Costello 2010	Insulin-sensitising drugs versus the combined oral contraceptive pill for hirsutism, acne and risk of diabetes, cardiovascular disease, and endometrial cancer in polycystic ovary syndrome	No update of search strategy since 2006
Dissemination	Overweight in polycystic ovary syndrome. An update on evidence based advice on diet, exercise and metformin use for weight loss	Didn't include number of articles extracted on search or quality assessment
Dissemination	N-acetylcysteine for treating women with clomiphene citrate resistant polycystic ovary syndrome: a systematic review	Not in English
Dissemination	Rosiglitazone versus metformin for polycystic ovary syndrome: a systematic review (Provisional abstract)	Not in English
Dissemination 2012	Efficacy of inositol in women with polycystic ovary syndrome and desire for children: systematic review and meta-analysis	Not in English
Dissemination 2015	Letrozole for ovulation induction in women with polycystic ovarian syndrome: a systematic analysis	Not in English
Dissemination 2015	Thiazolidinediones combined with metformin in treatment of polycystic ovary syndrome: a systematic review	Not in English
Dissemination 2016	Laparoscopic surgery versus laparotomy for women with polycystic ovarian syndrome: a systematic review.	Not in English
Dokas 2011	Increased risk for abnormal depression scores in women with polycystic ovary syndrome: a systematic review and meta-analysis	Didn't include quality assessment
Du 2010	Two FSHR variants, haplotypes and meta-analysis in Chinese women with premature ovarian failure and polycystic ovary syndrome	Didn't include search terms, quality assessment or number of articles extracted on search
Du 2013	The relationship between thyroiditis and polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Eckmann 2009	Aromatase inhibitors for ovulation and pregnancy in polycystic ovary syndrome	Didn't include number of articles extracted on search or quality assessment
Escobar-Morreale 2011	Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis	Didn't include quality assessment

Escobar-Morreale 2016	Health-related quality of life in polycystic ovary syndrome patients: A systematic review	did not address PCOS co-morbidities
Eyvazzadeh 2009	The role of the endogenous opioid system in polycystic ovary syndrome	Not a systematic review
Fan 2013	Association between the (TAAAA)n SHBG polymorphism and PCOS: a systematic review and meta-analysis	Didn't include quality assessment
Farquhar 2009	Laparoscopic ovarian diathermy versus metformin for women with polycystic ovarian syndrome	
Fernandez 2011	Ovarian drilling for surgical treatment of polycystic ovarian syndrome: a comprehensive review	Didn't include quality assessment
Frary 2016	The effect of dietary carbohydrates in women with polycystic ovary syndrome: a systematic review.	Didn't include quality assessment
Fu 2014	Association of methylenetetrahydrofolate reductase gene C677T polymorphism with polycystic ovary syndrome risk: a systematic review and meta-analysis update	Didn't include quality assessment
Galazis 2012	Proteomic biomarkers for ovarian cancer risk in women with polycystic ovary syndrome: a systematic review and biomarker database integration	Exclude, PCOS SR component update of Atiomo 2008/9 with no quality assessment
Galazis 2012	Metabolomic biomarkers of impaired glucose tolerance and type 2 diabetes mellitus with a potential for risk stratification in women with polycystic ovary syndrome	Not primarily focused on PCOS
Galazis 2013	Proteomic biomarkers of endometrial cancer risk in women with polycystic ovary syndrome: a systematic review and biomarker database integration	Exclude, PCOS SR component update of Atiomo 2008/9 with no quality assessment
Galazis 2013	Proteomic biomarkers of preterm birth risk in women with polycystic ovary syndrome (PCOS): a systematic review and biomarker database integration	Exclude, PCOS SR component update of Atiomo 2008/9 with no quality assessment
Gao 2012	Association of the T45G and G276T polymorphisms in the adiponectin gene with PCOS: A meta-analysis	Didn't include quality assessment or number of articles extracted on search
Groth 2010	Adiponectin and Polycystic Ovary Syndrome	Didn't include number of articles extracted on search or quality assessment
Haoula 2012	Evaluating the association between endometrial cancer and polycystic ovary syndrome	Didn't include quality assessment
He 2012	A meta-analysis on the association between PPAR-gamma Pro12Ala polymorphism and polycystic ovary syndrome	Didn't include quality assessment
Huang 2012	Four polymorphisms of the CAPN 10 gene and their relationship to polycystic ovary syndrome susceptibility: a meta-analysis	Didn't include quality assessment or number of articles extracted on search
Ioannidis 2010	Polymorphisms of the insulin receptor and the insulin receptor substrates genes in polycystic ovary syndrome: a Mendelian randomization meta-analysis	Didn't include quality assessment or number of articles extracted on search

Jalilian 2015	Prevalence of polycystic ovary syndrome and its associated complications in Iranian women: A meta-analysis	Didn't include quality assessment
Janci 2012	Polycystic Ovarian Syndrome: Metformin or Thiazolidinediones for Cardiovascular Risk Reduction?	Didn't include number of articles extracted on search or quality assessment
Jia 2013	Association of angiotensin-converting enzyme gene insertion/deletion polymorphism with polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Jia 2014	Association between retinol-binding protein 4 and polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Johnson 2011	Metformin is a reasonable first-line treatment option for non-obese women with infertility related to anovulatory polycystic ovary syndrome--a meta-analysis of randomised trials	Didn't include search terms, number of articles extracted on search or quality assessment
Kelly 2011	Insulin-like growth factor binding protein-1 in PCOS: a systematic review and meta-analysis	Didn't include quality assessment
Khan 2015	Overlap of proteomics biomarkers between women with pre-eclampsia and PCOS: a systematic review and biomarker database integration	Exclude, PCOS SR component update of Atiomo 2008/9 with no quality assessment
Kjerulff 2011	Pregnancy outcomes in women with polycystic ovary syndrome: a metaanalysis	Didn't include quality assessment
Kong 2015	Impact of Treatment with Metformin on Adipocytokines in Patients with Polycystic Ovary Syndrome: A Meta-Analysis	Didn't include quality assessment
Krul-Poel 2013	The role of vitamin D in metabolic disturbances in polycystic ovary syndrome: a systematic review	Didn't include quality assessment
Lai 2014	Chinese Herbal Medicine for Oligomenorrhoea and Amenorrhoea in Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis	Abstract only
Lakkakula 2013	Genetic variants associated with insulin signaling and glucose homeostasis in the pathogenesis of insulin resistance in polycystic ovary syndrome: a systematic review	Didn't include search terms, quality assessment or number of articles extracted on search
Lautatzis 2013	Efficacy and safety of metformin during pregnancy in women with gestational diabetes mellitus or polycystic ovary syndrome: a systematic review	Didn't include search terms
Lee 2014	Plasminogen activator inhibitor-1 4G/5G and the MTHFR 677C/T polymorphisms and susceptibility to polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment or number of articles extracted on search
Lim 2010	Current evidence of acupuncture on polycystic ovarian syndrome.	Didn't include search terms, number of articles extracted on search or quality assessment
Lin 2013	Androgen receptor gene polymorphism and polycystic ovary syndrome	Didn't include quality assessment
Lin 2014	Is a GnRH antagonist protocol better in PCOS patients? A meta-analysis of RCTs	Didn't include quality assessment
Liu 2014	Plasminogen activator inhibitor-1 -675 4G/5G polymorphism and polycystic ovary syndrome risk: a meta analysis	Didn't include quality assessment

Liu 2016	Meta-analysis of the correlation between the TNF-alpha308G/A polymorphism and polycystic ovary syndrome	Didn't include quality assessment
Louwers 2013	Cross-ethnic meta-analysis of genetic variants for polycystic ovary syndrome	Didn't include quality assessment or number of articles extracted on search
Mancini 2011	Gonadotrophin-releasing hormone-antagonists vs long agonist in in-vitro fertilization patients with polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Misso 2012	Status of clomiphene citrate and metformin for infertility in PCOS	Not a systematic review
Misso 2015	Metformin in women with PCOS, cons	Not a systematic review
Morris 2016	What does a diagnostic label of 'polycystic ovary syndrome' really mean in adolescence? A review of current practice recommendations	Didn't include quality assessment
Naderpoor 2015	Metformin and lifestyle modification in polycystic ovary syndrome: systematic review and meta-analysis	Didn't include search terms
Nahuis 2011	Metformin co-administration during follicle stimulating hormone ovulation induction with timed intercourse or intra-uterine insemination for subfertility associated with polycystic ovary syndrome	
Nahuis 2013	The basic fertility workup in women with polycystic ovary syndrome: a systematic review	Didn't include quality assessment
Niafar 2016	A systematic review of GLP-1 agonists on the metabolic syndrome in women with polycystic ovaries	Didn't include quality assessment
Nicholson 2010	Effectiveness of long-term (twelve months) nonsurgical weight loss interventions for obese women with polycystic ovary syndrome: a systematic review	Didn't include quality assessment
Palomba 2015	Pregnancy complications in women with polycystic ovary syndrome	Didn't include number of articles extracted on search or quality assessment
Parsanezhad 2009	Surgical ovulation induction in women with polycystic ovary syndrome: a sytematic review	Didn't include number of articles extracted on search or quality assessment
Peitsidis 2010	Role of vascular endothelial growth factor in women with PCO and PCOS: a systematic review	Didn't include quality assessment
Peng 2014	The association between androgen receptor gene CAG polymorphism and polycystic ovary syndrome: a case-control study and meta-analysis	Didn't include quality assessment
Qin 2013	Obstetric complications in women with polycystic ovary syndrome: a systematic review and meta-analysis	Didn't include quality assessment
Rajender 2013	Androgen receptor CAG repeats length polymorphism and the risk of polycystic ovarian syndrome (PCOS)	Didn't include quality assessment
Ren 2014	[A meta-analysis on acupuncture treatment of polycystic ovary syndrome]	Not in English

Rocca 2015	Polycystic ovary syndrome: chemical pharmacotherapy	Not a systematic review
Saha 2013	N-acetyl cysteine in clomiphene citrate resistant polycystic ovary syndrome: A review of reported outcomes	Didn't include quality assessment
San-Millan 2010	The role of genetic variation in peroxisome proliferator-activated receptors in the polycystic ovary syndrome (PCOS): an original case-control study followed by systematic review and meta-analysis of existing evidence	Didn't include quality assessment
Showell 2016	Inositol for subfertile women with polycystic ovary syndrome	
Sirmans 2012	Polycystic ovary syndrome and chronic inflammation: pharmacotherapeutic implications	Didn't include number of articles extracted on search or quality assessment
Song 2014	Lack of association of INS VNTR polymorphism with polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Sun 2013	Effect of metformin on ovulation and reproductive outcomes in women with polycystic ovary syndrome: a meta-analysis of randomized controlled trials	Didn't include quality assessment
Taghavi 2015	Type 1 Diabetes and Polycystic Ovary Syndrome: Systematic Review and Meta-analysis	Not primarily focused on PCOS
Tang 2009	WITHDRAWN: Insulin-sensitising drugs for polycystic ovary syndrome	Withdrawn from publication as error in citation
Tang 2010	Ultrasound-guided transvaginal ovarian needle drilling for clomiphene-resistant polycystic ovarian syndrome in subfertile women	
Tang 2012	Association of Pro12Ala polymorphism in peroxisome proliferator-activated receptor gamma with polycystic ovary syndrome: a meta-analysis	Didn't include quality assessment
Tang 2015	Insulin receptor substrate-1 (IRS-1) rs1801278G>A polymorphism is associated with polycystic ovary syndrome susceptibility: a meta-analysis	Didn't include quality assessment
Thethi 2015	Role of Insulin Sensitizers on Cardiovascular Risk Factors in Polycystic Ovarian Syndrome: A Meta-Analysis	"Reviewers worked independently and in duplicate to determine the methodological quality" but no details given
Tomlinson 2010	Type 2 diabetes and cardiovascular disease in polycystic ovary syndrome: what are the risks and can they be reduced?	Didn't include search terms or number of articles extracted on search
Toulis 2011	Meta-analysis of cardiovascular disease risk markers in women with polycystic ovary syndrome	Didn't include quality assessment
Tsikouras 2015	Features of Polycystic Ovary Syndrome in adolescence	Didn't include number of articles extracted on search or quality assessment
Unfer 2012	Effects of myo-inositol in women with PCOS: a systematic review of randomized controlled trials	Didn't include quality assessment

Wang 2012	[Therapeutic effect of metformin for clomiphene-resistant infertility patients with polycystic ovary syndrome: a systematic analysis]	Not in English
Wang 2012	Negative association between androgen receptor gene CAG repeat polymorphism and polycystic ovary syndrome? A systematic review and meta-analysis	Didn't include quality assessment
Wang 2015	4G/5G polymorphism of plasminogen activator inhibitor-1 gene is associated with polycystic ovary syndrome in Chinese patients: a meta-analysis	Didn't include quality assessment or number of articles extracted on search
Wild 2010	Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society	Not a systematic review
Wild 2011	Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis	Didn't include quality assessment
Wojciechowski 2012	Impact of FTO genotypes on BMI and weight in polycystic ovary syndrome: a systematic review and meta-analysis	Didn't include quality assessment
Wu 2016	Acupuncture for treating polycystic ovary syndrome: guidance for future randomized controlled trials	Didn't include number of articles extracted on search
Xian 2012	ADIPOQ gene polymorphisms and susceptibility to polycystic ovary syndrome: a HuGE survey and meta-analysis	Didn't include quality assessment
Xiao 2011	Effectiveness of GnRH antagonist in vitro fertilization-embryo transfer (IVF-ET) in PCOS patients: a systematic review	Not in English
Xie 2013	Microsatellite polymorphism in the fibrillin 3 gene and susceptibility to PCOS: a case-control study and meta-analysis	Didn't include quality assessment or number of articles extracted on search
Xu 2014	Effect of metformin on serum interleukin-6 levels in polycystic ovary syndrome: a systematic review	Didn't include quality assessment
Yu 2014	Polymorphisms of pentanucleotide repeats (tttta)n in the promoter of CYP11A1 and their relationships to polycystic ovary syndrome (PCOS) risk: a meta-analysis	Didn't include quality assessment
Zhang 2012	Association between the Pro12Ala polymorphism of PPAR-gamma gene and the polycystic ovary syndrome: a meta-analysis of case-control studies	Didn't include quality assessment
Zhang 2014	The -675 4G/5G polymorphism in the PAI-1 gene may not contribute to the risk of PCOS	Didn't include quality assessment or number of articles extracted on search
Zhang 2015	Peroxisome proliferator-activated receptor gamma rs1801282 C>G polymorphism is associated with polycystic ovary syndrome susceptibility: a meta-analysis involving 7,069 subjects	Didn't include quality assessment
Zheng 2013	The efficacy of metformin in pregnant women with polycystic ovary syndrome: a meta-analysis of clinical trials	Didn't include quality assessment

CHAPTER 4. GLOBAL DNA HYPO-METHYLATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM WOMEN WITH POLYCYSTIC OVARY SYNDROME IS CELL-TYPE SPECIFIC.

4.1 General background

There is apparent heritability in PCOS as demonstrated in genetic association studies and familial clustering as supported by twin and family studies (Legro, Driscoll et al. 1998, Legro, Bentley-Lewis et al. 2002, Kaminsky, Tang et al. 2009). Many genetic association studies have been undertaken as seen from chapter 3 that while genetics play a significant but minor role in the pathophysiology of PCOS there is still further work to be done. Genome wide association studies have only been able to account for 10% of heritability indicating other factors must be involved. Epigenetics is defined as changes in gene expression due to modifications of DNA without alterations to the DNA sequence (Qiu 2006). One such mechanism is DNA methylation that facilitates the molecular bridge by which the environment and genetics link together. Emerging evidence supports the role of inappropriate epigenetic programming in the aetiology of PCOS. However, large gaps in knowledge remain as to how epigenetic modifications relate to PCOS and its associated sequelae.

Therefore, in chapter 4 I started to investigate DNA methylation in immune cells of women with and without PCOS and whether the methylation profiles are associated with biomarkers of key clinical features of PCOS. DNA methylation. DNA methylation controls chromatin structure and gene expression (Tammen, Friso et al. 2013). Specifically, I explored the global methylation where I measured non-sequence dependent total methyl-cytosine content. This analysis does not establish any pathways, loci or genes that are affected by the methylation. However, global methylation status can be used as a biomarker of a disease potentially reflecting the internal pathophysiological environment. Cause and effect have yet to be determined however immune cells infiltrate many tissues (skeletal muscle, ovaries, adipose tissue) and

integrate any physiological and pathophysiological changes that are occurring in these tissues (Bukulmez and Arici 2000, Pate, Toyokawa et al. 2010). Or conversely the epigenome of immune cells can adapt to their environmental milieu and then influence the epigenome of surrounding tissues (Paparo, di Costanzo et al. 2014, Obata, Furusawa et al. 2015). The immune system is gaining more attention as important in metabolic dysfunction and links to the reproductive system are emerging. Immune cells are in "direct contact" with the environment and therefore can be affected by or affect the alterations in the environmental milieu (i.e. - higher circulating AMH levels, Testosterone, Glucose). To date there is limited literature in women with PCOS whether each immune cell have a distinct global or unique genome-wide DNA methylation patterns compared to women without PCOS. Furthermore, whether immune cell populations are generically methylated is unknown in women with PCOS. This could potentially be hiding cell-specific and disease-specific changes due to a disease which may play an important role in elucidating the aetiology of PCOS. Therefore, the aim of this chapter was to explore the global DNA methylation in specific immune cell populations (PBMC- T cells, Bells and monocytes) and if there are associations with key metabolic and reproductive features including BMI, insulin resistance and AMH in women with and without PCOS.

Chapters 4 (and 5) are based on a cross-sectional study that is collaboration between Victoria University, University of NSW, University of Copenhagen and Monash University. I lead this study design, data collection, write up as well as funding applications. As this chapter will be consolidated with chapter 5 for a manuscript, all listed collaborators have reviewed this chapter and consented to its inclusion in the thesis. In addition, they acknowledge and confirm my substantial compiling and

synthesis of this work for the thesis.

Collaborators

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Keywords: Polycystic ovary syndrome, DNA methylation, insulin resistance, inflammation, immune cells, lymphocytes, monocytes, Flow cytometry.

4.2 Abstract

Introduction: PCOS is a complex metabolic and reproductive disorder with a multifaceted aetiology. Emerging evidence suggests a role for inappropriate epigenetic programming in the complex aetiology of disease, including PCOS, indicating a link between genetics and lifestyle. A growing body of evidence illustrates the interaction of immune cell function with the reproductive system, and metabolic dysfunction in chronic conditions like PCOS. The immune system is known to be epigenetically regulated and this may have further implications in PCOS. There is increasing evidence to suggest that each type of immune cell within the peripheral blood mononuclear cells (PBMC) population is characterized by a specific methylation pattern. To date, no study has investigated the global DNA methylation profile in a cell-specific manner in PCOS. Therefore, in this study we aimed to investigate for the first time, the global DNA methylation profile in specific immune cell populations in women with and without PCOS. Secondly whether these methylation profiles are associated with biomarkers of the metabolic and reproductive features in women with and without PCOS.

Methods: Thirty-four women with and with PCOS were recruited from the community between 2014 and 2016. Women with PCOS were confirmed to have features consistent with the Rotterdam diagnostic criteria. A 75g oral glucose tolerance test (OGTT) and dual-energy X-ray absorptiometry were performed at Victoria University. Physical activity was monitored using a triaxial accelerometer and the international physical activity questionnaire (IPAQ). Food intake was assessed by a 7-day food diary. Blood draws prior to the OGTT were used to isolate PBMC which were analysed using multi-colour flow cytometry methods to determine cell-specific global DNA methylation levels. Reproductive markers, systemic methylation metabolites and cytokines were measured at baseline.

Results: There were no differences in age, height, weight or BMI between women with and without PCOS. Women with PCOS displayed significant hypo-methylation in monocytes (p=0.006), T helper (p=0.004) and T cytotoxic (p=0.004), and B cells (p=0.026) when compared to women without PCOS. Simple linear regression revealed PCOS status was negatively associated with global methylation in monocytes -28.5 [95%CI: -47.9, -8.9], T helper cells -7.5 [95%CI: -12.5, -2.6], T cytotoxic cells -7.3 [95%CI: -12.1, -2.5] and in B cells -13.3 [95%CI: -25, -1.7]. Anti-müllerian hormone (AMH) was negatively associated with global methylation in T helper cells -0.12 [95%CI: -0.19, -0.41] and T cytotoxic cells -0.97 [95%CI: -0.17, -0.02]. However, body composition, physical activity and dietary intake measurements were not associated with global DNA methylation in any of the PBMC populations.

Conclusion: For the first time we show using PBMC isolated into constituent populations that PCOS is associated with overall remodelling of the epigenome. Specifically, four immune cell population had reduced global DNA methylation in women with PCOS compared to women without PCOS. These data suggest that epigenetically regulated immune cells contribute to the aetiology of PCOS and warrant further investigation.

4.2.1 Introduction

PCOS is a metabolic and reproductive disorder with a complex and ill-defined aetiology. It is commonly characterised by increased levels of androgens, inflammatory cytokines, or insulin resistance and increased prevalence of sub-fertility (Cassar, Teede et al. 2014, Shorakae, Boyle et al. 2014, Dumesic, Oberfield et al. 2015). Familial clustering of PCOS is well documented, providing evidence for a genetic contribution to the condition (Legro, Driscoll et al. 1998, Legro, Bentley-Lewis et al. 2002). Investigations to identify the single nucleotide polymorphisms (SNPs) that may be involved in the genetic basis of PCOS have been inconclusive (chapter 3) despite important recent advances in the field from GWAS (Day, Hinds et al. 2015). There is emerging evidence suggesting the role of inappropriate epigenetic programming in the aetiology of chronic disease generally (Barres and Zierath 2011), including PCOS (Shen, Qiu et al. 2013, Wang, Wei et al. 2014, Li, Zhu et al. 2016), indicating an interaction of lifestyle, environment, and genetics.

The immune system is known to be epigenetically regulated and can interact with reproductive system and metabolically active tissue (Pate, Toyokawa et al. 2010, Figueroa, Davicino et al. 2012, Raghuraman, Donkin et al. 2016). Early seminal studies hypothesised that the ovulation of the ovarian follicles involves an inflammatory reaction (Espey 1980). Since evidence has accumulated, supporting that the ovaries can be modified by circulating immune cells and the cytokines they produce (Pate, Toyokawa et al. 2010, Clancy, Baerwald et al. 2013). Specifically follicular development has been associated with systemic and local inflammation, with further evidence shows that inflammation can suppress ovarian function (Clancy, Baerwald et al. 2013, Clancy, Klein et al. 2013, Clancy, Baerwald et al. 2016). In both animal

models and humans, altered immune responses and inflammatory markers have been found in various parts of ovarian tissue, suggesting a role in the pathophysiology of the reproductive features of PCOS (Pate, Toyokawa et al. 2010, Figueroa, Davicino et al. 2012, Schmidt, Weijdegard et al. 2014). Further, it is thought that impairment of immune cell function and the infiltration of these cells in insulin sensitive tissues may ultimately lead to metabolic impairment and type 2 diabetes mellitus (T2DM) (Delamaire, Maugendre et al. 1997, Zierath and Barres 2011). Evidence has shown that the immune system may reprogram metabolically active tissue through epigenetic modification, and that these modifications may result in the development of metabolic disease (Kintscher, Hartge et al. 2008, Wang, Zhu et al. 2010, Raghuraman, Donkin et al. 2016).

One type of epigenetic modification is DNA methylation which can control chromatin structure and gene expression (Barrès, Yan et al. 2012). Numerous techniques can be used to measure DNA methylation and see chapter 2.5.2. Briefly, global methylation is a non-sequence dependent measurement of methyl cytosine content but it unable to establish which pathways, loci or genes may be affected. Instead it gives an indication of whether the global methylation status is altered and/or related to environmental pathophysiology/stimuli. To date one study has investigated global DNA methylation in PCOS and found no differences in non-cell specific analysis of peripheral blood mononuclear cells (PBMC) (Xu, Azziz et al. 2010). There is increasing evidence to suggest that each type of immune cell within the PBMC population is characterised by different levels of DNA methylation. Therefore, non-cell-specific methylation analysis likely presents a significant limitation in elucidating the potential role of epigenetics in

the immune system in PCOS (Adalsteinsson, Gudnason et al. 2012, Glossop, Nixon et al. 2013, Simar, Versteyhe et al. 2014).

To date, no study has investigated the global DNA methylation in a cell-specific manner in PCOS. Therefore, in the present study we aimed test the hypothesis that women with PCOS will present with different levels of global DNA methylation in specific immune cell populations compared to women without PCOS. Secondly that these global methylation levels will be associated with biomarkers of the metabolic and reproductive features of PCOS.

4.3 Methods

4.3.1 Study population

Premenopausal women aged between 18-45 years with and without PCOS were recruited from the community, a non-clinical population. All participants provided informed written consent. Women with PCOS were confirmed by an endocrinologist (SS or AJ) to have features consistent with the Rotterdam diagnostic criteria based on participant's previous medical records. The Rotterdam criteria was used for confirmation of PCOS with two of the following (i) oligo- or anovulation (ii) clinical (hirsutism and acne) and/or biochemical hyperandrogenism (iii) polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Women without PCOS had no features of PCOS. Exclusion criteria were pregnancy, smoking, T2DM, known cardiovascular disease, asthma and medications affecting endpoint measures including: hormonal contraceptives, insulin sensitising drugs, anti-inflammatories and anti-androgens. This study was approved by the Victoria University Human Research Ethics Committee (HRE 14-138).

4.3.2 Clinical measures

All clinical measures were collected in the morning after an 8 hour fast. For women without PCOS, testing was conducted in the early follicular phase of the menstrual cycle (2-7days after beginning menses). All women with PCOS had irregular cycles and therefore the testing was conducted in early follicular phase when possible.

4.3.3 Lifestyle monitoring

In the seven days preceding the trial, participants recorded their dietary intake via a 7-day food diary. Food diaries were analysed by FoodWorks® (Xyris, Australia) for the major food groups (grains, fruit, vegetables, protein and dairy), total energy (macronutrients) and fat ratios. Physical activity was monitored by a triaxial accelerometer (Actigraph) that was worn around their waist on the right hip. Time spent in moderate to vigorous activity and METs was calculated by the Freedson VM3 (2011) algorithms in Actlife software (Sasaki, John et al. 2011). Participants also filled out the international physical activity questionnaire (IPAQ).

4.3.4 Anthropometric assessment

Participants were weighed lightly clothed and without shoes (HW-PW200, associated scales services, Australia). Height was taken without shoes using a calibrated stadiometer (Proscale Inductive Series I, Accurate Technology Inc., USA). BMI was calculated [weight (kilograms)/ height squared (squared metres)]. Waist and hip circumference measurements were taken (Swain 2014). Waist to hip ratio was calculated as waist/hip circumference. Fat mass, abdominal fat mass and fat free mass

were measured by dual-energy x-ray absorptiometry (DXA) [GE Lunar iDXA, United Kingdom] and analysed by a qualified DXA operator (DH).

4.3.5 Blood samples

After an overnight 8 hours fast, a sterile polyethylene catheter was inserted into the antecubital vein and baseline blood samples were collected. Serum collected was snap frozen in liquid nitrogen before being transferred into the -80C freezer for long-term storage. Plasma was collected in Eppendorf tubes before being placed on ice before transfer to -80 C freezer for long-term storage. Blood was collected in acid citrate dextrose tubes and left at room temperature until PBMC isolation by ficoll gradient separation on the same day (Simar, Versteyhe et al. 2014).

4.3.6 Oral glucose tolerance test

After collecting baseline bloods participants then ingested a 75g glucose drink within 5 minutes and samples of blood were collected at 30, 60, 90 and 120 minutes (Meyer, McGrath et al. 2007). Blood glucose in plasma was measured on the day by using an automated analyser (YSI 2300 STAT Plus). Blood cell counts were measured on the day by Sysmex automated analyser.

4.3.7 Biochemical analysis

Lipid profiles were quantified by automated enzymatic methods (Architect C18000 analyser), hs-CRP by immunoturbidimetric assay. Plasma insulin concentration was determined by radioimmunoassay according to manufacturer instructions (HI-14K, EMD Millipore). Results from the OGTT were used to determine both insulin sensitivity and resistance using area under the curve for plasma insulin and glucose

concentrations, the homeostasis assessment (HOMA) indices of insulin sensitivity calculated as (fasting insulin x fasting glucose/22.5) (Meyer, McGrath et al. 2007). IL-6 was measured using a high sensitivity ELISA (ab46042, Abcam). The access SHBG assay was performed using a sequential two-step immunoenzymatic ('sandwich') assay carried out on a Beckman Coulter Unicel DXI 800 (Beckman Coulter). Testosterone assay was performed by high performance liquid chromatography–mass spectrometry (HPLCMS/MS) method using a liquid sample extraction (AB Sciex Triple Quad 5500 LC/MS/MS system). Free androgen index (FAI) was calculated as (total testosterone x 100)/SHBG. S-adesylmethionine was determined by competitive enzyme immunoassay (STA-672, Cell Biolabs).

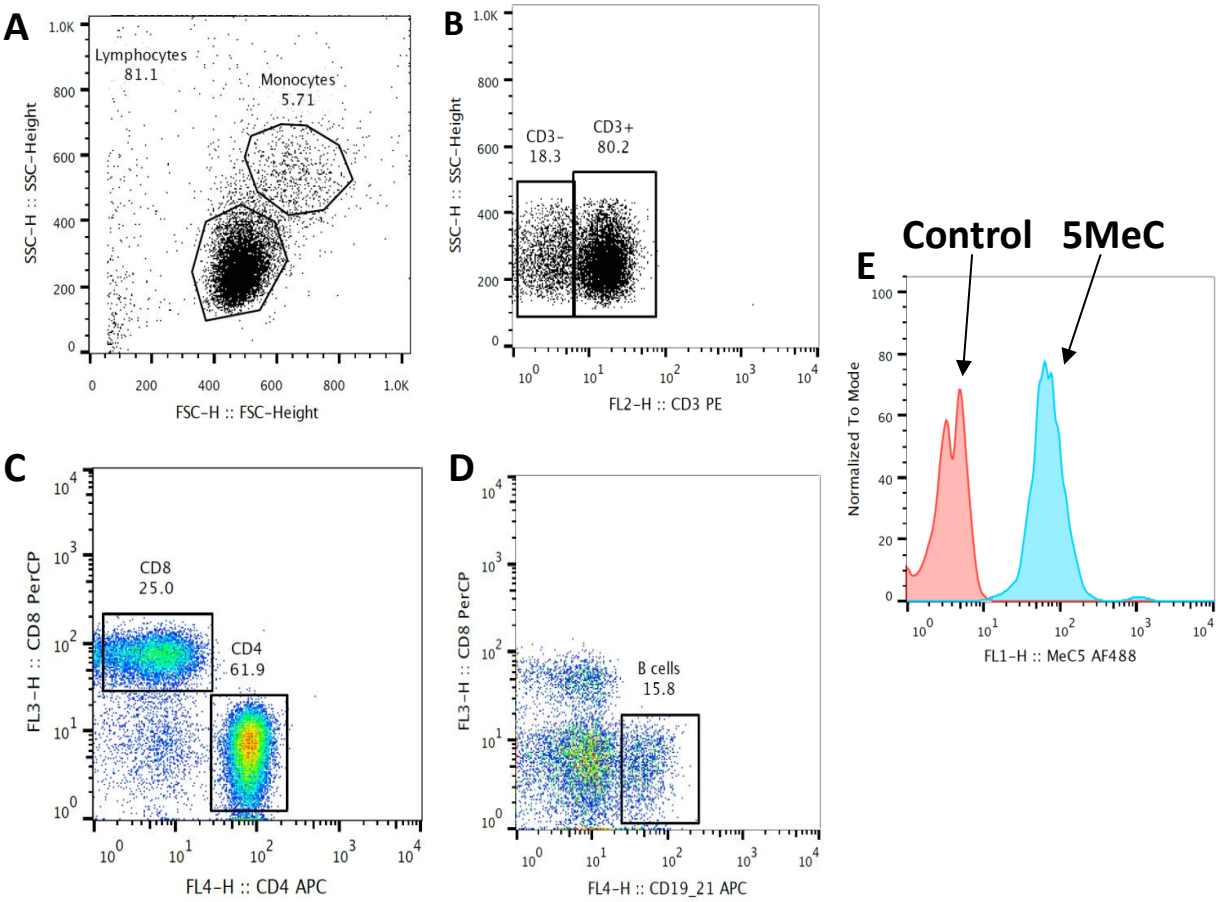
4.3.8 PBMC isolation

PBMC were isolated by ficoll gradient as previously described in Simar et al. (2014) with a few modifications. After centrifugation and removal of plasma, the cell suspension was diluted in RPMI, supplemented with penicillin/streptomycin and L-glutamine and carefully layered on ficoll in Sepmate^{TM} tubes (Stemcell technologies) in equal volumes and centrifuged. The buffy coat containing PBMC were collected, washed with Roswell Park Memorial Institute medium (RPMI) and resuspended in autologous plasma. Cells were counted on the automated cell counter (TC20^{TM} automated cell counter) before being frozen in 10% Cyropreservent DMSO in RPMI overnight in a controlled rate freezing container (CoolCell®, Biocision) and transferred to liquid nitrogen the following day for long-term storage.

4.3.9 Global DNA methylation

Global methylation in PBMC was quantified as previously described by (Simar, Versteyhe et al. 2014). Briefly, cryopreserved PBMC were quickly thawed at 37°C and transferred into culture medium (RPMI). Cells were washed twice in RPMI and 10% foetal calf serum (FCS) and cell viability was assessed and viable cells were diluted to a volume of 5 million per 1mL. Cells were incubated for 30mins at 37°C, 5% CO2 before being fixed in 2% paraformaldehyde (PFA, Sigma Aldrich,) in a solution of Dulbeco's Phosphate Buffered Saline (PBS), 1% BSA and 0.1% Tween-20 [PBS buffer] at 37°C for 10 minutes. After 2 washes, concentrated methanol (100%) was added to permeabilise the cells for 30 minutes at -20°C before being washed three times. Hydrochloric acid (2M HCl) was added and cells were incubated at 37°C for 30minutes before neutralising the pH using 0.1M borate buffer for 5minutes. Cells were washed 3 times in PBS buffer and were stained with an antibody cocktail of anti CD3-phycoerytrin conjugated, CD8- and CD14-peridinin chlorophyll conjugated, CD4- and CD19-allophycocyanin conjugated (BD). PBMC were then further stained with unconjugated anti-5-methylcytosine (5meC, AbD serotec, Bio-Rad, USA) or with its associated isotope control (Mouse, IgG1, BD,). The unconjugated antibodies were labelled according to manufacturer's instruction (Zenon Alexa Flour 488 Mouse IgG1 labelling kit, Molecular Probes, Life Technologies, Australia). Cells were incubated in the dark with the different antibodies or their corresponding control for 20 minutes at room temperature. Cells were washed twice in PBS wash buffer and then re-suspended in 400µL of 1% PFA in PBS and then read immediately on the flow cytometer (FACS Calibur, BD). A specific gating strategy was used to separate the different cell populations in the PBMC Figure 4.1. In each cell population the median fluorescence intensity (MFI) was measured and normalised by the MFI from the isotope controls.

343 Data were analysed using FlowJo version 10 (Tree Star, USA) and Cytobank (Cytobank
344 Inc., USA).
345



346

347 **Figure 4.1: Flow cytometry gating strategy**
348 **A**- Lymphocytes and monocytes were gated based on their size [forward scattered light
349 (FSC)] and granularity [side scattered light (SSC)]. **B**- Lymphocytes were further gated
350 based on CD3 (+ or -) CD3+ denotes T cells and CD3- are B cells. **C**- CD3+CD4+ were
351 T helper Cells and CD3+CD8+ are T cytotoxic cells. **D**- CD3-CD19/CD20+ were B
352 cells. Monocytes were further gated based on CD14+ (not shown) **E**- Red isotope
353 control and blue is the 5MeC antibody.
354

355 ***4.3.10 Statistical analysis***

356 Data were analysed using IBM SPSS Statistics, version 22 (Armonk, NY, USA).
357 Baseline characteristics are presented as mean ± SD or median (IQR), when data were
358 skewed. The baseline characteristics between PCOS and control groups were compared

using student t-tests. Data were tested for normality using the Shapiro-Wilk test and when deemed non-normal the Mann Whitney test was used to compare the baseline characteristics. Student t-test were used to examine the difference in global DNA methylation in women with and without PCOS.

Simple linear regression analysis was used to examine the association between global DNA methylation and body composition, physical activity or diet parameters. We also conducted linear regression analysis to assess any associations between metabolic and reproductive biomarkers (glucose, AMH and testosterone). Before each regression was performed linearity was confirmed by scatterplot, the variance of residuals was consistent across all independent variables and there was normality of residuals. Statistical significance was considered when $p \leq 0.05$.

4.4 Results

4.4.1 Clinical and biochemical characteristics

We recruited 34 women (n=17 PCOS; n=17 without PCOS); their detailed characteristics are reported in Table 4.1. There were no differences in age, height, weight, BMI, markers of body composition, and lifestyle factors between women with and without PCOS.

Table 4.1: Anthropometric data and lifestyle characteristics

Clinical features	Sample Size n	Controls Mean ± SD Median [IQR]	PCOS Mean ± SD Median [IQR]	p
General characteristics				
Age (years)	34	30.1 ± 6.6	28.9 ± 4.8	P=0.6
Height (cm)	34	165 ± 7.2	162 ± 4.7	P=0.3
Weight (kg)	34	70.5 ± 16.8	71.5 ± 18.2	P=0.9
BMI (kg/m^2)	34	25.5 ± 5.4	26.6 ± 6.9	P=0.6
WHR	32	0.85 [0.8, 1.0]	0.80 [0.8, 0.9]	P=0.2
Body fat (%)	31	32.3 [25.9, 40.5]	37.7 [31.8, 41.8]	P=0.3
Physical activity				
MVPA (IPAQ-mins.week)	31	172 [15.0, 427.5]	165 [40.0, 255.0]	P=0.6
MVPA (Accel-mins.day)	30	47.0 [45.5, 48.5]	47.0 [46.0, 50.5]	P=0.6
Food intake				
Energy (g)	31	8269 [7033, 11402]	7744 [5509, 8885]	P=0.2
Saturated fats (g)	31	28.0 [23.0, 50.0]	21.5 [17.2, 30.3]	P=0.07
Carbohydrates (g)	31	198 [157, 278]	171 [144, 220]	P=0.2
Sugar (g)	31	70.0 [56.5, 118]	67.5 [51.0, 123]	P=0.6

BMI, body mass index; WHR, waist-to-hip ratio; IPAQ, International Physical Activity Questionnaire; MVPA; Moderate Vigorous Physical Activity; Accel, Accelerometer.

There were no differences in the lipid profile, systematic methylation donors/substrates (SAM, homocysteine or folate) or cytokines (IL-6 or hsCRP) between with women with and without PCOS. There was no difference in the fasting or the postprandial response from the oral glucose tolerance test (OGTT) in either insulin or glucose or HOMA. Women with PCOS had higher levels of testosterone ($p<0.01$), free androgen index [FAI] ($p<0.01$) and anti-müllerian hormone [AMH] ($p<0.01$) Table 4.2.

In women with PCOS, all women had irregular cycles or amenorrhea (17/17); 11/17 had clinical or biochemical hyperandrogenism, and 15/17 had polycystic ovaries on ultrasound. Women without PCOS had no Rotterdam features including no signs of biochemical hyperandrogenism as assessed by testosterone and SHBG levels or using the FAI and all were regularly cycling (21 to 35 days is considered a regular cycle (Treloar, Boynton et al. 1967)).

Table 4.2: Metabolic and reproductive profile of participating women with and without PCOS.

Clinical features	Sample Size n	Controls Mean±SD Median [IQR]	PCOS Mean±SD Median [IQR]	p
Lipid profile				
Cholesterol (mmol/L)	30	4.2 ± 0.7	4.4 ± 0.7	P=0.5
Triglycerides (mmol/L)	30	0.6 [0.6, 0.9]	0.8 [0.5, 1.0]	P=0.6
HDL(mmol/L)	30	1.5 ± 0.3	1.5 ± 0.3	P=1.0
LDL (mmol/L)	30	2.5 ± 0.6	2.4 ± 0.7	P=0.6
OGTT				
Fasting glucose (mmol/L)	34	4.9 [4.8, 5.4]	4.9 [4.6, 5.3]	P=0.4
Fasting insulin (pmol/L)	34	72.8 [56.2, 89.4]	68.5 [47.2, 102.4]	P=0.9
2hr glucose (mmol/L)	34	4.75 [4.5, 5.4]	5.5 [4.6, 5.8]	P=0.2
2hr insulin (pmol/L)	34	278 [207, 399]	309 [165, 737]	P=0.5
HOMA	34	2.65 [2.0, 3.2]	2.41 [1.7, 4.4]	P=0.9
Reproductive markers				
Testosterone (nmol/L)	34	1.03 [0.8, 1.2]	1.72 [1.5, 2.1]	P<0.001
SHBG (nmol/L)	34	62.1±21.5	63.4±38.4	P=0.9
FAI	34	1.42 [1.4, 2.2]	3.17 [2.2, 5.3]	P<0.001
AMH (pmol/L)	33	18.9 [10.8, 31.1]	48.6 [37.7, 74.6]	P<0.001
Systemic methyl substrate/donors				
SAM (µg/mL)	32	7.7 [5.4, 8.3]	6.9 [6.0, 12.8]	P=0.7
Folate (nmol/L)	33	29.9 [23.2, 37.5]	37.3 [23.4, 45.8]	P=0.08
Homocysteine	33	9.0 [6.9, 10.1]	7.1 [6.4, 9.7]	P=0.3
Cytokines				
HsCRP (mg/L)	30	1.3 [0.5, 3.3]	1.4 [0.6, 4.5]	P=0.7
IL-6 (pg/ml)	31	1.8 [1.3, 3.0]	2.1 [1.5, 2.9]	P=0.3
Cell counts				
WBC (µL)	31	5335 ± 1229	5750 ± 1497	P=0.4

OGTT, oral glucose tolerance test; HOMA, homeostatic model assessment, SHBG, Sex Hormone Binding Globulin; FAI, Free Androgen Index; AMH, Anti-Müllerian Hormone; IL-6, Interleukin-6; HsCRP, High sensitivity C-reactive protein; SAM, S-adenosylmethionine.

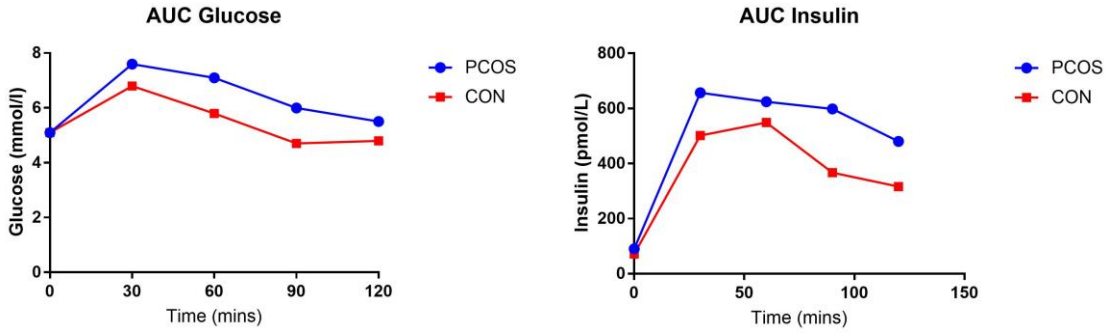


Figure 4.2: Area under the curve of glucose and insulin from OGTT
Women with PCOS had a high area under the glucose curve (p=0.02) but there was no difference in area under the insulin curve.

4.4.2 Global methylation analysis

Global DNA methylation analysis of the individual immune cell populations demonstrate that women with PCOS displayed significant hypo-methylation in monocytes (p=0.006), T helper (p=0.004) and T cytotoxic cells (p=0.004) and B Cells (p=0.03) when compared to women without PCOS (Figure 4.3).

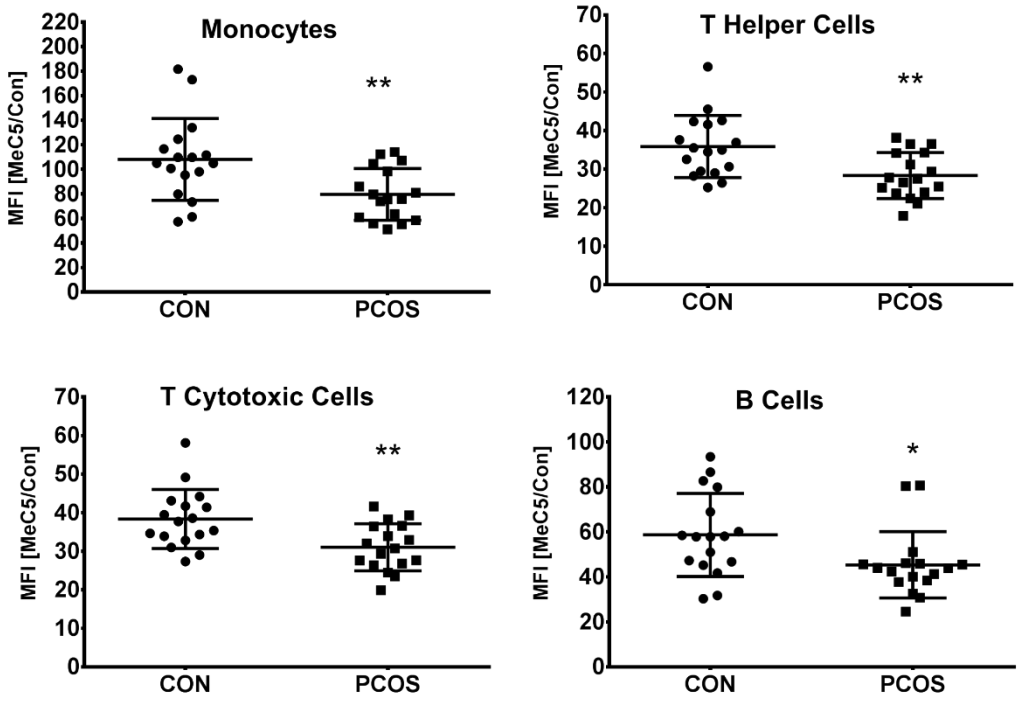


Figure 4.3: Analysis of 5-methylcytosine levels in monocytes, T helper cells, T cytotoxic cells and B cells between women with and without PCOS.
Comparison of 5-methylcytosine median fluorescence intensity (MFI) between women with PCOS (square) and women without PCOS (circle). MFI normalised by the MFI from the isotope control. Significantly different from control *p<0.05 **p<0.01.

Simple linear regression was conducted for women with and without PCOS combined in each population of PBMC, assessing the contribution of body composition, physical activity, dietary intake and PCOS status to global DNA methylation. PCOS status was negatively associated with global methylation in monocytes, T helper cells, T cytotoxic cells and in B cells (Table 4.3). We further explored any association between global DNA methylation and AMH, testosterone, and AUC glucose, all which are significantly higher in women with PCOS (see). AMH was associated with global DNA hypo-methylation in T helper cells and T cytotoxic cells, but not in in B cells or monocytes (Table 4.3).

Table 4.3: Simple linear regression analysis of *a-priori* co-variates in T helper cells, T cytotoxic cells, monocytes, B cells across all participants.

Covariate		Simple linear regression			
		T helper	**T cytotoxic**	**Monocytes**	**B Cells**
BMI	*Unadjusted B*	-0.29	0.007	0.29	0.32
	p-Value	0.897	0.98	0.97	0.531
	95% CI	[-0.491, 0.432]	[-0.45, 0.46]	[-1.77, 1.81]	[-0.71, 1.35]
Body Fat %	*Unadjusted B*	-0.97	-0.77	-0.304	0.33
	p-Value	0.569	0.63	0.63	0.36
	95% CI	[-.0441, 0.247]	[-0.40, 0.25]	[-1.59, 0.99]	[-0.39, 1.07]
Accel (MVPA mins.day)	*Unadjusted B*	0.054	-0.43	-1.62	-1.34
	p-Value	0.217	0.26	0.34	0.16
	95% CI	[-1.414,0.336]	[-1.3, 0.37]	[-5.03, 1.78]	[-3.3, 5.7]
IPAQ (MVPA mins.week)	*Unadjusted B*	0.004	0.003	-0.001	-0.14
	p-Value	0.459	0.57	0.96	0.25
	95% CI	[-0.006, 0.014]	[-0.008, 0.013]	[-0.43, 0.41]	[-0.37, 0.01]
Energy (g)	*Unadjusted B*	-326000	-4.80E-05	8.20E-05	0.00E+00
	p-Value	0.699	0.567	0.81	0.23
	95% CI	[0]	[0,0]	[-0.001, 0.001]	[0.00, 0.014]
PCOS status	*Unadjusted B*	**-7.52**	**-7.32**	**-28.45**	**-13.35**
	p-Value	**p<0.01**	**p<0.01**	**p<0.01**	**0.026**
	95% CI	**[-12.5, -2.5]**	**[-12.2, -2.5]**	**[-47.9, -8.9]**	**[-25.0, -1.7]**
AMH	*Unadjusted B*	**-0.12**	**-0.97**	-0.30	-0.03
	p-Value	**P<0.01**	**0.02**	0.06	0.78
	95% CI	**[-0.19, -.041]**	**[-0.17, -0.02]**	[-0.62, 0.18]	[-0.221,0.167]
Testosterone	*Unadjusted B*	-2.56	-1.58	-13.01	-2.20
	p-Value	0.17	0.39	0.07	0.61
	95% CI	[-6.29, 1.18]	[-5.29, 2.132]	[-27.26, 1.245]	[-10.76, 6.37]
AUC glucose	*Unadjusted B*	-0.01	-0.01	-0.06	-0.03
	p-Value	0.24	0.25	0.10	0.17
	95% CI	[-0.031, 0.008]	[-0.03, 0.008]	[-0.140, 0.015]	[-0.076, 0.014]

BMI, Body Mass Index; IPAQ, International Physical Activity Questionnaire; MVPA, Moderate Vigorous Physical Activity; Accel, Accelerometer; AMH, Anti-Müllerian Hormone; AUC, area under the curve.

Subset analysis revealed that AMH was associated with the global hypo-methylation in T helper cells in women with PCOS but not in women without PCOS (Figure 4.4). In

T cytotoxic cells the association between global DNA hypo-methylation and AMH was not specific to PCOS status (Figure 4.4).

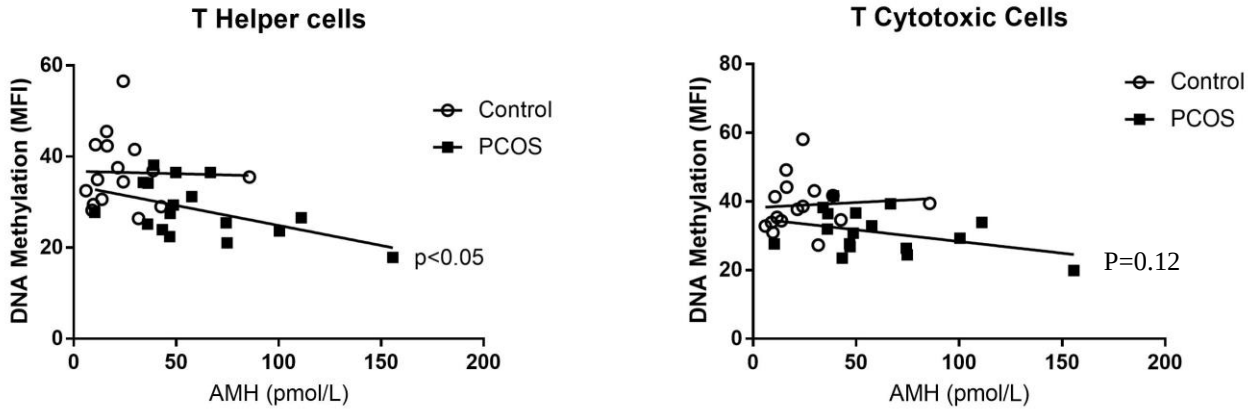


Figure 4.4: Regression analysis of AMH and association with 5-methylcytosine levels, a comparison between women with and without PCOS in T helper cells, T-cytotoxic cells.

4.5 Discussion

Summary of key findings

We report for the first time that women with PCOS display hypo-methylation in T helper cells, T cytotoxic cells, B cells and monocytes. This hypo-methylation was significantly associated with AMH in T cells (T helper cells and T cytotoxic cells) but not body composition, dietary intake or physical activity, despite these being well established regulators of DNA methylation. Interestingly, when subset analysis was conducted in T helper cells, the association between global DNA hypo-methylation and AMH only appeared relevant in women with PCOS, suggesting a cell-specific relationship with the PCOS hormonal milieu.

To the best of our knowledge, only one other study has investigated global DNA methylation in PBMC from women with and without PCOS and found no difference between those two groups (Xu, Azziz et al. 2010). However, it has been shown that in women with PCOS each immune cell displays distinct levels of global DNA methylation compared to women without PCOS (Adalsteinsson, Gudnason et al. 2012, Glossop, Nixon et al. 2013). Therefore, analysing PBMC as a whole may mask the differences in methylation between cell types and hide disease-specific levels of methylation and related signatures (Glossop, Nixon et al. 2013). Our data demonstrates that each immune cell has its own level of DNA methylation and highlights the importance of cell-specific methylation analysis. This has also been shown in previous work in T2DM, a common and related co-morbidity of PCOS. When fractionated into composite subpopulations, B cells showed differences in global methylation between lean and obese males and the natural killer (NK) cells showed a difference between lean men and men with T2DM (Simar, Versteyhe et al. 2014).

The S-adenosylmethionine (SAM) cycle is responsible for the transfer of the methyl group to DNA and therefore disruption to this cycle has been shown to alter DNA methylation levels (Crider, Yang et al. 2012). Methylation substrates; homocysteine, methionine, S-adenosylhomocysteine (SAH) and SAM are a part of this cycle. The cycle relies on the principle dietary source, folate, that regenerates methionine from homocysteine and is the rate limiting step in the SAM cycle (Crider, Yang et al. 2012). Multiple studies in animal models have shown low folate levels lead to an accumulation of homocysteine, impeding the cycle leading to low global methylation levels (Kim, Hong et al. 2009, Crider, Yang et al. 2012, Kim, Kim et al. 2013). Therefore, we measured key methylation substrates SAM, homocysteine and the methyl donor folate to detect differences in these substrates that could account for differences in DNA methylation levels observed between women with and without PCOS. There was no significant difference between women with and without PCOS in methylation substrates SAM or homocysteine, or the methyl donor folate. This suggests the global DNA hypo-methylation of immune cells in women with PCOS is not due to issues with donor or substrate availability of the SAM cycle.

It is well known that environmental factors such as physical activity habits and dietary intake can alter DNA methylation patterns including global methylation levels (Feil and Fraga 2012, Rasmussen, Zierath et al. 2014, Mendelson, Marioni et al. 2017). Obesity, as classified by BMI, is also known to alter DNA methylation patterns and levels (Wang, Zhu et al. 2010). Therefore, we examined whether these environmental factors were associated with the differences in global methylation in immune cell-subsets. We conducted simple linear regression with the aim to include any variables with a $p<0.1$

into a multiple regression model. We found that no measures of body composition (BMI and body fat percentage), physical activity measurements (subjective or objective measures) or diet (total energy) were associated with the global methylation in any of cell subsets. PCOS status, whether a women had PCOS or not, was significantly associated with global DNA methylation across the different cell types. This indicates that the observed hypo-methylation in cell-subsets was not due to common confounding variables.

Epigenetic programming such as DNA methylation plays a role in the immune cell differentiation, function, and recruitment (Lawson, Eleftheriadis et al. 2012). It has been hypothesized that infiltrating immune cells in various tissues (skeletal muscle and adipose tissue) could cause dramatic changes as seen in chronic diseases (Barres and Zierath 2011, Raghuraman, Donkin et al. 2016). In PCOS, altered DNA methylation in whole blood may be affecting the functioning of immune cells (Shen, Qiu et al. 2013, Li, Zhu et al. 2016). We further explored associations between global DNA methylation within each immune cell population and signs of inflammation, metabolic profile and reproductive hormones that are commonly found to be altered in women with PCOS. Anti-müllerian hormone (AMH) is a member of the transforming growth factor beta (TGF-β) superfamily that is predominantly expressed in ovaries but released into circulation at elevated levels in PCOS (Cassar, Teede et al. 2014). The TGF-β ligand superfamily functions in growth, embryonic development and immunity. Specifically, AMH is a negative regulator of follicle growth and is involved in follicular arrest of the pre-antral and antral follicles in the ovary (Dewailly, Andersen et al. 2014). AMH was elevated in women with PCOS consistent with other studies and further establishes that a potential biomarker for ovarian dysfunction and the reproductive phenotype displayed

517 in PCOS (Cassar, Teede et al. 2014). Interestingly, members of the TGF-β superfamily
518 are known to interact with the epigenome and control a variety regulatory epigenetic
519 signals such as chromatin remodelling, histone modification and DNA methylation (Bai
520 and Xi 2017). We found a negative association between AMH and global hypo-
521 methylation in T helper and T cytotoxic cells, suggesting AMH may play a role in the
522 aberrant epigenetic programming in these immune cells. Collectively, our results show
523 a cell-type specific alteration of global DNA methylation in immune cells in PCOS and
524 suggest an association with specific clinical features.
525
526 *Strengths and limitations*
527 The strengths of this study were that we used a well characterised, community recruited
528 women with and without PCOS, who were otherwise healthy. We used validated
529 methods to assess all parameters from glucose metabolism by OGTT and flow
530 cytometry. We also did not observe any significant difference in blood cell counts
531 between women with and without PCOS this rules out the potential masking affect by
532 altered blood counts. However, there were a number of limitations of our study include
533 the inability to distinguish the causality of the hypo-methylation in cell subsets. This
534 limits our capacity to clearly conclude whether methylation changes have any causal
535 impacts on molecular pathways. Our limited sample size affected our capacity to fully
536 explore potential confounding variables in order not to violate the assumptions of the
537 statistical model. Furthermore, sample size may have been underpowered to detect
538 more subtle associations between other variables and global DNA methylations.
539

4.6 Conclusion

Despite study limitations this study provided clear insights into specific immune cell global DNA methylation and is associated with PCOS. The influence of epigenetic factors in the development of PCOS is of increasing interest to explain the complexity and challenges of the inheritance and pathophysiology of the syndrome. This study has shown a clear role for immune cell-specific global DNA methylation in PCOS and requires further research to better understand epigenetics in PCOS including genome-wide DNA methylation profiling in immune cells.

CHAPTER 5. GENOME WIDE DNA METHYLATION AND TRANSCRIPTOME ANALYSIS IN T HELPER CELLS OF WOMEN WITH POLYCYSTIC OVARY SYNDROME

5.1 General background

In chapter 4, I provided evidence that women with PCOS are characterised by a global reduction of DNA methylation in specific immune cells (T Helper cells, T cytotoxic cells, monocytes and B cells). As global DNA methylation status can reflect environmental factors I explored global DNA methylation status and their relationships with established clinical/pathophysiological measures. Circulating AMH was negatively associated with global hypo-methylation in T cells (T helper and T cytotoxic cells) but not B cells or monocytes. Interestingly, when a subset analysis was conducted in the T helper cells, the association between global DNA hypo-methylation and AMH was only significant in women with PCOS, suggesting this may be an intrinsic effect of PCOS. While these findings show that immune cells are epigenetically regulated in PCOS, global DNA methylation does not provide any information on possible pathways, genes or loci that are affected or implicated in the pathophysiology and biological origins of PCOS.

Therefore, I expanded this analysis to further explore the altered global hypo-methylation by seeking to understand a concomitant change of genome-wide DNA methylation and the transcriptome in immune cells. Due to financial, and time constraints of my PhD timeframe, I focused on investigating the genome-wide transcriptome and methylome in T helper cells from women with and without PCOS. Firstly, due to finding a strong PCOS-specific relationship between global DNA methylation and AMH and secondly, T helper cells are crucial to the coordination of the adaptive immune response by regulating macrophages, B cells and T cytotoxic and have been implicated in chronic inflammatory diseases (Hirahara and Nakayama 2016).

Differential patterns of genome-wide DNA methylation have previously been found in whole blood between women with and without PCOS with many of sites related to immunity and immune cell function (Shen, Qiu et al. 2013, Li, Zhu et al. 2016). In this chapter I aim to significantly expand the knowledge of this data and our prior findings by focusing on the transcriptome and DNA methylome profile in one specific population of immune cells (T helper cells). This is important for analysing blood as it is a heterogeneous tissue and therefore analysing without any discrimination could potentially be hiding cell-specific and disease-specific changes that may play a significant role in the molecular mechanisms of PCOS (Adalsteinsson, Gudnason et al. 2012, Glossop, Nixon et al. 2013, Simar, Versteyhe et al. 2014). This was shown in healthy women, where it was found that there are unique genome-wide DNA methylation patterns when comparing T cells and B cells (Glossop, Nixon et al. 2013), a finding confirmed by (Mamrut, Avidan et al. 2015). Mumrut (2015) also found unique DNA methylation patterns seen in other populations of immune cells (B cells and monocytes). To date there is no literature whether each immune cell type displays a unique global or genome-wide DNA methylation pattern in PCOS. The aim of chapter 5 was to discover cell-specific transcriptome and DNA methylome markers that will inform on novel molecular pathways implicated in the biological origins and/or pathophysiology of PCOS. Ultimately informing further hypotheses for future mechanistic research in this complex condition.

The work contained in this chapter was funded by two NHMRC-CRE grants that I was awarded. I travelled to Copenhagen to work in laboratory of Professor Romain Barres lab at the Novo Nordisk Foundation Centre for Basic Metabolic Research at the University of Copenhagen. I worked in collaboration with Dr Rhianna Laker who

supervised my laboratory work to measure the DNA methylome and transcriptome using next generation sequencing techniques. As bioinformatics analysis of these data are complex and specialised, I worked in collaboration with the bioinformatician Dr Ali Altintas in the Barres laboratory.

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5.2 Abstract

Introduction: A number of studies have demonstrated differences in genome-wide DNA methylation patterns between women with and without PCOS in various tissues including adipose, granulosa cells and ovaries. Differential patterns of genome-wide DNA methylation in whole blood have demonstrated methylation in many sites related to immunity and immune cell function. To date, no study has investigated the genome-wide DNA methylation profile of specific cell populations in blood in PCOS. T helper cells are crucial to the coordination of the adaptive immune response by regulating macrophages, B cells and T cytotoxic cells. Therefore, we investigated the transcriptome and methylome in of T helper cells of women with and without PCOS to further elucidate the role of epigenetics in the aetiology of PCOS and potential novel molecular pathways affected.

Methods: Transcriptome and methylome analysis of T helper cells were conducted from the previously described cross-sectional study participants (chapter 4). Cryopreserved peripheral blood mononuclear cells (PBMC) were stained and sorted to collect T helper cells by fluorescence activated cell sorting (FACs) before RNA and DNA was extracted. RNA sequencing was performed according to Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold protocol. Genome-wide DNA methylation was assessed using reduced representation bisulphite sequencing.

Results: Thirty-seven genes were differentially expressed between women with and without PCOS. Thirty-three of these genes were down-regulated in women with PCOS and the remaining four were upregulated. KEGG analysis revealed that two pathways, cGMP signalling and BMP signalling, that were functionally enriched. 5 581 CpGs, 8 promoters and 5 genes were identified as differentially methylated between women with and without PCOS. Functional GO enrichment identified nnumerous differentially

methylated CpGs in T helper cells in pathways related to immune function and immune-mediated inflammation.

Conclusion: This is the first study to analyse the transcriptome and DNA methylome profile in one specific population of immune cells. These data show that having PCOS differentially impacts the transcriptome and methylome of T-helper cells compared to women without PCOS, which may be associated with the aberrant hormonal and metabolic milieu in PCOS. Further research is required for a better understanding of these mechanisms and their downstream effects in the aetiology of PCOS.

5.3 Introduction

The pathophysiology of PCOS is a complex endocrine condition with clinical features such as insulin resistance, hyperandrogenism and inflammation. However, the nuances of the relationships between these features are yet to be fully elucidated. It is hypothesised that these interactions are driven by the polygenic predisposition that is exacerbated by environmental factors such as obesity (Franks, McCarthy et al. 2006). Familial clustering of PCOS is well documented, providing evidence for a genetic contribution to the condition (Legro, Driscoll et al. 1998). Monozygotic twin studies have demonstrated the heritability of PCOS to be approximately 70% (Vink, Sadrzadeh et al. 2006). Genome wide association studies conducted in Chinese and European cohorts have identified fifteen significant PCOS risk loci in the genome. However, these account for only a small portion of the heritability (Chen, Zhao et al. 2011, Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015).

While genetics plays a role in the pathophysiology of PCOS, the environment (obesity and diet) and its interactions with genetics seems to be as important in PCOS pathogenesis. Gene-environmental interaction are termed epigenetics, which includes sustained modifications of the genome, such as DNA methylation, to facilitate the molecular mechanisms by which the environment interacts with the genome (Qiu 2006, Abbott, Nicol et al. 2013). There is emerging evidence supporting the role of inappropriate epigenetic programming in the aetiology of PCOS (Shen, Qiu et al. 2013, Wang, Wei et al. 2014). However, large gaps in knowledge remain as to how epigenetic modifications relate to PCOS and its associated sequelae.

Genome-wide DNA methylation analysis establishes differentially methylated loci or genes across the whole genome and can elucidate functional pathways affected by a condition. This is particularly useful for identifying biological origins and/or understanding the pathophysiological of complex conditions like PCOS. Recent studies have demonstrated differences in genome-wide DNA methylation patterns between women with and without PCOS in adipose tissue, granulosa cells, the ovaries, and whole blood (Wang, Wei et al. 2014, Yu, Sun et al. 2015, Kokosar, Benrick et al. 2016, Xu, Bao et al. 2016). Chapter 4 provided evidence that PCOS is characterised by a unique *global* hypo-methylation in T helper cells, T cytotoxic cells, monocytes and B cells. These findings suggest that epigenetically regulated immune cells may be implicated in/or be impacted by the pathophysiology of PCOS. Taken together with the whole blood study by Li et al. (2016) the data links epigenetic modifications to PCOS but did not elucidate cell specific changes to the methylome or potential consequences of these modifications. Each population of immune cell has a unique role in the immune system. T helper cells have a crucial role in the coordination of the adaptive immune response by regulating macrophages, B cells and T cytotoxic cells as well as being implicated in chronic low-grade inflammation in many diseases (Hirahara and Nakayama 2016). Therefore, the aim of this study was to explore if there is any cell-specific transcriptome and DNA methylome markers in T helper cells unique to PCOS that will inform novel molecular pathways implicated in the biological origins and/or pathophysiology of the syndrome. Ultimately this may inform novel hypotheses for future mechanistic research in this complex condition.

5.4 Methods

5.4.1 Study overview

Transcriptome and methylome analysis of T helper cells were undertaken on blood samples collected from the participants described in the cross-sectional study (details found in chapter four). Briefly, thirty-four women with and without PCOS were recruited from the community between 2014 and 2016. PCOS diagnosis was based on the Rotterdam criteria and were confirmed to have features consistent with the criteria (SS). A 75g oral glucose tolerance test (OGTT) and dual-energy X-ray absorptiometry were performed at Victoria University. Physical activity was monitored using a triaxial accelerometer and the international physical activity questionnaire (IPAQ). Food intake was assessed by a 7-day food diary. Blood was drawn immediately prior to the OGTT for the isolation of PBMC which, were stored in liquid nitrogen for fluorescence activated cell sorting (FACS) sorting and later analysis of genome-wide transcriptome and methylome. Reproductive markers, cytokines and systemic methyl donors, and substrates were also measured at baseline. This study was approved by the Victoria University Human Research Ethics Committee (HRE 14-138).

5.4.2 Cell sorting

Cryopreserved PBMC were quickly thawed and resuspended in RPMI before being washed and stained with anti-CD3 (APC), anti-CD4 (PE), anti-CD8 (BV510), anti-CD19 (BV421), anti-CD20 (PE-Cy7) and anti-CD14 (APC). All antibodies were sourced from BD Biosciences (Australia). The stained PBMC were washed twice in sort buffer [PBS 1%BSA, 25mM HEPES (Ph7), 1mM EDTA] before being sorted into four populations (monocytes, T-Helper Cells, T cytotoxic cells and B Cells) using the FACS-Aria (BD Biosciences, Europe) (

727 **Figure 5.1**). For the purpose of this thesis and the fact that T helper cells coordinate the
728 adaptive immune response (Hirahara and Nakayama 2016) only this cell population's
729 transcriptome and methylome analysis was conducted.
730

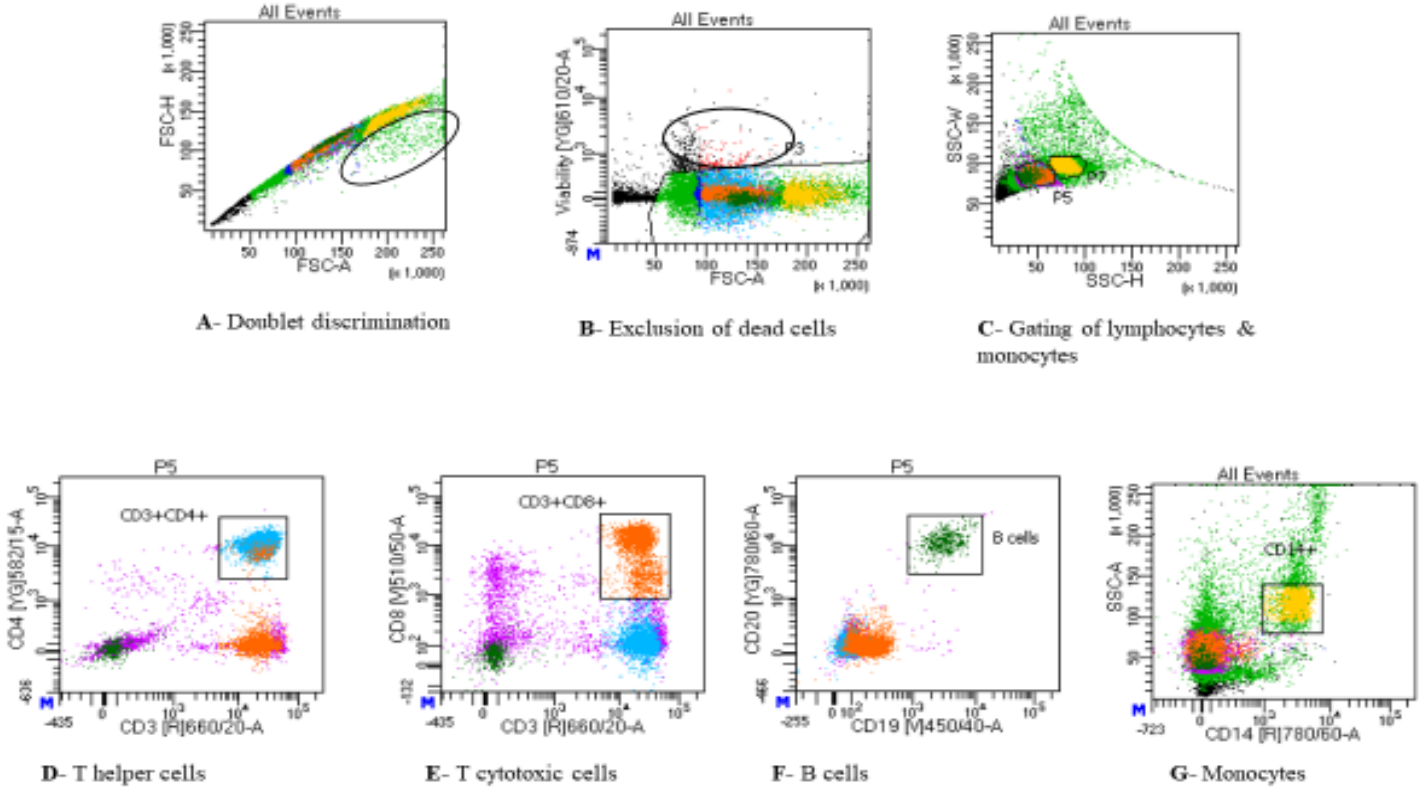


Figure 5.1: Representative FACS sort report of PBMC.
A- Doublet discrimination, doublets (circled) have double the area but the same height as single cells, **B-** Viability stain was used to exclude dead cells which are circled and shown in red, **C-** Lymphocytes (P5) and monocytes (P7) were gated based on their size [forward scattered light (FSC)] and granularity [side scattered light (SSC), **D, E** and **F** are from the P5 gate **D-** CD3+CD4+ T helper cells, **E-** CD3+CD8+ T cytotoxic cells, **F-** CD19+CD20+ B cells, **G-** is from the P7 gate and CD14+ were denoted as monocytes.

5.4.3 RNA sequencing

Total RNA was extracted using the Qiagen all prep DNA/RNA/miRNA universal kit (#80224) following manufacturer's instructions. Quality of RNA was established using the Agilent RNA 600 Nano kit and Bioanalyser instrument (Agilent Technologies). RNA sequencing was performed according to Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold protocol (Illumina) as previously described (Laker, Garde et al. 2017). Briefly, sample libraries (cDNA) were generated from total RNA. First the RNA was depleted for ribosomal RNA followed by a clean up with AMPure beads. The depleted RNA was fragmented for 4 min at 94 °C in Elute, Prime, Fragment High Mix (Illumina), and cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The cDNA was subjected to a clean up with AMPure beads (Beckman Coulter) and primed for adapter ligation. Adapters were diluted 1:20. After additional clean ups, with AMPure beads, cDNA fragments were amplified using polymerase chain reaction (PCR) techniques. Using a pre-defined cycle number this was based on the Ct threshold of the pre-PCR of each individual sample, samples were then subjected to 98 °C for 30mins then Ct threshold number of cycles of 98 °C for 10 secs, 60 °C for 30 secs and 72 °C for 30 secs and finally 72 °C for 5min followed by a final clean up. Each library was quantified to ensure optimum cluster densities across every lane of the flow cell using the Qubit dsDNA HS assay kit (Invitrogen). Quality control for base pair size and purity was assessed using the Agilent High Sensitivity DNA chip and Bioanalyser instrument (Agilent Technologies). Each library was diluted to 1nM before being pooled and measured on the Illumina Next Seq 500 (Illumina, San Franscisco).

5.4.4 DNA methylation sequencing

Genomic DNA was extracted using the Qiagen all prep DNA/RNA/miRNA universal kit (#80224) following manufacturer's instructions. Reduced representation bisulphite sequencing (RRBS) was performed using Diagenode Premium RRBS Kit (#C02030033) following manufacturer's instructions. Briefly, sample libraries were generated from 100ng of genomic DNA subjected to enzymatic digestion and then primed for adapter ligation. Ligated DNA fragments were subjected to a clean up using AMPure beads (Beckman Coulter). DNA fragments were amplified using the following PCR protocol: initial denaturation at 98°C for 3 minutes, 25 cycles of denaturation at 95°C for 15seconds followed by annealing at 60°C for 30seconds and finally extension at 72°C for 30seconds. The libraries (8 per lane) were then pooled using a programmed excel spreadsheet to ensure the libraries were of a similar concentration. The pooled libraries underwent bisulphite conversion before undergoing PCR enrichment and additional clean up using the AMPure beads. Once again, each library was quantified using the Qubit dsDNA HS assay kit (Invitrogen) and quality controlled for base pair size and purity using the Agilent High Sensitivity DNA chip and Bioanalyser instrument (Agilent Technologies). Genome-wide DNA methylation of T helper cells was measured on the Illumina Next Seq 500.

5.5 Bioinformatics analysis

All bioinformatics analysis was conducted with established laboratory algorithms using R software (https://www.r-project.org/about.html), as outlined below.

5.5.1 Transcriptome analysis

RNAseq raw reads were aligned to human genome (hg38) using STAR (Dobin, Davis et al. 2013) and gene coverages were computed by featureCounts (Liao, Smyth et al. 2014) using Gencode annotation (Harrow, Frankish et al. 2012). The ribosomal RNA counts were excluded from the downstream analysis. Libraries with less than 15 million assigned reads were removed from analysis. A generalised linear model (*y ~ 0 + disease*) was fitted for disease factor by using DEseq2 pipeline (Love, Huber et al. 2014). Genes with a false discovery rate (FDR) $q<0.1$ were considered differentially expressed.

Approximately 15 million reads/sample were assigned to genes with 38 genes surviving the expression threshold ($q<0.1$). Molecular functions and biological processes were established from the Universal Protein Resource (UniProt) which is a comprehensive resource for protein sequence and annotation data. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) database investigated the interaction of molecular pathways that were associated with the identified differentially expressed genes. This provided an *in-silico* indication of the molecular pathways that may be affected in PCOS.

5.5.2 DNA methylome analysis

Reduced representation bisulphite sequencing (RRBS) reads were processed with the 'rrbs' setting of Trim Galore v0.3.7 and Cutadapt v1.4.2. Processed reads were mapped to hg38 followed by derivation of CpG methylation using Bismark (Krueger and

Andrews 2011). Mappings to Y chromosome was removed for the differential methylation analysis. Differential methylation analysis was conducted on site and region level according to the sample groups (PCOS v CON) by using RnBeads pipeline (Assenov, Muller et al. 2014). The RRBS libraries with less than 1 million detected sites were excluded from downstream analysis. For each library, SNP-enriched sites were removed and sites with less than 10 counts were masked. Gene ontology (GO) enrichment analysis was conducted significant GO terms as determined by a hypergeometric test and a rank cut-off applied for the top 100 best ranking regions in RnBeads pipeline.

5.6 Results

The detailed information of PCOS phenotypes, metabolic profile, lifestyle and reproductive profile can be found in chapter 4 (Table 4.1 and 4.2).

5.6.1 Transcriptome analysis of T helper cells

Transcriptome analysis of T helper cells from the participants of the previously described cross-sectional study was conducted. A subset (n=11) of the original 34 participant samples survived the threshold (15 million reads/sample). Thirty-seven genes were differentially expressed between women with and without PCOS (Figure 5.2). Thirty-three of these genes were down-regulated and the remaining four were upregulated in women with PCOS. Molecular functions and biological processes were established from the Universal Protein Resource (UniProt). Details of the molecular functions and biological processes of the differentially expressed genes are detailed in Table 5.1. Seventeen of the genes were long non-coding genes (lncRNA) including

pseudogenes, long intergenic non-coding RNA (lincRNA) and Y RNA. Twenty were protein coding genes and were related to inflammatory and immune cell function.

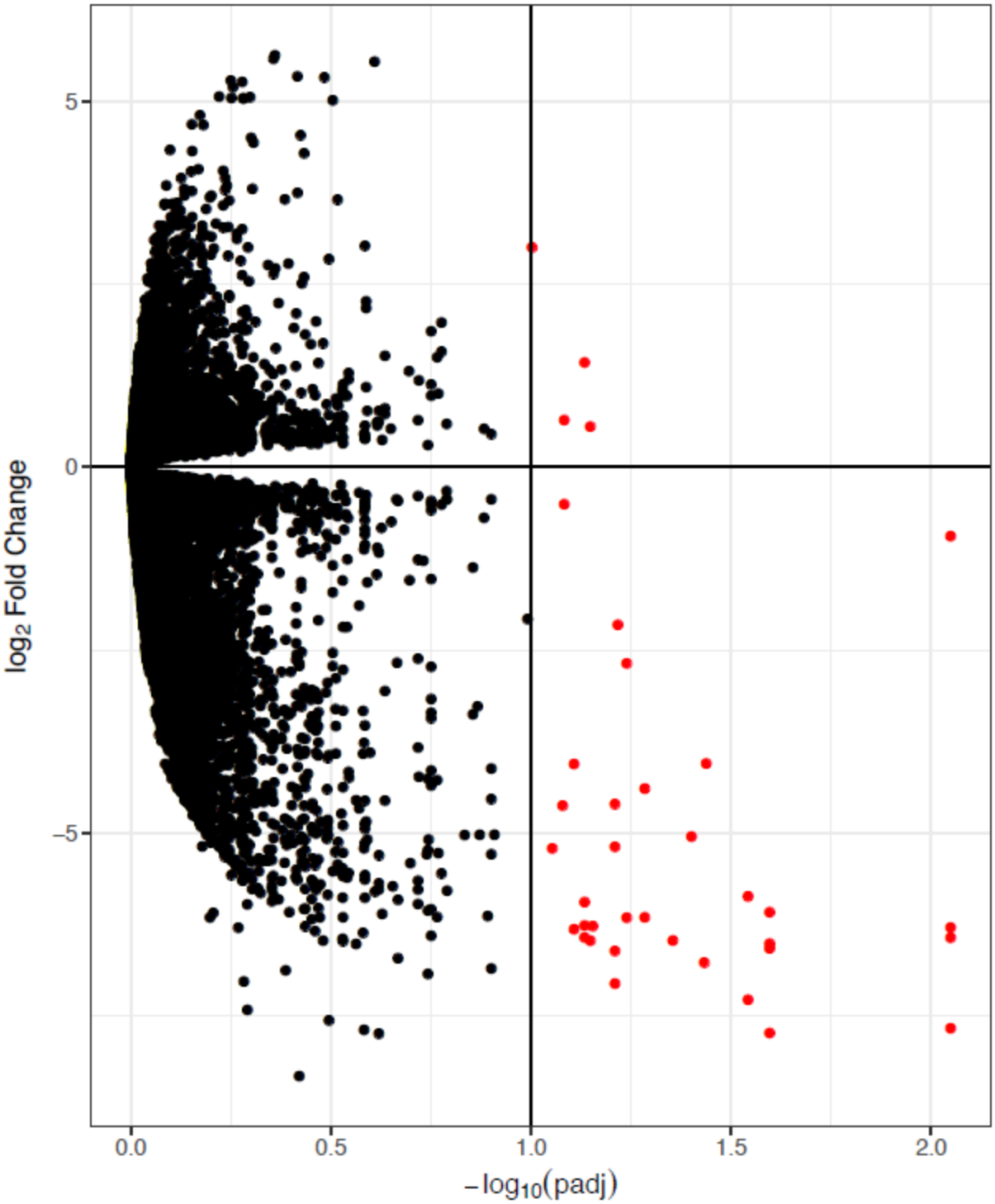


Figure 5.2: Volcano plots representing differentially expressed genes in T helper cells of women with PCOS compared to women without PCOS.
Red circles are statistically significant differentially expressed genes. False discovery rate (FDR) q<0.1

Table 5.1: Genes with significant differential gene expression listed in order of magnitude of log change in T Helper cells from women with PCOS compared to women without PCOS.

Down regulated genes in women with PCOS						
Gene name	**Gene ID**	**log2Fold Change**	**padj**	**Gene type**	**Molecular function**	**Molecular process**
ENSG00000227827	ENSG00000227827	-7.7	0.025	Pseudogene	Unknown	Unknown
Ring Finger Protein 217	RNF217	-7.7	0.009	Protein coding	metal ion binding, ubiquitin-protein transferase activity	Unknown
FK506 Binding Protein 1B	FKBP1B	-7.3	0.029	Protein coding	peptidyl-prolyl cis-trans isomerase activity	Unknown
Guanylate Cyclase 1 Soluble Subunit Alpha 2	GUCY1A2	-7.1	0.062	Protein coding	GTP binding, phosphorus-oxygen lyase activity guanylate cyclase activity, heme binding	Intracellular signal transduction, cyclic nucleotide biosynthetic process, positive regulation of cGMP biosynthetic process, adenylate cyclase activity
Caveolae Associated Protein 2	CAVIN2	-6.8	0.037	Protein coding	phosphatidylserine binding, phospholipid binding, protein kinase C binding	plasma membrane tubulation
Alcohol Dehydrogenase 4 (Class II), Pi Polypeptide	ADH4	-6.6	0.062	Protein coding	alcohol dehydrogenase (NAD) activity, all-trans retinal binding, ethanol binding, NAD binding	alcohol catabolic process, cellular aldehyde metabolic process, ethanol oxidation
Solute Carrier Family 5 Member 11	SLC5A11	-6.6	0.025	Protein coding	transporter activity	transmembrane transport
VWA8 Antisense RNA 1 (Non-Protein Coding)	VWA8-AS1	-6.5	0.025	lncRNA	Unknown	Unknown
ENSG00000234271	ENSG00000234271	-6.5	0.071	Processed pseudogene	Unknown	Unknown
ENSG00000257449	ENSG00000257449	-6.5	0.044	Antisense RNA	Unknown	Unknown
ENSG00000272787	ENSG00000272787	-6.4	0.009	LincRNA	Unknown	Unknown
Atypical Chemokine Receptor 2	ACKR2	-6.4	0.074	Protein coding	C-C chemokine receptor activity, scavenger receptor activity, G-protein coupled receptor activity	chemotaxis, inflammatory response, receptor-mediated endocytosis
Keratin 74	KRT74	-6.3	0.078	Protein coding	keratin filament binding, structural molecule activity	intermediate filament cytoskeleton organization, cornification, keratinization
ENSG00000252652	ENSG00000252652	-6.3	0.009	Y RNA	Unknown	Unknown
Protocadherin Gamma Subfamily B, 8 Pseudogene	PCDHGB8P	-6.3	0.070	Transcribed pseudogene	Unknown	Unknown
LOC101928238	LOC101928238	-6.3	0.074	LincRNA	Unknown	Unknown

Transmembrane And Coiled-Coil Domains 2	TMCO2	-6.2	0.058	Protein coding	Unknown	Unknown
ENSG00000237986	ENSG00000237986	-6.2	0.052	lncRNA	Unknown	Unknown
ENSG00000234193	ENSG00000234193	-6.1	0.025	lncRNA	Unknown	Unknown
Pannexin 3	PANX3	-5.9	0.074	Protein coding	wide pore channel activity, gap junction hemi-channel activity	cell-cell signalling, transmembrane transport, cation transport
ENSG00000202222	ENSG00000202222	-5.9	0.029	Y RNA	Unknown	Unknown
LOC101927851	LOC101927851	-5.2	0.088	LincRNA	Unknown	Unknown
Leucine Rich Repeat Containing 9	LRRC9	-5.2	0.062	Protein coding	Unknown	Unknown
EXTL3 Antisense RNA 1	EXTL3-AS1	-5.1	0.040	lncRNA	Unknown	Unknown
KIAA2012	KIAA2012	-4.6	0.083	Protein coding	Unknown	Unknown
ENSG00000253875	ENSG00000253875	-4.6	0.062	lncRNA	Unknown	Unknown
Cytochrome B Reductase 1	CYBRD1	-4.4	0.052	Protein coding	ferric-chelate reductase activity, protein binding, oxidoreductase activity, oxidizing metal ions	cellular iron ion homeostasis, response to iron ion, oxidation-reduction process
ENSG00000270147	ENSG00000270147	-4.1	0.078	LincRNA	Unknown	Unknown
ENSG00000229979	ENSG00000229979	-4.1	0.037	Processed pseudogene	Unknown	Unknown
Stearoyl-CoA Desaturase	SCD	-2.7	0.058	Protein coding	stearoyl-CoA 9-desaturase activity, iron ion binding, oxidoreductase activity	lipid metabolic process, fatty acid biosynthetic process, regulation of cholesterol biosynthetic process, oxidation-reduction process
NIMA Related Kinase 10	NEK10	-2.2	0.061	Protein coding	nucleotide binding, protein serine/threonine kinase activity, ATP binding, transferase activity	protein phosphorylation, positive regulation of protein autophosphorylation, positive regulation of MAP kinase activity, regulation of ERK1 and ERK2 cascade, regulation of cell cycle G2/M phase transition
Thymocyte Selection Associated Family Member 2	THEMIS2	-0.9	0.009	Protein coding	Protein Binding	immune system process, inflammatory response, cell adhesion, T cell receptor signalling pathway
CAMP-Dependent Protein Kinase Inhibitor Alpha	PKIA	-0.5	0.083	Protein coding	protein kinase inhibitor activity, cAMP-dependent protein kinase inhibitor activity,	negative regulation of transcription by RNA polymerase II, negative regulation of protein kinase activity, regulation of G2/M transition of mitotic cell cycle, negative regulation of protein import into nucleus, negative regulation of catalytic activity

1

Upregulated genes in women with PCOS						
Gene name	**Gene ID**	**log2Fold Change**	**padj**	**Gene name**	**Molecular function**	**Molecular process**
MX Dynamin Like GTPase 2	MX2	0.6	0.0711	Protein coding	nucleotide binding, GTPase activity, protein binding, microtubule binding	mitochondrial fission, immune system process, defence response, response to virus, response to interferon-alpha, innate immune response, mRNA transport
Small Cajal Body-Specific RNA 21	SCARNA21	0.6	0.082652	ScaRNA	Unknown	Unknown
Histone Cluster 1 H3 Family Member C	HIST1H3C	1.4	0.073504	Protein coding	DNA binding, protein binding, cadherin binding, protein heterodimerization activity	chromatin silencing at rDNA, nucleosome assembly, telomere organization, interleukin-7-mediated signalling pathway, negative regulation of gene expression, epigenetic, regulation of hematopoietic stem cell differentiation, regulation of gene silencing
SMAD Family Member 1	SMAD1	3.0	0.099453	Protein coding	RNA polymerase II proximal promoter sequence-specific DNA binding, signal transducer activity, downstream of receptor, transforming growth factor beta receptor, pathway-specific cytoplasmic mediator activity, protein homodimerization activity, co-SMAD binding, I-SMAD binding, primary miRNA binding	MAPK cascade, mesodermal cell fate commitment, osteoblast fate commitment, inflammatory response, transforming growth factor beta receptor signalling pathway, SMAD protein complex assembly, gamete generation, embryonic pattern specification, BMP signalling pathway

Molecular functions and biological processes were established from the Universal Protein Resource (UniProt). False discovery rate (FDR) $q<0.1$.

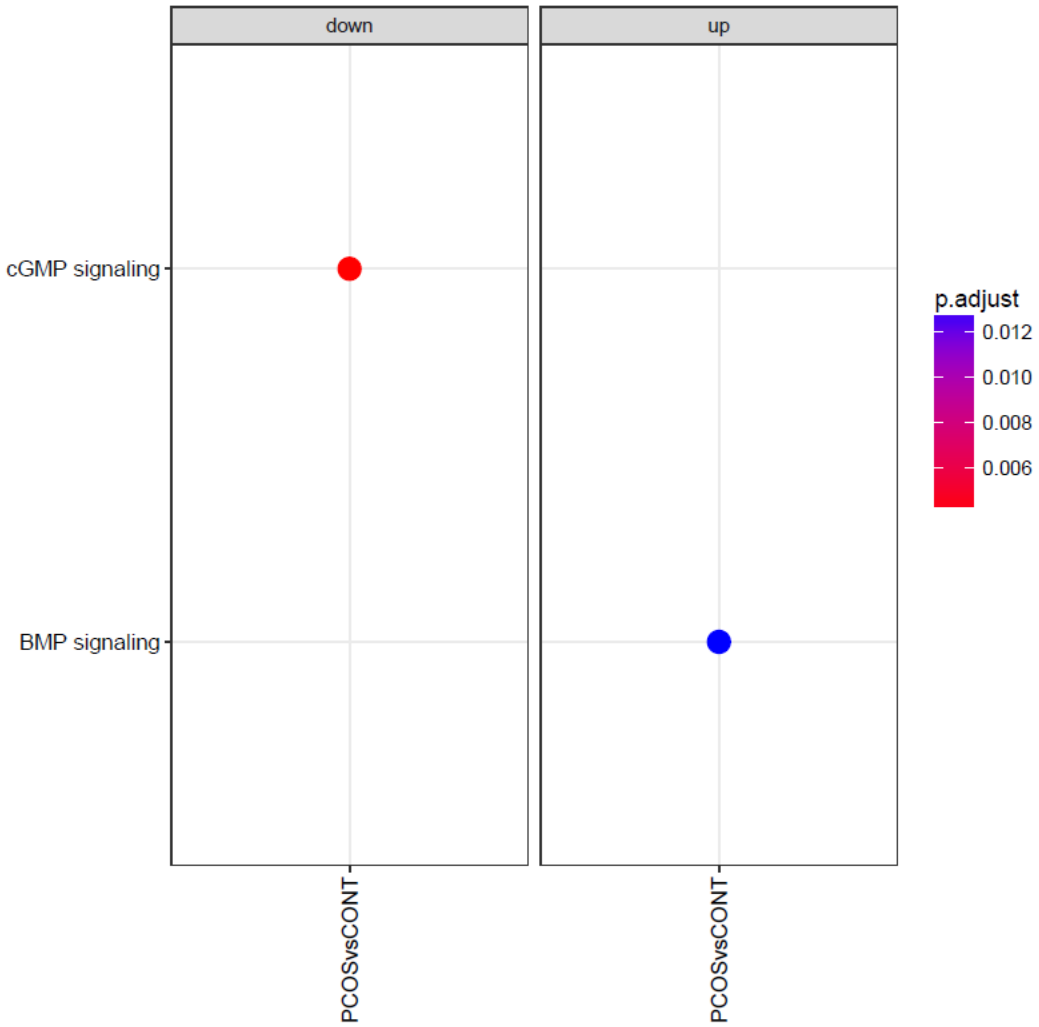
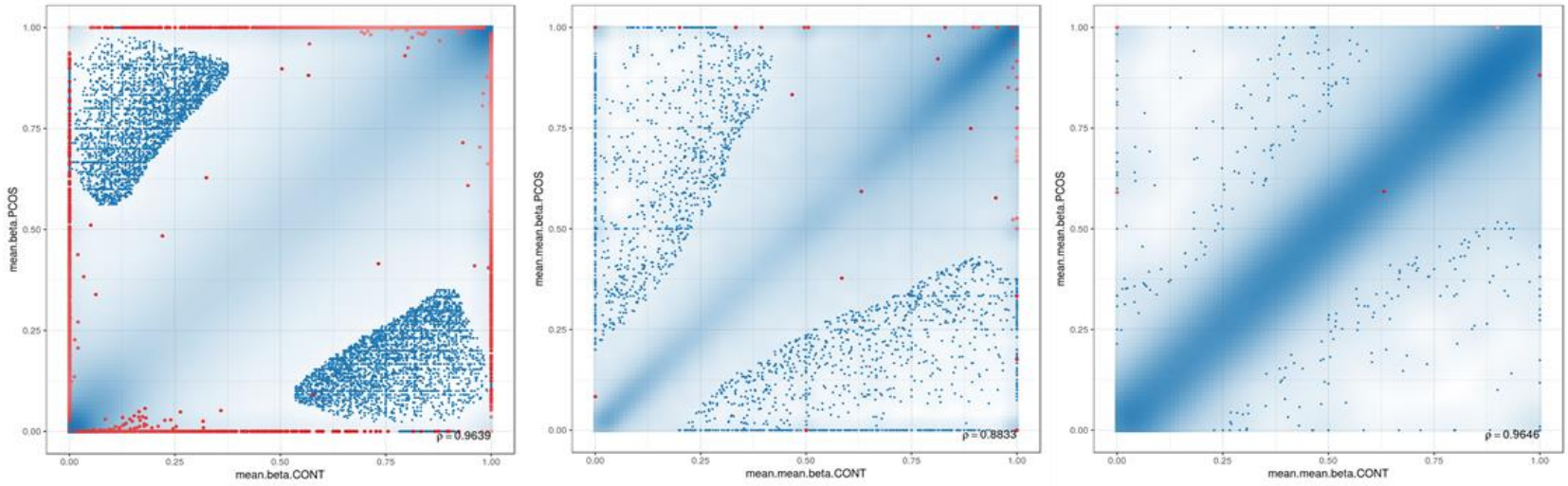


Figure 5.3: KEGG analysis of enriched pathways.
Kyoto encyclopaedia of genes and genomes (KEGG) analysis revealed that two enriched pathways, cGMP signalling and BMP signalling, were functionally enriched (Figure 5.3). BMP signalling were upregulated in women with PCOS whereas cGMP signalling was downregulated compared to women without PCOS.

5.6.2 Reduced representation bisulphite sequencing

Based on RRBS analysis we found 5581 CpGs, 8 promoters and 5 genes that were differently methylated in T helper cells between women with and without PCOS. Figure 5.4 is a visual display of the significant differentially methylated regions. In Table 5.2 the name, gene type and the GO annotations of function associated with the promoters and genes were displayed.



A- CpG sites **B- Promoters** **C- Genes**

Figure 5.4: Scatterplot displaying significant differentially methylated regions in T helper cells in women with PCOS compared to women without PCOS.
A- Differentially methylated CpG sites, **B-** Differentially methylated CpGs in promoters, **C-** Differentially methylated CpGs in genes. The X axis is percentage of methylated region in women without PCOS (CONT). The Y axis is percentage of methylated region in women with PCOS. Red circles were differentially methylated regions, FDR q<0.05.

Table 5.2: Genes and promoters of genes that had differentially methylated CpGs in T helper cells in women with PCOS compared to women without PCOS.

Genes			
symbol	**Gene Name**	**Chromosome**	**Function and gene type**
COX6CP15	cytochrome c oxidase subunit 6C pseudogene 15	chr10	pseudogene
SCGB1D4	Secretoglobin family 1D member 4	chr11	Regulation of chemotactic cell migration and invasion.
NA	AC025678.3	chr15	Tec protein tyrosine kinase- involved in T cell signalling and activation
NA	AP006565.1	chr18	Anti-sense RNA
NA	AC104301.2	chr20	ncRNA
Promoter of genes			
symbol	**Gene Name**	**Chromosome**	**Function and gene type**
NA	WW Domain Binding Protein 11 Pseudogene	chr2	pseudogene
SCGB3A2	secretoglobin family 3A member 2	chr5	receptor-mediated endocytosis
COX6CP15	Cytochrome C Oxidase Subunit 6C Pseudogene 15	chr10	pseudogene
SCGB1D4	secretoglobin family 1D member 4	chr11	regulation of chemotactic cell migration and invasion.
OVCH1	ovochymase 1	chr12	serine-type endopeptidase activity, hydrolase activity, metal ion binding, proteolysis
NA	AP006565.1	chr18	Anti-sense RNA
NA	AC104301.2	chr20	ncRNA
IGLJ2	Immunoglobulin Lambda Joining 2	chr22	Immunoglobin

GO Enrichment Analysis was then conducted with the differentially methylated CpGs, genes and promoters by identifying GO terms that are over-represented (or under-represented) by a hypergeometric testing. The Table 5.3, Table 5.4, Table 5.5 and Table 5.6 contain significant GO terms that were enriched as determined. Genes or promoters associated with differentially methylated CpGs in the gene ontology pathway are listed in order of magnitude of log change in T helper cells from women with PCOS compared to women without PCOS. Pregnancy, T cell proliferation or function, immune response, cytokine production, response to prolactin, responses to steroid hormones and inflammatory were functionally enriched pathways in the methylome.

Table 5.3: Hyper-methylated CpGs associated with genes in the gene ontology pathways in women with PCOS compared with women without PCOS.

GOMFID	Pvalue	OddsRatio	Term
GO:0045917	0.0022	741.4091	positive regulation of complement activation
GO:1990637	0.0029	494.2424	response to prolactin
GO:0033087	0.0037	370.6591	negative regulation of immature T cell proliferation
GO:0010519	0.0044	296.5091	negative regulation of phospholipase activity
GO:0060087	0.0044	296.5091	relaxation of vascular smooth muscle
GO:2000323	0.0044	296.5091	negative regulation of glucocorticoid receptor signalling pathway
GO:2000354	0.0044	296.5091	regulation of ovarian follicle development
GO:0043951	0.0051	247.0758	negative regulation of cAMP-mediated signalling
GO:0050847	0.0059	211.7662	progesterone receptor signalling pathway
GO:0071877	0.0059	211.7662	regulation of adrenergic receptor signalling pathway
GO:0010944	0.0066	185.2841	negative regulation of transcription by competitive promoter binding
GO:2001223	0.0066	185.2841	negative regulation of neuron migration
GO:0001787	0.0088	134.7273	natural killer cell proliferation
GO:0032740	0.0088	134.7273	positive regulation of interleukin-17 production
GO:0060452	0.0088	134.7273	positive regulation of cardiac muscle contraction
GO:0060766	0.0088	134.7273	negative regulation of androgen receptor signalling pathway
GO:0030238	0.0095	123.4924	male sex determination
GO:0034472	0.0095	123.4924	snRNA 3'-end processing
GO:0072540	0.0095	123.4924	T-helper 17 cell lineage commitment
GO:0007565	3e-04	29.0595	female pregnancy
GO:0002526	0.0051	21.9946	acute inflammatory response
GO:0043409	0.0058	20.5809	negative regulation of MAPK cascade
GO:0007186	0.0049	7.5918	G-protein coupled receptor signalling pathway
GO:0002376	0.0076	5.0598	immune system process

GOMFID, Go ontology categories in molecular function

Table 5.4: Hypo-methylated CpGs associated with genes in the gene ontology pathways in women with PCOS compared with women without PCOS.

GOMFID	Pvalue	OddsRatio	Term
GO:0034021	0.0017	1254.6154	response to silicon dioxide
GO:0071673	0.0026	627.2692	positive regulation of smooth muscle cell chemotaxis
GO:0032747	0.0034	418.1538	positive regulation of interleukin-23 production
GO:0045918	0.0034	418.1538	negative regulation of cytolysis
GO:0071315	0.0034	418.1538	cellular response to morphine
GO:0071672	0.0043	313.5962	negative regulation of smooth muscle cell chemotaxis
GO:1900227	0.0043	313.5962	positive regulation of NLRP3 inflammasome complex assembly
GO:0030223	0.006	209.0385	neutrophil differentiation
GO:0042045	0.0068	179.1648	epithelial fluid transport
GO:0071447	0.0068	179.1648	cellular response to hydroperoxide
GO:1902715	0.0068	179.1648	positive regulation of interferon-gamma secretion
GO:0097011	0.0077	156.7596	cellular response to granulocyte macrophage colony-stimulating factor stimulus
GO:0001821	0.0085	139.3333	histamine secretion
GO:0071803	0.0085	139.3333	positive regulation of podosome assembly
GO:0042268	0.0095	123.4621	regulation of cytolysis
GO:0042116	0.0013	43.6801	macrophage activation
GO:0051384	2e-04	31.5019	response to glucocorticoid
GO:0071346	0.0056	20.5852	cellular response to interferon-gamma
GO:0031424	0.0086	16.4096	keratinization
GO:0048545	0.004	11.2817	response to steroid hormone
GO:0006954	0.002	9.4855	inflammatory response
GO:0034097	9e-04	8.8543	response to cytokine
GO:0042221	0.0089	4.1296	response to chemical
GO:0014739	9e-04	Inf	positive regulation of muscle hyperplasia
GO:0072616	9e-04	Inf	interleukin-18 secretion

GOMFID, Go ontology categories in molecular function

Table 5.5: Hyper-methylated CpGs associated with promoters in the gene ontology pathways in women with PCOS compared with women without PCOS.

GOMFID	Pvalue	OddsRatio	Term
GO:0061402	0.0031	667.5217	positive regulation of transcription from RNA polymerase II promoter in response to acidic pH
GO:1900005	0.0031	667.5217	positive regulation of serine-type endopeptidase activity
GO:0002249	0.0047	333.7391	lymphocyte anergy
GO:0002667	0.0047	333.7391	regulation of T cell anergy
GO:0051365	0.0047	333.7391	cellular response to potassium ion starvation
GO:0060005	0.0047	333.7391	vestibular reflex
GO:1901318	0.0047	333.7391	negative regulation of flagellated sperm motility
GO:1904640	0.0047	333.7391	response to methionine
GO:0007598	0.0062	222.4783	blood coagulation, extrinsic pathway
GO:0046952	0.0062	222.4783	ketone body catabolic process
GO:0019401	0.0078	166.8478	alditol biosynthetic process
GO:0038170	0.0078	166.8478	somatostatin signalling pathway
GO:0071332	0.0078	166.8478	cellular response to fructose stimulus
GO:0031338	0	50.141	regulation of vesicle fusion
GO:0090630	0	37.7111	activation of GTPase activity
GO:0090174	1e-04	20.272	organelle membrane fusion
GO:0007218	0.0092	15.2478	neuropeptide signalling pathway
GO:0016050	0.0024	8.1902	vesicle organization
GO:0002250	0.0096	7.6078	adaptive immune response
GO:0051345	5e-04	7.3514	positive regulation of hydrolase activity
GO:0043087	0.0044	6.8756	regulation of GTPase activity
GO:0009617	0.0049	6.6545	response to bacterium
GO:0051049	1e-04	5.6843	regulation of transport
GO:0006886	0.001	5.4259	intracellular protein transport
GO:0061024	0.0073	4.7686	membrane organization
GO:0015833	0.0064	3.5469	peptide transport
GO:0045184	0.0076	3.4329	establishment of protein localization
GO:0046327	0.0016	Inf	glycerol biosynthetic process from pyruvate

GOMFID, Go ontology categories in molecular function

Table 5.6: Hypo-methylated CpGs associated with promoters in the gene ontology pathways in women with PCOS compared with women without PCOS.

GOMFID	Pvalue	OddsRatio	Term
GO:0019516	0.0022	960	lactate oxidation
GO:0060697	0.0022	960	positive regulation of phospholipid catabolic process
GO:0010387	0.0033	479.9688	COP9 signalosome assembly
GO:0044375	0.0033	479.9688	regulation of peroxisome size
GO:0010916	0.0044	319.9583	negative regulation of very-low-density lipoprotein particle clearance
GO:0034447	0.0052	255.9	very-low-density lipoprotein particle clearance
GO:0010898	0.0066	191.95	positive regulation of triglyceride catabolic process
GO:0010890	0.0077	159.9479	positive regulation of sequestering of triglyceride
GO:0001561	0.0088	137.0893	fatty acid alpha-oxidation
GO:0016559	0.0088	137.0893	peroxisome fission
GO:0033089	0.0099	119.9453	positive regulation of T cell differentiation in thymus
GO:0034371	0.0099	119.9453	chylomicron remodelling
GO:0034382	0.0099	119.9453	chylomicron remnant clearance
GO:0051006	0.0099	119.9453	positive regulation of lipoprotein lipase activity
GO:0034377	4e-04	85.2056	plasma lipoprotein particle assembly
GO:0070328	4e-04	78.641	triglyceride homeostasis
GO:0071825	0.0011	47.4977	protein-lipid complex subunit organization
GO:0031424	7e-04	20.886	keratinization
GO:0055088	0.0059	19.7515	lipid homeostasis
GO:1905952	0.0084	16.3839	regulation of lipid localization
GO:0009913	0.0028	12.544	epidermal cell differentiation
GO:0043588	0.0052	9.9451	skin development
GO:0010902	0.0011	Inf	positive regulation of very-low-density lipoprotein particle remodelling
GO:0019244	0.0011	Inf	lactate biosynthetic process from pyruvate
GO:0046296	0.0011	Inf	glycolate catabolic process

GOMFID, Go ontology categories in molecular function

5.7 Discussion

In chapter 4 we found that women with PCOS displayed hypo-methylation in monocytes, T helper, T cytotoxic and B cells compared to women without PCOS. From this analysis alone, we were not able to conclude whether these differences have any causal impacts on molecular pathways. Therefore, we investigated the transcriptome and methylome of T helper cells in both women with and without PCOS. These data show that having PCOS differentially impacts the transcriptome and methylome of T-helper cells compared to women without PCOS. Specifically, cGMP signalling and BMP signalling were functionally enriched in the transcriptome. Genes were differentially methylated in pathways relating to pregnancy, T cell proliferation or function, immune response, cytokine production, response to prolactin, responses to steroid hormones and inflammation.

5.7.1 Transcriptome Analysis

Thirty-seven genes were differentially expressed between women with and without PCOS in T helper cells. To our knowledge only one other study has investigated the transcriptome in peripheral blood (Su, Ma et al. 2017) similarly to our results the differential genes expressed were functionally enriched in inflammatory response and immune response. Our data supports Su et al. findings that inflammatory response and indeed the immune system may be associated with PCOS.

KEGG analysis identified that cGMP signalling and the BMP signalling pathway were functionally enriched based on the differential genes expressed. Specifically, the BMP signalling pathway was upregulated in PCOS compared to women without PCOS. In these women with PCOS we found circulating AMH levels which was associated with the global

hypo-methylation in T cells (T helper and T cytotoxic cells) chapter 4. Interestingly, AMH and BMP are both members of the TGF-β ligand superfamily (Akhurst and Hata 2012). AMH's specific receptors are not ubiquitously expressed, current literature indicates that the receptors are solely located in ovarian tissue. However, AMH can signal through other TGF-β ligand receptors that may provide an alternate signalling pathway in non-reproductive tissue (Josso and Clemente 2003, Kuczma, Kurczewska et al. 2014, Martinez, Sacedon et al. 2015). The elevated circulating AMH in women with PCOS, may be upregulating the BMP signalling in T helper cells via bone morphogenic protein receptors (BMPR). Both AMH and BMPs activate the same downstream intracellular signalling molecules Smads 1/5/8. These Smads are collectively termed bone morphogenic protein receptors (BR-Smads), that assemble into a complex with Smad 4 (Co-Smad) and translocate into the nucleus and activate a diverse range of genes that is dependent on the cellular context (Nohe, Hassel et al. 2002, Josso and Clemente 2003, Martinez, Sacedon et al. 2015). In T cells, the BMP canonical signalling role is to regulate activation and homeostasis of circulating naïve immune cells, (Martinez, Sacedon et al. 2015). Therefore, it is possible that AMH may upregulate BMP signalling pathway and impact proliferation and homeostasis of T helper cells, which may lead to inflammation due to hyperactivity of T cells, however further investigation is required (Josso and Clemente 2003, Yoshioka, Ono et al. 2012).

cGMP signalling was down-regulated according to KEGG analysis. cGMP is a intracellular secondary messenger that has a diverse array of protein targets resulting in wide-ranging effects that can differ by cell and tissue type (Denninger and Marletta 1999). Interestingly, the nitric oxide/cGMP/cGMP-activated protein kinase (cGK) pathway is a negative regulator of T cell activation and regulation by inhibiting the release of IL-2. IL-2 regulates T cells by suppressing

or activating the cells dependent on environmental milieu to prevent hyperactivity of these cells (Fischer, Palmetshofer et al. 2001, Ahluwalia, Foster et al. 2004). Down-regulation of cGMP signalling genes and its downstream effects in T helper cells provides a new avenue to investigate inflammation and its potential roles in the pathophysiology of PCOS.

Interestingly, many of the genes identified from the transcriptome analysis are non-protein-coding genes, the majority of which are collectively termed long non-coding RNAs (lncRNAs) (Johnsson, Lipovich et al. 2014). Our analysis identified a range of lncRNAs including; pseudogenes, anti-sense RNA and long intergenic non-coding protein RNAs (lincRNA). The function of lncRNAs are only beginning to be characterised but they appear to modulate gene expression via RNA:RNA, RNA:DNA and RNA:protein interactions (Atianand and Fitzgerald 2014). LncRNAs have been shown to play a role in related co-morbidities of PCOS including T2DM (Kornfeld and Bruning 2014, Knoll, Lodish et al. 2015) and inflammatory disorders (Carpenter, Aiello et al. 2013). In immune cells, lncRNAs have been shown to be widely expressed and regulate gene expression particularly during development, differentiation and activation (Atianand and Fitzgerald 2014). There is also increasing evidence that suggests that lncRNAs can act as a molecular scaffold for epigenetic modifications including DNA methylation and therefore play a role in epigenetic programming (Spitale, Tsai et al. 2011, Rinn and Chang 2012). The finding that many of the differential genes expressed in T helper cells are lncRNAs suggests a potential molecular mechanism for epigenetic programming in immune cells. LncRNAs gene expression is specific to cell type further highlighting the importance of cell-specific analysis (Knoll, Lodish et al. 2015). As the role LncRNAs in regulation is still in infancy further research is required to elucidate the role of these genes in PCOS both at a cellular and tissue level.

5.7.2 DNA methylome analysis

To the best of our knowledge, this is the first study to examine the genome-wide DNA methylome within a single population (T helper cells) of peripheral blood cells. This allows us to identify molecular and cellular pathways related to PCOS that are specific to T helper cells. From 30 participants, 5581 CpGs, 8 promoters and 5 genes were identified as differentially methylated between women with and without PCOS in T helper cells. Consistent with our enrichment analysis for the methylome in T helper cells, Li et al (2016) found enrichment in GO terms related to immune function and immune-mediated inflammation from their DNA methylation analysis in whole blood. Further, Li et al. (2016) also provided the first evidence linking DNA methylation and altered prolactin regulation in PCOS (Li, Zhu et al. 2016). Women with PCOS have lower levels of circulating prolactin (independent of BMI) and these can be associated with adverse metabolic profile however the literature is somewhat controversial in regards to prolactin in PCOS, further investigation is still required. (Glintborg, Altinok et al. 2014). Prolactin is responsible for proliferation, differentiation and maintenance of immune cells numbers (Yu-Lee 2002). We found hyper-methylation of the CpGs near genes related to prolactin regulation providing further evidence that there may be dysregulation of prolactin and this may have a functional role in PCOS and its aetiology.

Functional gene ontology enrichment indicated that promoter regions were hypo-methylated in pathways that related to lipid transport and metabolism, and regulation of T cell proliferation. Altered lipid homeostasis underlies many chronic diseases, such as obesity, insulin resistance, non-alcoholic fatty liver disease and cardiovascular disease (CVD) all of which are co-morbidities of PCOS (Hubler and Kennedy 2016, Narayanan, Surette et al. 2016). Further, endogenous lipid metabolism can regulate T helper cell activation, differentiation and function

(Hubler and Kennedy 2016, Howie, Ten Bokum et al. 2017). Metabolic reprogramming in response to the tissue environment can ultimately influence immune polarisation and can lead to a pro- or anti-inflammatory phenotype (Berod, Friedrich et al. 2014, Hubler and Kennedy 2016). While represented in GO enrichment of T helper methylome we did not identify any genes that were dysregulated in the transcriptome analysis that were associated with lipid homeostasis.

Many of the hyper-methylated promoters were enriched in cell-cell communication processes, and vesicle trafficking, and regulation. Vesicle trafficking regulates chemotaxis (Traynor and Kay 2007, Colvin and Luster 2011), an essential process that directs the immune cells and inflammatory processes (Colvin and Luster 2011). Interestingly the gene body and promoter region of secretoglobin family 1D member 4 (SCGB1D4) were differentially methylated and according to gene ontology annotation play a role in chemotaxis and cell-cell signalling. Functional analysis of this differentially methylated gene and its associated affects (i.e. Chemotaxis) in T helper cells needs to be investigated.

5.7.3 Strengths and Limitations

The strengths of this study are that the participants were well characterised and recruited from a community population. Further, this is the first study to provide evidence of a link between an alerted T helper cell methylome, and transcriptome in PCOS. While these results provide novel pathways in both the methylome and transcriptome, these data should be interpreted with caution. There was a lack of alignment between the transcriptome and methylome analysis and the need for validation of both the transcriptome and DNA methylome analysis results. The lack of overlap between the transcriptome and methylome may be for one of two reasons. The

first being the limited samples size in the transcriptome analysis. Many participants samples had to be excluded from the analysis due not meeting quality control threshold (15million reads/sample). Most likely due a low starting input for the RNA sequencing that caused technical issues. However, from the samples that survived quality control threshold we can assume the findings are of high quality. This smaller sample size may result in a under representation in the differential genes expressed in T helper cells but do set a platform to explore these relationships in future larger scale studies. Secondly, we identified many lncRNAs that have not been characterised therefore may be why there is lack of overlap between the transcriptome and methylome. Characterisation of the lncRNAs identified and their potential down-stream effects would provide additional clues as to the molecular mechanisms of PCOS.

5.8 Conclusion

This is the first study to analyse the transcriptome and DNA methylome profile in one specific population of immune cells. These data show that having PCOS differentially impacts the transcriptome and methylome of T-helper cells compared to women without PCOS. Specifically, cGMP signalling and BMP signalling were functionally enriched in the transcriptome. Many functionally enriched pathways in the methylome related to immune response and function and were specific to T cells. Demonstrating that PCOS has an altered methylome and transcriptome in immune cells, that may be associated with the aberrant hormonal and metabolic milieu in PCOS. Further research will be required for a better understanding of these mechanisms and their downstream effects in the aetiology of PCOS. This work is the first step in advancing our understanding of the role of epigenetics in the pathophysiology of PCOS.

CHAPTER 6. MOLECULAR MECHANISMS OF INSULIN RESISTANCE INVOLVING INSULIN SIGNALLING AND TGFB IN SKELETAL MUSCLE OF WOMEN WITH PCOS: THE IMPACT OF EXERCISE.

6.1 General background

The previous chapters have explored the contribution of genetics and DNA methylation to biological origins and pathophysiology to PCOS. While these provide partial explanation and are indeed involved, there is a compelling case that insulin resistance may play a leading role in the complex aetiology of PCOS. Insulin resistance has been postulated to play a central aetiological role in PCOS and contributes to both the metabolic and reproductive features of PCOS (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Harrison, Stepto et al. 2012, Stepto, Cassar et al. 2013, Shorakae, Boyle et al. 2014, Moran, Norman et al. 2015, Cassar, Misso et al. 2016). Specifically, it is thought that the intrinsic insulin resistance or obesity-independent insulin resistance to be the potential mechanism in the multifactorial aetiology of PCOS (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Stepto, Cassar et al. 2013, Moran, Norman et al. 2015, Cassar, Misso et al. 2016). Previous studies have implicated impaired insulin signalling within the skeletal muscle of women with PCOS. However, additional *in vivo* and *in vitro* human research is warranted to better understand the molecular mechanisms of insulin resistance in PCOS.

In chapter 6 I will explore this in a separate but related series of studies on the molecular mechanisms of PCOS. I will explore the mechanisms of insulin resistance in skeletal muscle and the impact of obesity and exercise. Specifically, I will investigate:

1. The potential insulin signalling defects that may help explain the intrinsic insulin resistance associated with PCOS.
2. A new hypothesis that excess stromal deposition regulated by the TGFβ ligands may apply to metabolic tissues like skeletal muscle and interfere with signalling and

increased extracellular matrix, limiting insulin and glucose movement across the interstitial space.

3. Impact of exercise training on the mechanisms of insulin resistance in PCOS.

The cross-sectional study and randomised control trial reported in this chapter were the final analysis from a larger study undertaken by my supervisor (Professor Nigel Stepto) and was a collaboration between Monash, Adelaide and Victoria Universities, the study was funded by the NHMRC APP606553. My role in this study was collecting data (immuno-blotting), data analysis and interpretation. I co-wrote the manuscript and therefore as a major contributor I am co-first author. This chapter is written as manuscript and is under review in the Journal of Endocrinology and Metabolism.

Collaborators

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6.2 Abstract:

Context. The aetiology of insulin resistance in Polycystic Ovary Syndrome (PCOS) remains ill-defined, contributing to controversy over diagnostic criteria, and a lack of optimal therapies.

Objective. To investigate whether women with PCOS have altered early insulin signalling and its association with aberrant TGFβ network gene expression. To clarify the effect of exercise therapy on these pathways.

Design. A clinical trial comprising a cross-sectional study with a prospective controlled intensive exercise intervention sub-study. The clinical trial registration number is ISRCTN84763265.

Setting. University and hospital setting with women recruited from the community.

Participants. Women with or without PCOS.

Intervention. A subset of overweight participants also undertook 12 weeks of intensified supervised exercise training.

Main Outcome Measure. We examined the activation of proteins in the insulin signalling pathway before and 30 min into a euglycaemic-hyperinsulinaemic clamp and TGFβ ligand signalling network gene expression in skeletal muscle.

Results. We found dysfunctional signalling at protein kinase B/Akt and signalling proteins up and downstream, including AS160, mTOR and atypical PKC. A 12-week program of intensified aerobic exercise improved but did not rescue insulin stimulated signalling at Akt, AS160, and mTOR in overweight women with PCOS. A number of genes in the TGFβ signalling pathway were upregulated but unresponsive to exercise training.

Conclusions. Our data provide new insights regarding defects in early insulin signalling in skeletal muscle of women with PCOS. The data support a significant role of aberrant signalling via Akt/PKB and its associated activator and inhibitor proteins in PCOS-specific insulin

resistance. In addition, our data indicates a potential role of the TGFβ superfamily ligands and resulting tissue fibrosis in the molecular mechanisms of PCOS-specific insulin resistance. It also demonstrates suboptimal responses of women with PCOS to exercise therapy, and highlight a novel pathway for investigation.

Key words: Akt/PKB, exercise training, insulin resistance, polycystic ovary syndrome, obesity, skeletal muscle.

6.3 INTRODUCTION

Polycystic ovary syndrome (PCOS) affects 6-10% of reproductive aged women (Bozdag, Mumusoglu et al. 2016) and has major metabolic [increased type 2 diabetes mellitus and cardiovascular risk factors] (Moran, Lombard et al. 2010), reproductive [leading cause of anovulatory infertility] (Teede, Misso et al. 2011) and psychological [anxiety and depression] (Cooney, Lee et al. 2017) impacts, representing a substantial health burden. On meta-analysis the risk of type 2 diabetes in PCOS is increased 4.4 fold independent of BMI (Moran, Misso et al. 2010). Despite a high prevalence of PCOS and considerable health implications, the aetiology and ideal therapies to treat the metabolic and reproductive consequences of PCOS remain unclear. Insulin resistance is a central characteristic in the majority of affected women (Stepto, Cassar et al. 2013, Cassar, Misso et al. 2016), driving both hyperandrogenism and clinical features, yet the underlying mechanisms of insulin resistance in PCOS remain ill-defined (Diamanti-Kandarakis and Dunaif 2012). Therapeutic strategies in PCOS include medical therapy (metformin) and weight management via exercise and diet (Teede, Misso et al. 2011, Legro, Arslanian et al. 2013), these all reduce but do not reverse insulin resistance and fail to optimally treat PCOS. In this context, greater insight into aetiology of insulin resistance in PCOS is needed.

Based on euglycaemic-hyperinsulinaemic clamp data, prevalence of insulin resistance has been reported to range from 75 to 95% in women with PCOS diagnosed by Rotterdam criteria (Stepto, Cassar et al. 2013). PCOS has been shown to comprise of a unique PCOS related insulin resistance (intrinsic insulin resistance) that is compounded by BMI-related insulin resistance (extrinsic insulin resistance) (Diamanti-Kandarakis and Dunaif 2012, Stepto, Cassar et al. 2013, Cassar, Misso et al. 2016). Intrinsic insulin resistance in PCOS is likely due to a

dysfunctional response to insulin in metabolically active peripheral tissues including adipose tissue and skeletal muscle. As skeletal muscle accounts for 70-80% of insulin stimulated glucose uptake (Lundsgaard and Kiens 2014) any defect in this tissue may have profound effects on whole body insulin sensitivity. Recently Raja-Khan et.al. (2014) proposed an alternative hypothesis that dysfunctional TGFβ signalling regulated by fibrillin's and latent TGFβ binding proteins may lead to increased organ stroma or fibrosis predisposing women with PCOS to insulin resistance (Hatzirodos, Bayne et al. 2011, Tal, Seifer et al. 2013, Yang, Zhong et al. 2015, Bastian, Bayne et al. 2016, Roh, Yoon et al. 2017). The role of this aberrant extracellular matrix (ECM) remodelling or tissue fibrosis and TGFβ in the aetiology of PCOS related insulin resistance has not been investigated.

In this context, we hypothesised that the women with PCOS will have altered early insulin signal transduction compared to BMI matched controls and this would be associated with remodelling of the ECM and TGFβ signalling network gene expression. We aimed to examine the activation/phosphorylation of proteins in both the proximal and distal parts of the insulin signalling cascade before and 30 minutes into a euglycemic hyperinsulinaemic insulin clamp in women with or without PCOS (spanning lean and obese BMIs). Our secondary aim was to investigate if exercise training impacted any aberrant skeletal muscle insulin signalling and gene expression of the ECM and TGFβ ligand signalling network.

6.4 Methods

6.4.1 Participants:

The participants from this study are a subset of women who participated in our previously published studies (Hutchison, Stepto et al. 2011, Hutchison, Teede et al. 2012, Harrison,

Lombard et al. 2013, Stepto, Cassar et al. 2013). Specifically, we included fifty-nine of the original cohort (n=79) of premenopausal adult women with or without PCOS. The women were categorised according to PCOS status and matched for BMI. Confirmation of PCOS diagnosis was undertaken by expert endocrinologists (SKH, AEJ and HJT) based on Rotterdam criteria. The Southern Health Research Advisory and Ethics Committee approved the study and participants gave written informed consent. The clinical trial registration number is ISRCTN84763265.

6.4.2 Study Design:

Data were collected at baseline in all women (after three-month washout of medications where appropriate) and following 12 weeks of exercise training (subgroup of overweight women with [n=8] or without PCOS [n=8]) in the follicular phase of the menstrual cycle wherever feasible.

6.4.3 Exercise Intervention:

The subgroup of overweight to obese participants consisting of women without PCOS (controls) (n=8) and women with PCOS (n=8) undertook 12 weeks of supervised, progressive, intensified exercise training on a motorised treadmill as described previously (Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012, Hutchison, Teede et al. 2012).

6.4.4 Clinical and Biochemical Measurements

Participants were assessed for anthropometric measures including body weight, height, body composition, abdominal visceral fat (VF) and subcutaneous fat (SCF) and, waist and hip circumference as previously reported (Hutchison, Stepto et al. 2011, Stepto, Cassar et al. 2013). Insulin sensitivity was assessed by the insulin clamp technique as previously reported (Stepto,

Cassar et al. 2013). Stored blood samples were batch analysed for fasting glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, insulin, testosterone and HbA1c. Low-density lipoprotein and the homeostatic model insulin resistance assessment (HOMA) were calculated as previously described (Meyer, McGrath et al. 2005).

6.4.5 Muscle Samples:

Thigh vastus lateralis muscle samples were obtained by percutaneous biopsy under local anaesthesia (Hutchison, Teede et al. 2012) immediately prior to and 30 minutes into the insulin clamp. Muscle biopsies were immediately frozen in liquid nitrogen and then stored at -80°C for subsequent analysis.

6.4.6 Muscle Protein Extraction and Analyses (Western blots):

Protein extraction and western blotting was performed as previously described (Hutchison, Teede et al. 2012). Briefly equal quantities of protein were resolved by SDS-PAGE (Bio-Rad, Criterion TGX Gels), transferred to a PVDF membranes (Bio-Rad, Turbo-Blot) using optimised protocols, blocked with TBST (10mM Tris; 10% Tween 20) containing 5% skim milk washed for 4 by 5 mins in TBST and immunoblotted overnight at 4°C with primary antibodies. Specific targets were insulin receptor (IR; abcam ab5500), tyrosine phospho-IR $tyr^{1162/1163}$ (Santa Cruz sc-25103), insulin receptor substrate 1 (IRS; Millipore #06-248) phospho-IRS 1 ser^{307} (Cell signalling #2384) protein kinase B/Akt (Cell Signalling #9272), phospho-Akt ser^{473} (Cell Signalling #9271), phospho-Akt thr^{308} (Cell Signalling #9275), Akt substrate 160kDA (AS160; Cell signalling #2447), phospho-AS160 thr^{642} (Cell Signalling #4288), typical phospho-protein kinase C $ser^{643/676}$ (PKCδ/θ; Cell Signalling #9376), atypical

phospho-PKC thr$^{410/403}$ (PKCλ/ζ; Cell Signalling #9378), mechanistic target of rapamycin (mTOR; Cell Signalling #2972), phospho-mTOR ser^{2448} (Cell Signalling #2971), glyceraldehyde phosphate dehydrogenase (GAPDH; Santa Cruz sc-25778) glycogen synthase kinase 3α (GSK; Cell Signalling #9338) and phospho-GSK 3α/β ser$^{21/9}$(Cell Signalling #9331). After washing and incubation with horseradish peroxidase-conjugated secondary antibody (Perkin Elmer) in 5% skim milk and TBST, the immune-reactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) on the Versadoc MP4000 (Bio-Rad) and quantified by densitometry (Quantity-One; Bio-Rad).

6.4.7 RNA extraction and TGFβ network/tissue fibrosis gene expression analysis:

Total RNA was isolated from the muscle (15–20 mg) using the Trizol and clean up with RNeasy Total RNA Kit columns (Qiagen). The total RNA content and purity were established by measuring absorbance at 260 and 280 nm (NanoDrop; Eppendorf). Ten micrograms of each RNA sample were then DNAse treated using DNase 1 (Thermo Fisher Scientific) described in detail in (Prodoehl, Hatzirodos et al. 2009).

Relative gene expression was quantified by real-time PCR using the Qiagen RT2 custom profiler array for fibrosis pathway related genes. 100ng of DNAse-treated RNA was used for cDNA, diluted 1 in 10 and amplified for 14 genes plus 2 housekeeping genes. cDNA was generated according to manufacturer's guidelines with modifications (Prodoehl, Hatzirodos et al. 2009) using 40 U of Superscript RT III (Thermo Fisher Scientific). Custom array primers were designed against the human mRNA sequences for the corresponding genes in the Ref Seq database listed in Table 6.1.

Table 6.1: Genes assessed using the Fibrosis Pathway Qiagen RT2 profiler array platform

Gene	Name	Primers/Accession #
House keeping		
ACTB	Actin, beta	NM_001101
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046
TGFβ ligand pathway genes		
COL1A2	Collagen, type I, alpha 2	NM_000089
COL3A1	Collagen, type III, alpha 1	NM_000090
DCN	Decorin	NM_001920
IGF1	Insulin like growth factor 1	NM_000618
LOX	Lysosome oxidase	NM_002317
LTBP1	Latent transforming growth factor beta binding protein 1	NM_000627
MMP2	Matrix Metaloproteinase 2	NM_004530
MMP9	Matrix Metaloproteinase 9	NM_004994
SMAD2	SMAD family member 2	NM_005901
TGF1l1	Transforming growth factor beta 1 induced transcript 1	NM_015927
TGFB1	Transforming growth factor, beta 1	NM_000660
TGFB2	Transforming growth factor, beta 2	NM_003238
TGFB3	Transforming growth factor, beta 3	NM_003239
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	NM_003242

All reactions were performed according to the Sybr-Green™ cycle threshold (Ct) method using a Biorad CFX 384 real-time PCR detection. Thermocycling conditions for the PCR included 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Melt curve analysis from 72 to 95°C (5 seconds per C) was performed to ensure a single defined peak for each amplified product. Comparative Ct calculations for the expression of the studied genes were performed by subtracting the mean GAPDH and ACTB Ct values from Ct values of the gene of interest to derive a ΔCt value. The expression of the genes was then calculated according to the formula: $2^{-\Delta Ct}$.

6.5 Statistical analysis

All statistical analyses were conducted using mixed modelling procedures (PROC MIXED) in Statistical Analysis System (Version 9.4, SAS Institute). Data, unless otherwise stated, were log transformed before analysis to overcome heteroscedastic issues and presented as a back-transformed mean with standard deviation (SD) as a coefficient of variation (%). For the cross-sectional study separate models were generated to compare differences in baseline variables between groups including participant characteristics, protein abundance and phosphorylation and gene expression, and the fold changes in normalised protein phosphorylation induced by 30 minutes of insulin infusion. These models also included an adjustment for age, change in insulin and age plus change in insulin where appropriate. For the exercise training sub-study, a similar approach was adopted to compare exercise induced changes in variables. Differences in fold change in insulin-stimulated protein phosphorylation were modelled but without adjustment for age. All models estimated changes as a percentage and are presented with 90% confidence intervals (90% CI). Significance was accepted when $P<0.05$.

6.6 Results:

Table 6.2: Participant characteristics

Parameter	Lean Control	Lean PCOS	Overweight Control	Overweight PCOS
Age (y)	28 ± 6[a]	26 ± 3[ab]	35 ± 4	31 ± 6
BMI (kg.m^{-2})	22 ± 2[ab]	23 ± 2[ab]	36 ± 5	37 ± 7
WHR	0.83 (5.9)	0.84 (4.7)	0.84 (12.1)	0.85 (6.7)
DXA				
%Body Fat	27 (28)[ab]	30 (28)[ab]	50 (11)	47 (8)
Fat mass (kg)	16.4 ± 5.0[ab]	20.1 ± 6.7[ab]	48.3 ± 10.6	46.3 ± 10.4
CT				
Abdominal SCF (cm^2)	185 ± 74[ab]	229 ± 74[ab]	571 ± 155	582 ± 174
Abdominal VF (cm^2)	32 ± 22[ab]	35 ± 10[ab]	117 ± 31	135 ± 58
Glucose Homeostasis				
Fasting blood glucose (mmol.L^{-1})	4.5 ± 0.3[bc]	4.6 ±0.4	4.8 ± 0.3	5.0 ± 0.6
Fasting plasma insulin (pmol.L^{-1})	5.0 ± 4.1[ab]	4.5 ± 1.7 [ab]	16.6 ± 6.0 [b]	28.5 ± 12.8
Clamp 30min plasma insulin (pmol.L^{-1})	59.1 ± 7.5[ab]	58.2 ± 12.4[ab]	84.7 ± 20	99.6 ± 34
HOMA-IR	0.85 (65)[ab]	0.85 (44)[ab]	3.32 (41)[b]	5.78 (64)
Clamp Glucose infusion rate (mg.min^{-1}.m^{-2})	332 (29)[ab]	270 (26)[b]	250 (29)[b]	131(105)
Lipid profiles				
Cholesterol (mmol.L^{-1})	4.76 ± 0.64	4.93 ± 0.66	4.80 ± 0.80	4.94 ± 1.25
Triglycerides (mmol.L^{-1})	0.86 ± 0.70[b]	0.71 ± 0.32[b]	1.17 ± 0.61[b]	1.61 ±0.96
LDL/HDL cholesterol ratio	1.65 (38)[ab]	1.61 (29)[ab]	2.37 (24)	3.27 (54)
Androgens				
Testosterone (µmol.L^{-1})	1.67 ± 0.46[b]	2.14 ± 0.80	1.61 ± 0.79[b]	2.66 ± 0.63
SHBG (mmol.L^{-1})	78 ± 21[ab]	72 ± 33[ab]	45 ± 30	28 ± 2
FAI (%)	2.2 (52)[ab]	3.14 (82)[b]	3.64 (101)[b]	9.47(51)
PCOS Diagnosis				
Number of participants	16	16	13	14
NIH (n)		4		13
Rotterdam (n)		12		1

Data are presented as mean±SD or when data were log transformed they are presented as a back transformed mean (±SD as a CV%). BMI, body mass index; CT, Computer Tomography; DXA, dual x-ray absorptiometry; WHR, waist to hip ratio, HOMA-IR, homeostasis model of insulin resistance(Meyer, McGrath et al. 2007); LDL, low density lipoprotein; HDL, high density lipoprotein; SHBG, steroid hormone binding globulin; FAI, free androgen index; n/a, not applicable; SCF, subcutaneous fat; VF, visceral fat.

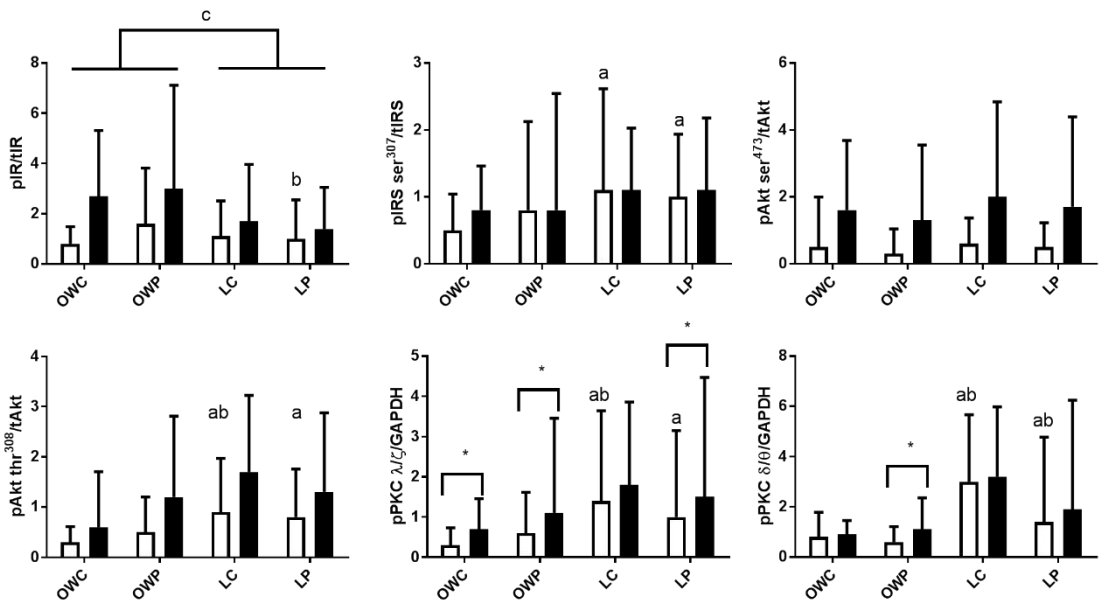
Statistical differences are reported after adjusting for age: a–significantly different from overweight controls P≤0.05, b–significantly different from overweight PCOS P≤0.05, c-significantly different from lean PCOS P≤0.01.

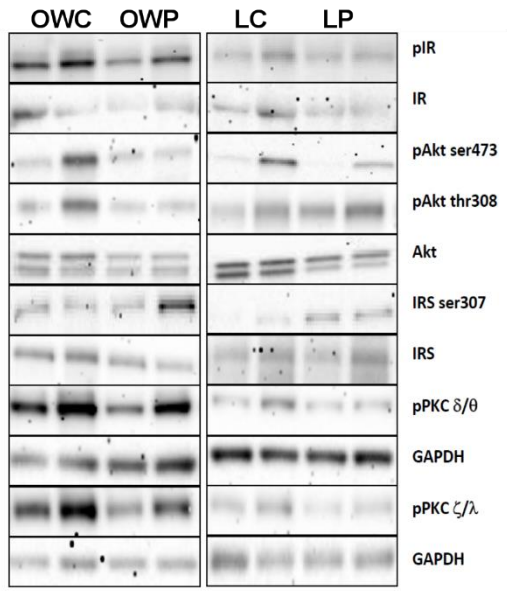
6.6.1 Insulin signalling:

We analysed the phosphorylation of key proteins in the insulin signalling pathway across the proximal (IR, IRS, Akt/PKB) and distal (AS160, GSK, mTOR) components of the pathway, as well as the insulin signalling pathway regulators (PKC ζ/λ and θ/δ). We compared the protein and phospho-protein abundance across the 4 groups of women to identify possible early insulin signalling defects in skeletal muscle that align with intrinsic PCOS-specific insulin resistance.

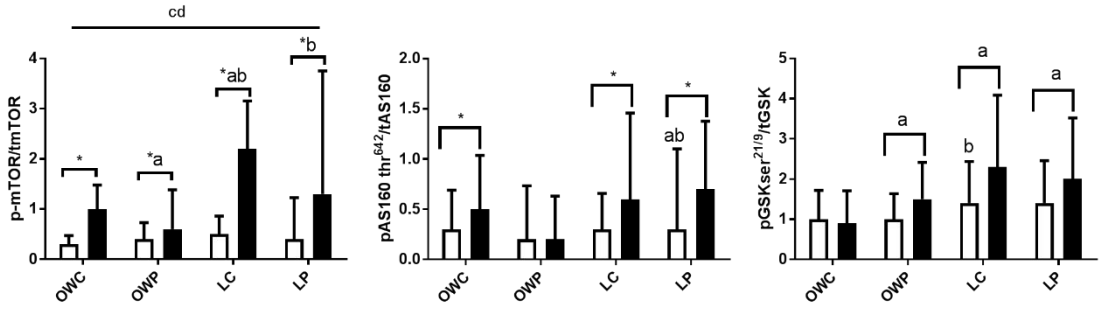
411

Proximal insulin signalling subtrates





Distal insulin signalling subtrates



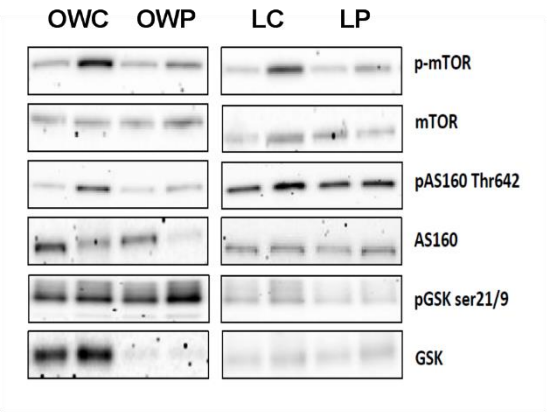


Figure 6.1: Normalised phosphorylation of key proteins in the insulin signalling pathway and insulin signalling pathway regulators. Statistical difference reported: **a**- significantly different from overweight control, **b**- significantly different from overweight PCOS, **c**- insulin stimulation of lean groups significantly different from overweight groups, **d**-insulin stimulation significantly different by PCOS status *****- significant difference between baseline and 30 minutes into clamp. Presented as mean ± SD, as a coefficient of variation (%), $p<0.05$. White indicates pre-clamp, black indicates 30 minutes into clamp. (N=59)

6.6.1.1 Proximal insulin signalling

At baseline, pIR tyr$^{1162/1163}$ in lean women with PCOS (LP) was 49% ([90% CI: -69, -17%; P=0.03) lower than overweight with PCOS (OWP). Baseline phospho-IRS ser^{307} was elevated by ~90% ($p<0.05$) in the LP and lean women without PCOS (LC) groups compared to overweight women without PCOS (OWC). Baseline phospho-Akt ser^{473} was not impacted by obesity or PCOS status. Phospho-Akt thr^{308} was impacted by both obesity and PCOS status. Phospho-Akt thr^{308} was significantly higher in LC (177%; [90%CI: 84, 319%], P<0.001) and LP (163%, [90%CI: 66, 319%], P=0.001) compared to OWC. Akt thr^{308} phosphorylation in the LC was 181% ([90%CI: 71, 359%], P=0.001) and 93% ([90%CI: 13, 230%], P=0.04) higher than in OWC and OWP groups respectively, furthermore LP was 160% ([90%CI 59, 324%]; P=0.003) higher than the OWC group. Thirty minutes of insulin stimulation significantly increased the phosphorylation of the IR (70%), Akt ser^{473} (250%) and Akt thr^{308} (80%) but had no effect on IRS ser^{307}phosphorylation. Obesity influenced insulin activation of the IR tyr$^{1162/1163}$ where the insulin stimulated a 35% ([90%CI: 7, 49%]; P=0.034) greater phosphorylation in the overweight (OWC, OWP) compared to the lean groups (LC, LP).

6.6.1.2 Insulin signalling pathway regulators

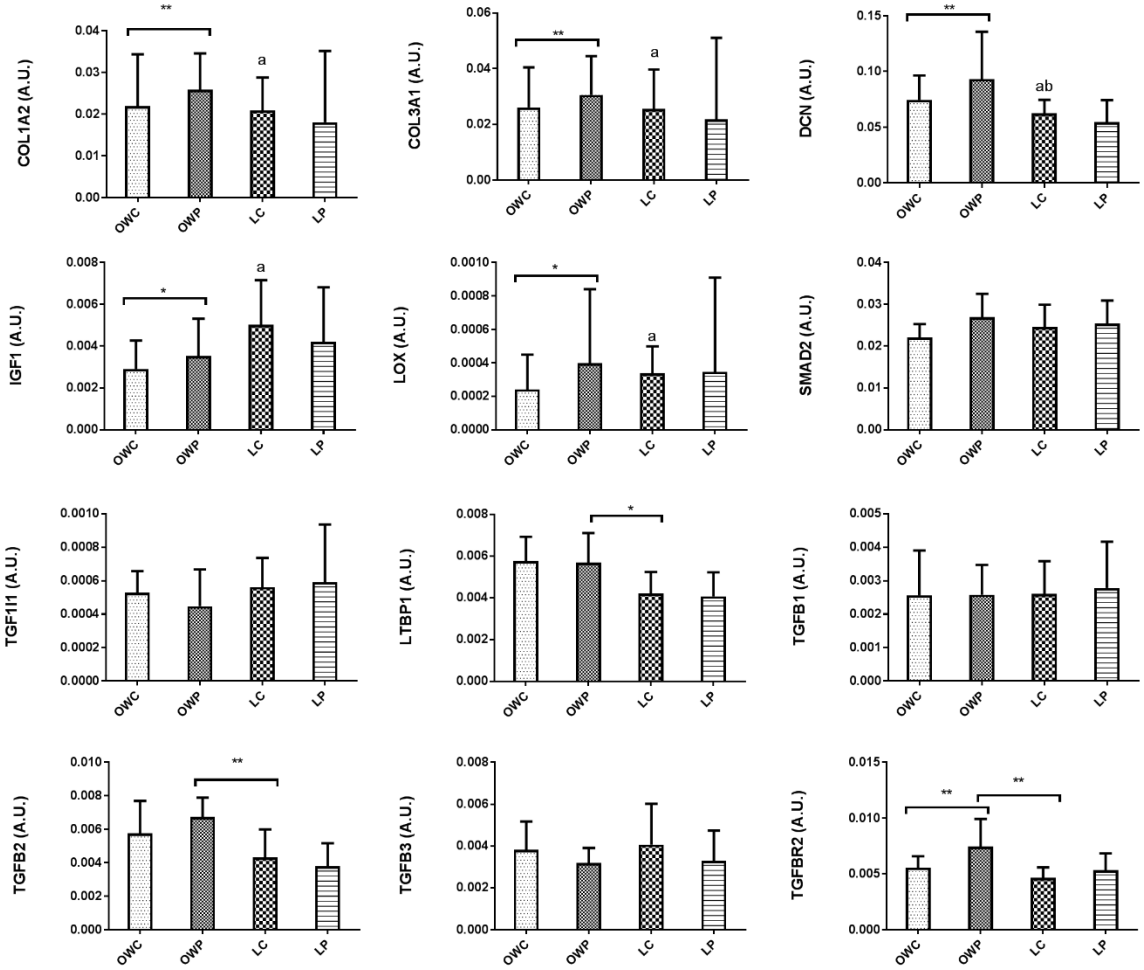
PKC δ/θ phosphorylation at baseline was higher in LC and LP compared to both OWC and OWP. Insulin infusion increased phospho-PKC δ/θ by 77% (136%; P=0.03) in OWP group, with no changes for the LC, LP and OWC. The baseline phospho- PKCζ/λ was elevated in LC and LP compared to OWC (P<0.01) and in the LC vs. OWP (P<0.05). Thirty minutes of insulin infusion induced increases of 55% (43%; P=<0.01), 134% (114%; P<0.01) and 90% (137%; P=0.016) in the LP, OWC and OWP, respectively. PCOS appears to have had no impact on insulin stimulated phosphorylation in the proximal proteins that were assessed.

6.6.1.3 Distal insulin signalling

Baseline phosphorylation of phospho-AS160 thr^{642} in LP was higher compared to OWC (92% [90% CI: 19, 210%, P=0.03) and OWP (133% [90% CI: 20, 350%, P=0.04). Insulin infusion induced an 80 to 104% increase in phospho-AS160 thr^{642} in LC, LP and OWC (P<0.05), but only a 13% increase in OWP. When comparing the insulin induced changes in phospho-AS160 thr^{642} across groups, obesity reduced (-35% [90% CI: -54, -10%; P = 0.03) insulin impacts on phosphorylation. mTOR, is a potential IRS serine kinase, LC had higher baseline phosphorylation compared to overweight controls (P<0.05). Insulin stimulation increased phosphorylation of mTORser^{2448} by 339% (CV=60%) (mean change as a percentage ± SD (as a CV%), 211% (CV=74%), 189% (CV=68%) and 65% (CV=58%) for the LC, LP, OWC and OWP groups respectively (P≤0.001). Both obesity and PCOS status significantly impacted insulin induced changes in phosphorylation of mTOR ser^{2448} where overall obesity (-41% [90% CI: -53, -26%]; P=0.00031) and PCOS status (-37%, [90% CI: -49, -20%]; P=0.002) reduced insulin stimulated phosphorylation. At baseline phospho-GSK3α $ser^{21/9}$ was higher in the LC compared with the OWP group (P<0.05). Obesity significantly reduced (-35% [90% CI: -50, -16%]; P=0.008) insulin stimulation of phospho-GSK3α $ser^{21/9}$.

6.6.2 Gene expression of tissue fibrosis genes

The relative gene expression of COL1A2, COL3A1, DCN, LOX, LTBP1, TGFB2 and TGFBR2 was highest in the OWP cohort, while IGF1 was lowest in both OWP and OWC (Figure 6.2), suggesting aberrant signalling via the TGFβ ligand signalling network, establishing a pro-fibrotic gene program. Specifically COL1A2 and 3A1 were 57% (90%CI:-69,-40%; P=0.001) and 56% (90%CI:-71,-33%; P=0.005) lower in OWC compared to OWP. The LC group had higher (83%; P<0.05) expression of both COL1A2 and 3A1 compared to OWC. The LTBP1 gene expression was highest in the overweight groups, with LC having significantly lower (-25% 90%CI:-36,-12%; P=0.008) expression than OWP. For DCN (-47% 90%CI:-61,-28%; P=0.005) and LOX (-72% 90%CI:-88,-34%; P=0.021) the OWC group had lower gene expression than OWP. While DCN in LC was 34% lower for (P=0.031), LOX was 198% higher (P=0.025) compared to OWP. The TGFβ signalling related gene expression was higher in the OWP group where TGFB2 and TGFBR2 expression were 40% (90%CI:-52,-26%; P=0.0009) and 38% (90%CI:-51,-22%; P=0.004) lower in LC compared to OWP respectively. TGFBR2 gene expression in OWC was a 39% lower in expression (P=0.005). In contrast, IGF1 gene expression was highest in LC being 157% (90%CI: 61,311%; P=0.006) compared to OWC, with OWC also being lower (-52% 90%CI:-71,-20%; P=0.026) than OWP.



474

475 **Figure 6.2: Relative levels of gene expression of the tissue fibrosis (TGFβ) pathway.**
476 These data are from a subset of women (n=59) (Stepto, Cassar et al. 2013). Statistical
477 difference: *P<0.05, **P<0.01, Data are presented as mean with SD as a coefficient of
478 variation (%).
479

480 ***6.6.3 Exercise response***

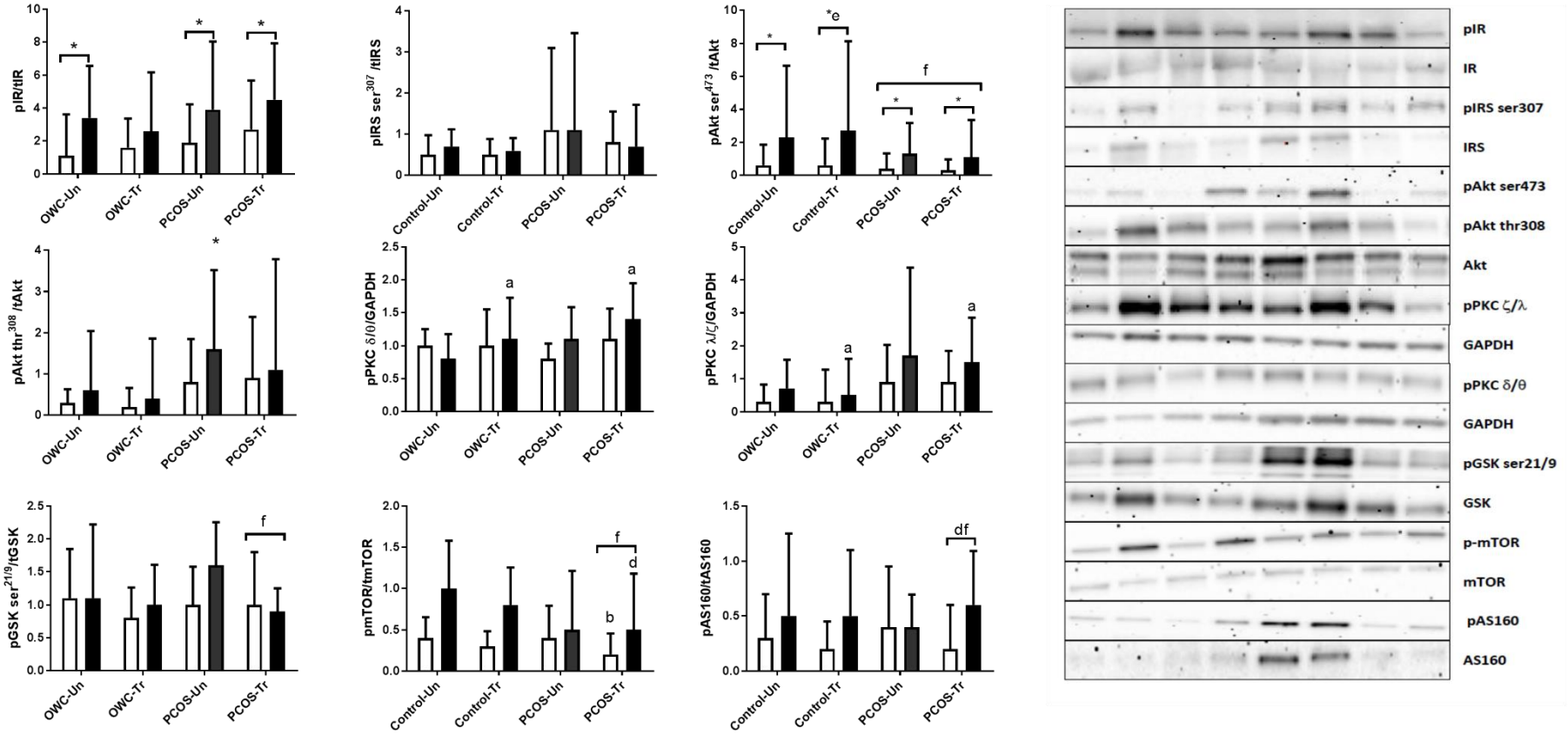
481 A subset of overweight women from the cross-sectional [OWC (n=8) and OWP (n=8)]
482 participated in a 12-week exercise intervention. The participant characteristics and responses
483 to 12 weeks of treadmill exercise training are summarised in Table 6.3.

Table 6.3 Training study participant characteristics

Parameter	Control Untrained (n=8)	Control Trained (n=8)	PCOS Untrained (n=8)	PCOS Trained (n=8)
Age (y)	35 ± 4	-	31 ± 6	-
BMI ($kg.m^{-2}$)	37 ± 6	36 ± 5	36 ± 6	36 ± 7
WHR	0.8 (3.2)	0.8 (6.9)	0.9 (5.2)	0.9 (6.4)
DXA				
%Body Fat	50 (12)	48 (13)	45 (8)	45 (10)
Fat mass (kg)	48 (30)	46 (27)[d]	42 (26)	41 (30)
CT				
Abdominal VF (cm^2)	120 (47)	125(49)	123 (69)	110 (77)[d]
Abdominal SCF (cm^2)	528 (49)	525 (52)[b]	520 (34)	498 (34)
PCOS Phenotype				
Irregular cycles +HA+PCO	0	0	8	8
Glucose Homeostasis				
Fasting blood glucose ($mmol.L^{-1}$)	4.7 (3.8)	4.8 (6.4)	5.0 (10.2)	5.0 (5.1)
Fasting plasma insulin ($pmol.L^{-1}$)	17 (46)*	18 (55)	27 (59)	21 (86)[b]
Clamp 30min plasma insulin ($pmol.L^{-1}$)	83 (34)	77 (25)	91 (50)	88 (73)
HOMA-IR	3.5 (47)*	3.3 (56)	6.1 (63)	4.5 (92)[b]
Clamp Glucose infusion rate ($mg.min^{-1}.m^{-2}$)	245 (57)*	293 (36)	117 (141)	170 (75)[b]
Lipid profiles				
Cholesterol ($mmol.L^{-1}$)	4.7 (29)	4.9 (23)	4.5 (33)	4.4 (22)
Triglycerides ($mmol.L^{-1}$)	1.0 (35)	1.2 (42)	1.2 (68)	0.9 (67)[b] [c]
LDL/HDL cholesterol ratio	2.4 (31)**	2.5 (42)	3.2 (75)	3.0 (51)
Androgens				
Testosterone ($\mu mol.L^{-1}$)	1.4 (48)*	1.5 (86)	2.6 (27)	2.5 (53)
SHBG ($mmol.L^{-1}$)	44 (78)**	47 (79)	26 (32)	29 (38)
FAI (%)	3.1 (86)*	3.3 (103)	10.1 (39)	8.6(66)
Fitness				
VO2max ($mL.kg^{-1}.min^{-1}$)	25.7 (12.5)	30.3 (11.3)[a]	25.3 (29.6)	31.1(27.0)[a]

Data are presented as mean ±SD or when data were log transformed they are presented as a back transformed mean with SD (CV%). BMI- body mass index; CT- Computer Tomography;

DXA- dual x-ray absorptiometry; WHR- waist to hip ratio, HOMA-IR- homeostasis model of insulin resistance; LDL- low density lipoprotein; HDL – high density lipoprotein; SHBG- steroid hormone binding globulin; FAI – free androgen index; n/a- not applicable; SCF subcutaneous fat; VF- visceral fat. * – significantly different from PCOS (untrained values) $P \leq 0.01$, **– significantly different from PCOS (untrained values) $P \leq 0.05$, a – significantly different from pre-training $P \leq 0.01$, b – significantly different from pre-training $P \leq 0.05$, c - significantly different change between groups $P \leq 0.01$, d- trend for difference from pre-training $P < 0.1$


pIR/tIR
pIRS ser307/tIRS
pAkt ser473/tAkt
pAkt thr308/tAkt
pPKC δ/θ/GAPDH
pPKC λ/ζ/GAPDH
pGSK ser21/9/tGSK
pmTOR/tmTOR
pAS160/tAS160
OWC-Un
OWC-Tr
PCOS-Un
PCOS-Tr
Control-Un
Control-Tr
OWC-Untr
OWC-Tr
OWP-UnTr
OWP-Tr
pIR
IR
pIRS ser307
IRS
pAkt ser473
pAkt thr308
Akt
pPKC ζ/λ
GAPDH
pPKC δ/θ
GAPDH
pGSK ser21/9
GSK
p-mTOR
mTOR
pAS160
AS160

495

Figure 6.3: Phosphorylation of key proteins in the insulin signalling pathway after 12 weeks of intensified exercise training in overweight women with and with PCOS.

Statistical difference reported as: **a**–significantly different from pre-training, **b**–significantly different PCOS baseline from pre-training, **c**-PCOS significantly different to control baseline, **d**-significantly different insulin stimulated response from untrained PCOS, **e**-significantly different insulin stimulated response from trained PCOS, **f**-significantly different change between groups (PCOS v control) Presented as mean with SD as a coefficient of variation (%), $P<0.05$. White indicates pre-clamp, black indicates 30 minutes into clamp.

Twelve weeks of treadmill exercise training did not significantly modify baseline or insulin induced changes in IR, IRS or Akt thr^{308} phosphorylation. Exercise training did increase insulin stimulated phospho-Akt ser^{473}, and this increase was greater in the OWC compared to the OWP. Baseline and insulin stimulated phosphorylation were more impacted by 12 weeks of treadmill exercise training in OWP compared with OWC women. Training increased insulin stimulated phosphorylation of AS160 by 82% (p=0.03) from 41% ([90%CI: 5, 109%]; P=0.143) pre-training to 156% ([90%CI: 126, 192%]; P<0.001) post-training in the OWP women. Training reduced baseline phospho-mTOR by 38% (77.6%; P=0.05) in OWP compared to 14% (38%; P=0.24) in OWC women but this was not significant. Training enhanced insulin stimulation of phospho-mTOR by 65% (P=0.04) in OWP women but was unchanged by training in OWC women.

Exercise training enhanced the phosho-PKC δ/θ isoform such that post training insulin stimulated phosphorylation increased by 13% ([90% CI: 6, 20%]; P=0.011) and 29% (7, 55%, P=0.035) in OWC and OWP respectively. Training did reduce insulin stimulated activation of phospho-PKCζ/λ from 104% ([90% CI: 20, 245%]; P=0.038) and 126% ([90% CI: 51, 241%]; P=0.006) pre-training to 51% ([90% CI: 13, 103%]; P=0.031) and 95% ([90% CI: 54, 146%]; P=0.00064) post-training for OWC and OWP respectively.

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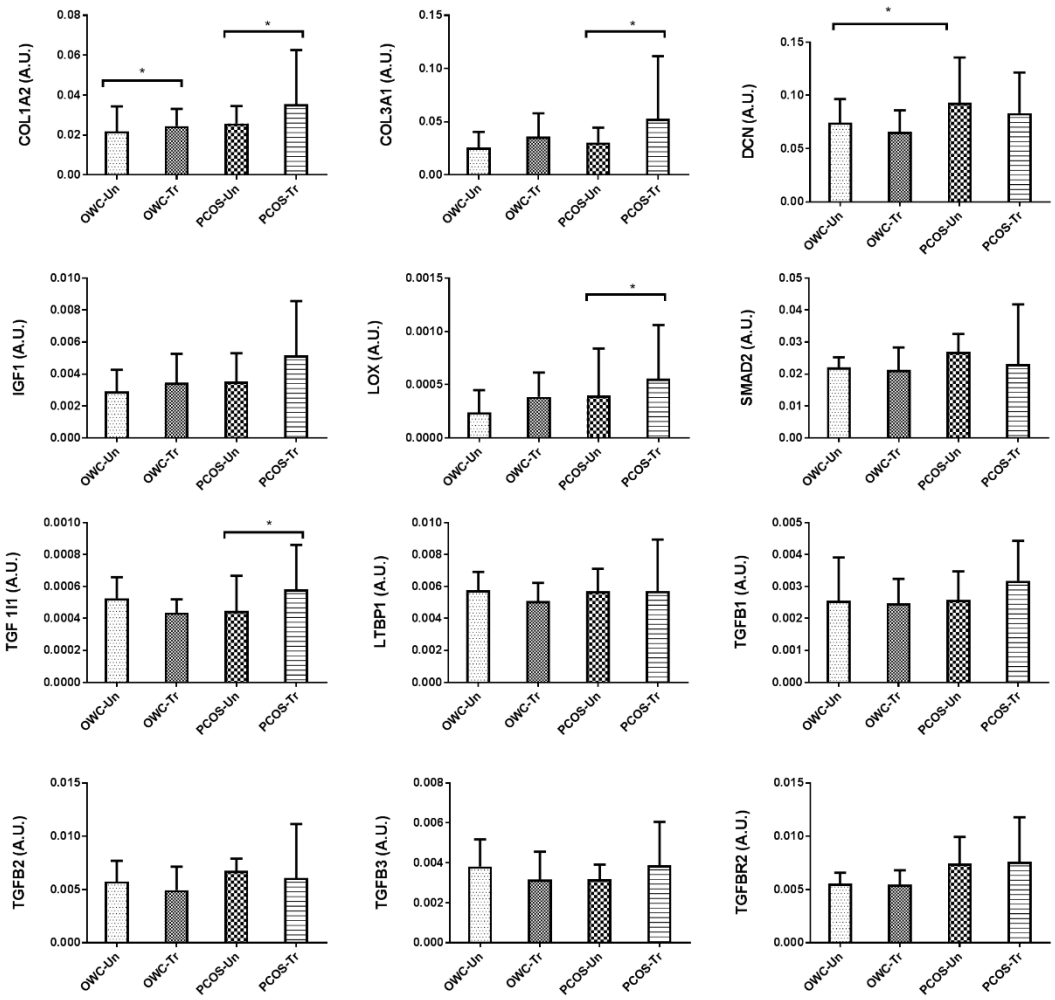


524

525 **Figure 6.4: Training response of relative gene expression in the tissue fibrosis**
526 **(TGFβ) pathway for women with and without PCOS after 12 weeks of intensified**
527 **exercise training.**
528 These data are from a subset of women (n=8 PCOS, n=8 control). Presented as mean
529 with SD as a coefficient of variation (%). Statistical difference reported *p<0.05.
530

We explored changes in relative gene expression of the tissue fibrosis (TGFβ) pathway (Figure 6.4). Four key genes in extra-cellular matrix deposition, COL1A2, COL3A1, DCN and LOX, were differentially affected by the training protocol between OWP and OWC. COL1A2 gene expression increased by 65% after training in PCOS ([90%CI: 23, 123%]; P=0.02) and -39% ([90%CI: -58, -12%]; P=0.03) in OWC. Exercise training induced COL3A1 and LOX gene expression to increase 94% ([90%CI: 11, 235%; P=0.05) and 95% ([90%CI: 26, 202%]; P=0.03) in the OWP, respectively. On the other hand, DCN was reduced by 23% ([90%CI: -38, -5%; P=0.03) in OWC compared to OWP, but training had no significant effect.

6.7 Discussion:

This study compared early insulin signalling events in skeletal muscle across four groups of women, including lean and obese women with or without PCOS. The novel findings demonstrated dysfunction in insulin signalling events at or distal to Akt/PKB after 30 minutes of insulin infusion during an insulin clamp. Specifically, phospho-mTOR, was negatively impacted by PCOS status and obesity, while phospho-AS160 and phosho-GSK3 were mainly affected by obesity. Interestingly the typical PKC (PKC δ/θ) had an obesity related loss of insulin activation, while the novel regulator of GLUT4 translocation atypical PKC (PKCζ/λ) tended to have loss of insulin activation in PCOS. A 12-week exercise intervention improved but did not rescue insulin sensitivity in the OWP compared to OWC and these were accompanied by improved but not normalised phospho-AS160, phospho-mTOR and phospho-Aktser^{473}. This suggests that this length of exercise intervention without weight loss may not improve

554 insulin signalling significantly. Further, other factors such as excess stromal deposition
555 in combination with the hyperandrogenic milieu may contribute to insulin resistance
556 and insulin signalling defects in PCOS skeletal muscle.
557
558 Emerging data (Böhm, Hoffmann et al. 2016, Seong, Manoharan et al. 2018) for direct
559 TGFβ signalling, via the SMAD proteins, and our new hypothesis (Raja-Khan, Urbanek
560 et al. 2014) of TGFβ ligand mediated excess stromal deposition or fibrosis, may apply
561 beyond the ovary to metabolic tissues like skeletal muscle in women with PCOS,
562 predisposing them to insulin resistance. We found that a number of genes in the TGFβ
563 regulated tissue fibrosis pathway that encode extracellular matrix components
564 (collagen: COL1A2, COL3A1), key enzymes in the collagen deposition (LOX, DCN),
565 ligands (TGFB2) and its receptor were elevated in OWP. After 12-weeks of exercise
566 training 4 genes (COL1A2, COL3A1, DCN and LOX) were differentially regulated in
567 the women with PCOS showing a pattern conducive to greater extracellular matrix
568 deposition or fibrosis after the exercise training intervention.
569
570 Defects in insulin signalling in skeletal muscle are well documented for insulin resistant
571 conditions (Krook, Bjornholm et al. 2000), including PCOS (Corbould, Kim et al. 2005,
572 Diamanti-Kandarakis and Dunaif 2012). Our data significantly expands this work
573 exploring possible defects in both proximal and distal components of this pathway. In
574 contrast to the literature (Corbould, Kim et al. 2005, Diamanti-Kandarakis and Dunaif
575 2012) our data suggests a defect in the insulin receptor activation of signalling due to
576 differential phosphorylation of $tyr^{1162/1163}$ between groups. Insulin concentrations were
577 significantly higher in the overweight groups (OWC & OWP) and when this was

accounted for in our statistical modelling, the obesity driven difference was negated, highlighting the extrinsic insulin resistance that is commonly associated with higher BMIs. After this adjustment our data aligns with the current literature (Corbould, Kim et al. 2005, Diamanti-Kandarakis and Dunaif 2012) where there appears to be no functional defect in the insulin receptor in PCOS.

Our data also allowed us to explore the hypothesis of Dunaif et al. (2012) that postulates that there may be a PCOS-specific serine kinase targeting IR and IRS1/2. Our *in vivo* data contrasts with this, as phospho IRS Ser^{307} could not explain the insulin clamp measured PCOS-specific insulin resistance, at least in skeletal muscle, but suggest a signalling defect more distal of the IR and IRS1/2.

mTOR signalling is traditionally associated with nutrition regulated anabolic processes, especially in skeletal muscle (Bodine, Stitt et al. 2001), but has now been linked to insulin resistance in skeletal muscle (Kleinert, Sylow et al. 2014). The key finding of this study, was that our data implicates reduced phosphorylation of mTOR as a possible PCOS-specific mechanism for reduced insulin signalling through Akt activation. The women with PCOS from the exercise training sub-group shows improved but not rescued mTOR signalling responses to insulin. Clearly, more research is needed to understand the mTOR-Akt relationship in PCOS-specific insulin resistance in muscle and other metabolic tissues.

GSK3 and AS160 were impacted by PCOS and/or obesity. GSK3 is a key enzyme limiting glycogen synthesis (Parker, Shaw et al. 2017). In our cohort of women, obesity

appears to be the main driver of lower phospho-GSK3 to impact early insulin signalling. Exercise training attenuated some of the insulin stimulated GSK-3 phosphorylation differences in the overweight women with or without PCOS. This was not unexpected as exercise has a direct impact on GSK3 phosphorylation (Krook, Roth et al. 1998). Linking GSK3 activity to improved muscle glycogen synthesis and storage which are well-established adaptions to exercise training in health and disease (Benziane, Burton et al. 2008).

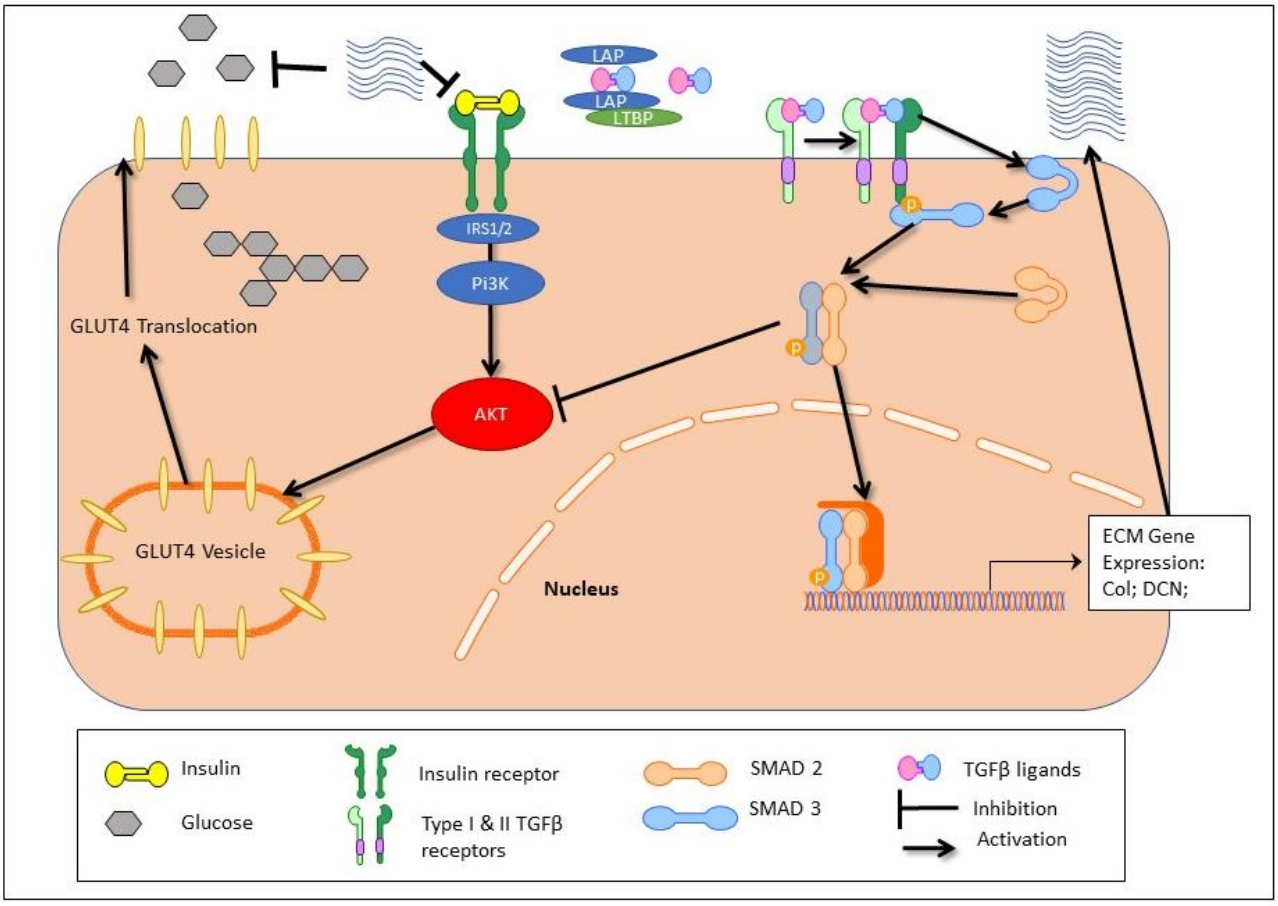
AS160, a substrate of Akt and integral protein in the Glut4 vesicle (Peck, Chavez et al. 2009), has been found that reduced signalling via this protein is impacted by both obesity and PCOS status. Here 12-weeks of treadmill exercise rescued the dysfunction, but did not normalise it. Taken together these data suggest that reduced AS160 activation by insulin may play a role in PCOS-specific insulin resistance. But it is more likely a consequence of an upstream signalling defect at Akt driven by PCOS and its synergy with obesity (Stepto, Cassar et al. 2013, Cassar, Misso et al. 2016).

PKC δ/θ is activated by diacyl-triglycerides (DAGs) and implicated in insulin-mediated glucose uptake (Yu, Chen et al. 2002). Contrary to the proposed role, our insulin signalling data found reduced phosphorylation of PKC δ/θ after 30 minutes of insulin infusion. Exercise training as used in this study is well known to improve skeletal muscle lipid metabolism likely altering lipid species profiles, and content including DAGs reducing its impact on insulin signalling (Goto-Inoue, Yamada et al. 2013). This may explain the absolute increase of phospho-PKCδ/θ or improved insulin responsiveness to insulin within the trained cohorts. A second PKC has been implicated

in insulin resistance, specifically a reduction in activation of PKC λ/ξ which reduces Glut4 vesicle translocation and docking to the membrane (Newton 2003). PKC λ/ξ demonstrated the expected reduction of phosphorylation at baseline and in response to insulin stimulation in the insulin resistant groups (OWP, LP and OWC) (Stepto, Cassar et al. 2013). While little is known about the overall function of PKCλ/ξ, it is responsive to insulin sensitising stimuli like exercise (Perrini, Henriksson et al. 2004). Our data from the subgroup of overweight women with or without PCOS showed reduced phosphorylation at baseline and in response to 30 minutes of insulin stimulation that was reduced by exercise training to a similar extent post training in both groups.

The fibrosis of the PCOS ovary suggests that dysfunctional TGFβ network signalling may lead to increased organ stroma or fibrosis predisposing these women to the morbidities of PCOS (Raja-Khan, Urbanek et al. 2014). Dysfunctional TGFβ or TGFβ superfamily ligand signalling may be involved as anti-müllerian hormone (AMH (Cassar, Teede et al. 2014)) and TGFβ1 (Tal, Seifer et al. 2013) are elevated in women with PCOS. These ligands act via their respective receptors to activate the Smad signalling proteins that are not only negative regulators of Akt (Chen, Colgan et al. 2016) but key signals in extracellular matrix deposition (Figure 6.5). Thus this pathway is a plausible contributor to dysfunctional insulin signalling in PCOS and reduced response to insulin sensitising therapy like exercise (Böhm, Hoffmann et al. 2016). In this context, our gene expression data of elevated collagen, extra-cellular matrix deposition enzymes and TGFβ2R gene expression (pro-fibrotic gene profile), elevated ligands (AMH and TGFβ1) and previously reported elevated Hounsfield units in thigh skeletal muscle from CT analysis (Hutchison, Teede et al. 2012), support the notion

650 that dysfunctional TGFβ signalling networks and tissue fibrosis may be involved in
651 PCOS-specific skeletal muscle insulin resistance. This may occur not only via Akt
652 signalling interference but also via increased extracellular matrix limiting insulin and



653 glucose movement across the interstitial space (Figure 6.5).

654 **Figure 6.5: Hypothetical signalling pathway showing that dysfunctional TGFβ**
655 **network signalling regulates tissue fibrosis and may play a role in this PCOS-**
656 **specific insulin resistance and its limited response to exercise training.**
657

658 We acknowledge that our study has a number of limitations including sample size being
659 limited (Hutchison, Stepto et al. 2011, Hutchison, Teede et al. 2012, Stepto, Cassar et
660 al. 2013) due to the invasive nature of the procedures used in this study. We biopsied
661 the vastus lateralis muscle, which when exercise trained using a treadmill protocol, is

not the primary muscle group trained. Additional *in vivo* and *in vitro* human research is warranted to better understand the link between the dysfunctional insulin signalling, TGFβ ligand signalling networks and extracellular matrix deposition in the aetiology PCOS and its intrinsic insulin resistance. The strengths of this study were that we utilised gold-standard methods to assess insulin sensitivity in a community-recruited, well-characterised population of lean and overweight women with or without PCOS.

6.8 Conclusions:

In conclusion, our data provides new insights into PCOS-specific insulin resistance and the associated early signalling events both proximal and distal of the insulin receptor in skeletal muscle. We could not confirm previously postulated aberrant signalling at IR or IRS1/2 in PCOS-specific insulin resistance, at least in skeletal muscle (Corbould, Kim et al. 2005, Corbould, Zhao et al. 2006). We found that there was a significant role of aberrant signalling in AS160, mTOR and PKC λ/ξ in PCOS-specific insulin resistance. Intensified aerobic exercise training did not normalise insulin sensitivity, despite improving insulin stimulated signalling at Akt, AS160, and mTOR in overweight women with PCOS. Our data supports the hypothesis of the potential role of the TGFβ superfamily ligands, their signalling and resultant tissue fibrosis in PCOS-specific insulin resistance and response to exercise therapy.

CHAPTER 7. STUDY PROTOCOL FOR THE HIGH INTENSITY INTERMITTENT TRAINING PCOS RANDOMISED CONTROLLED TRIAL

7.1 General background

Weight loss or weight management (by exercise, and/or diet), is one of the first line therapies for women with PCOS (Teede, Misso et al. 2011) as it improves the clinical symptoms by improving insulin sensitivity in these women. Gaps remain in understanding of the molecular mechanisms of the positive response in insulin sensitivity from an exercise intervention. In the preceding chapters, and in particular chapter 6, I established that PCOS has unique molecular signatures that may contribute to insulin resistance and resistance to exercise therapy. There is a need for a high quality, mechanistic but clinically relevant randomised control trials to further explore these findings. In Chapter 7, I develop a clinical trial methodology to address the effectiveness of HIIT against moderate intensity exercise or best practice standard care to demonstrate comparative efficacy of improvements in insulin sensitivity, to inform clinical practice. I also aimed to explore the impact of different exercise regimes on reproductive hormone profiles (anti-müllerian hormone [AMH] and steroid profiles), psychosocial health and new molecular mechanisms of PCOS-specific insulin resistance.

This trial has successfully completed (n=8) as of March 2018 and is expected to be completed in 2019. It is being run in accordance with the CONSORT guidelines in which the SPIRIT initiative (Standard Protocol Items: Recommendations for Interventional Trials) recommends the publishing of the protocol of randomised control trials. As this study extends beyond my PhD timeframe I present the protocol paper manuscript and some preliminary data. This paper will be published in BMC trials journal and I will be first author. Post PhD I will still be involved in the analysis of the

711 DNA methylation analysis from the trials and qualify as an author in most publications
712 that arise from this study as per international guidelines for authorship in biomedical
713 scientific journals.
714

715 "**The impact of high intensity intermittent training on metabolic, reproductive and**
716 **mental health in women with Polycystic Ovary Syndrome: Study protocol for the**
717 **iHIT-PCOS randomised controlled trial."**
718
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721 Levinger I[1], Stepto NK[1,2]
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729 **Keywords**:
730 Polycystic ovary syndrome, high-intensity interval training, cardiorespiratory fitness,
731 cardiometabolic health, insulin, mental health, overweight, exercise, exercise therapy
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734

7.3 Abstract

Background: Polycystic ovary syndrome (PCOS) is a reproductive-metabolic condition. Insulin resistance is thought to underpin PCOS and to increase hyperandrogenism that drives inherent metabolic, reproductive, and psychological features of the condition. To improve the clinical features of PCOS, insulin resistance is currently treated by weight loss via exercise, diet and insulin-sensitising medications. A deeper understanding of the insulin resistance mechanisms in women with PCOS is required to improve its treatment, as efficacy is limited with neither medications nor lifestyle completely normalising insulin resistance. This manuscript describes the protocol of a study evaluating the effectiveness of high intensity intermittent training (HIIT) or moderate intensity exercise on cardio-metabolic, reproductive and psychosocial health in overweight women with PCOS.

Methods: A parallel-group, three-arm, randomised controlled trial was employed to recruit sixty women diagnosed with PCOS, aged between 18 and 45 years and with a body mass index (BMI) greater than 25kg/m^2. Following screening and baseline testing, women will be randomised to undergo one of two 12-week supervised interventions: either HIIT or standard supervised exercise (SSE), or to best practice standard care [Con] (unsupervised lifestyle advice). The primary outcome for this trial is to measure the improvements in metabolic health specifically changes in insulin sensitivity in response to different exercise protocols. Baseline and post intervention testing include anthropometric measurements (BMI, dual X-ray absorptiometry), cardiorespiratory fitness testing (symptom-limited graded exercise test), reproductive hormone profiles (anti-müllerian hormone and steroid profiles), metabolic health (oral glucose tolerance test and a hyperinsulinemic-euglycemic clamp), mental health (quality of life using SF-

36), Depression, Anxiety, Stress Scale questionnaires) and lifestyle monitoring (Actigraph^{TM} accelerometer, Australian Physical Activity Questionnaire and a 3-day food diary).

Discussion: This trial aims to demonstrate comparative efficacy of different exercise protocols to inform clinical practice in treatment of PCOS. It will advance the understanding of PCOS management by providing insights into the optimal exercise programme to improve insulin sensitivity. Finally, as a secondary outcome we will explore the impact of different exercise protocols on reproductive hormone profiles (anti-müllerian hormone and steroid profiles), psychosocial health and new molecular mechanisms of PCOS-specific insulin resistance.

Trial Registration: Trial is registered with the Australian New Zealand Clinical Trials Registry (ACTRN12615000242527).

7.4 Introduction

Polycystic ovary syndrome (PCOS) is a major public health concern affecting 6-10% of reproductive aged women worldwide (Bozdag, Mumusoglu et al. 2016). Insulin resistance is strongly implicated in the PCOS aetiology and is associated with the reproductive and metabolic consequences of the syndrome (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Harrison, Stepto et al. 2012, Stepto, Cassar et al. 2013, Shorakae, Boyle et al. 2014, Moran, Norman et al. 2015). It has been illustrated that there is a PCOS-specific insulin resistance that is independent of BMI, yet can be exacerbated by obesity (Dunaif, Segal et al. 1989, Stepto, Cassar et al. 2013, Cassar, Misso et al. 2016).

Exercise, along with diet, is one of the first line therapies for women with PCOS as it improves the clinical symptoms by improving insulin sensitivity in these women (Teede, Misso et al. 2011, Harrison, Stepto et al. 2012). Moderate intensity aerobic exercise improves metabolic, and reproductive features, body composition and improvements to psychological well-being in overweight women with PCOS (Harrison, Lombard et al. 2011, Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012). However, in response to a standard exercise intervention, insulin sensitivity in women with PCOS is not normalised compared to women without PCOS (Harrison, Stepto et al. 2012). HIIT is a popular fitness trend (Thompson 2016) that addresses general barriers to exercise such as time limitations but also results in greater positive metabolic health outcomes. Despite this, there is limited comprehensive research on the efficacy of different exercise intensities and the underlying molecular mechanisms of improvements in insulin resistance following exercise in PCOS.

As gaps remain in understanding the mechanisms of insulin resistance in PCOS and optimal exercise interventions, we aimed to perform a randomised control trial in overweight women with PCOS. We will compare the effectiveness of HIIT against moderate intensity exercise or best practice standard care to demonstrate efficacy on insulin sensitivity, in different exercise regimes. Secondary outcomes we will also explore include the impact of different exercise regimes in reproductive hormone profiles (anti-müllerian hormone [AMH] and steroid profiles), psychosocial health and molecular mechanisms of insulin resistance. With the aim to ultimately inform best practice in managing and treating PCOS.

7.5 Methods

7.5.1 Design

We will employ a parallel-group, three-arm, randomised controlled trial. We will recruit sixty women diagnosed with PCOS who will be randomised to undergo one of two 12-week supervised interventions: either HIIT or standard supervised exercise (SSE), or to best practice standard care [Con] (unsupervised lifestyle advice).

7.5.2 Inclusion Criteria

To participate in this study, women must be aged between 18 and 45 years with a BMI greater than 25kg.m^2 and diagnosed with PCOS but otherwise healthy. PCOS will be previously diagnosed by their medical practitioner and a research endocrinologist will confirm diagnosis using the Rotterdam criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). For diagnosis of PCOS the Rotterdam criteria requires confirmation of two of the following (i) oligo- or

anovulation (ii) clinical (hirsutism and acne) and/or biochemical hyperandrogenism (iii) polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Features of PCOS will be recorded to allow phenotyping as recommended by the National Institutes of Health (The National Institutes of Health 2012).

7.5.3 Exclusion Criteria

Exclusion criteria are other causes of menstrual disturbance and hyperandrogenism, known cardiovascular or respiratory conditions (e.g. asthma, cardiac arrhythmias), hypertension (resting blood pressure >160/105), bleeding disorders, eating disorders, skin or anaesthetic allergies, musculoskeletal injuries that may be aggravated by the exercise protocol, pregnancy, type 1 or 2 diabetes, or taking anti-hypertensive, insulin sensitising, anti-obesity or hormonal contraceptive medications.

7.5.4 Screening

Prospective participants will be screened to check eligibility and will also complete a risk factor questionnaire to establish any risk of experiencing an adverse event during exercise testing and training. If they are identified as having multiple risk factors (family history [first-degree] of cardio-metabolic risk factors, chronic conditions [that do not exclude from participation] but could interfere with testing or exercise, if they have in the past experienced cardiovascular symptoms during exercise or have a BMI >40kg.m^2) exercise clearance will be required from their general practitioner. Written informed consent will be obtained from all participants.

7.5.5 Ethics

The study has been approved by the Victoria University Human Research Ethics Committee (Reference- HRE15-298) and is registered with the Australian New Zealand Clinical Trials Registry (ACTRN12615000242527). Reporting of the study will follow the CONSORT statement (Schulz, Altman et al. 2010).

7.5.6 Baseline Assessment

Baseline assessment will involve three sessions. Two sessions will be to complete a symptom-limited graded exercise protocol (the first session being a familiarisation of the test). During the 7 days between sessions two and three participants will wear an Actigraph^{TM} accelerometer around their waist to establish levels of habitual physical activity. The third baseline session will involve a muscle biopsy from the vastus lateralis, adipose tissue biopsies from the subcutaneous abdominal tissues, an oral glucose tolerance test (OGTT) and a hyperinsulinemic-euglycemic clamp. Participants will also undergo a dual X-ray absorptiometry scan (DXA) [GE Lunar iDXA] and be asked to complete validated questionnaires to assess physical activity (Australian Physical Activity Questionnaire [APAQ]), quality of life (SF-36) (Ware and Sherbourne 1992), depression, anxiety and stress (Depression, Anxiety, Stress Scale [DASS]) (Henry and Crawford 2005) and a 3-day food diary.

7.5.7 Randomisation

Following screening and baseline testing, participants will be randomised to HIIT, SSE or Con. An independent biostatistician will provide concealed group allocations stratified according to BMI. Participants and staff (accredited exercise physiologists)

implementing the intervention will not be blinded. Staff undertaking sample analysis and endpoint data processing will be blinded to group allocation.

7.5.8 Intervention

Every participant will receive a menstrual diary to monitor menstrual cyclicity and a FitBit™ to support physical activity throughout the 12-week intervention. As recommended in the Australian evidence-based PCOS guideline all women will receive behaviour change coaching (Teede, Misso et al. 2011). This will involve a 2-hour session on lifestyle behavioural modification techniques including goal setting, goal striving, physical activity, media and resources, healthy eating and diet (Lombard, Harrison et al. 2016).

881 **Table 7.1: Details of the three treatment arms**

Interventions	Details
Con	As described above. We do not anticipate the control group will engage in a significant amount of exercise compared to their baseline physical activity levels without the addition of a structured exercise program (Harrison, Lombard et al. 2011).
SSE Group	Minimum physical activity recommendations (150 min per week) (Department of Health and Aging 2014), in three supervised sessions/week of continuous low to moderate intensity exercise sessions (building up to 50 min sessions of cycling/walking at 3.5 METs or 50-60% HRR).
HIIT Group:	Minimum vigorous physical activity recommendations (~75 min per week) (Department of Health and Aging 2014), in three supervised sessions/week of HIIT exercise (cycling/running). Based on existing literature, pilot data (Parker, Shaw et al. 2017) and patient consultation, we will use a practical weekly training program encompassing two successful HIIT protocols (Little, Gillen et al. 2011, Kessler, Sisson et al. 2012, Weston, Wisloff et al. 2013): • Two sessions/week of short constant load cycling of 8–12 x 1 min at ~10METs (or 90-95% HRR; [1 min HIIT]) with 1 min passive recovery. • One session/week of cycling/jogging/running 4–8x4 min at 8 METS (or 70-85%HRR; [4 min HIIT]) with 1 min passive recovery.

882 Con, control; SSE, Supervised standard exercise; HIIT, High-intensity intermittent
883 training; min, minutes; METs, metabolic equivalent task; HRR, heart rate reserve.
884

885 **Exercise treatments:**

886 Exercise will be conducted on stationary bikes or treadmills according to each
887 participant's preference in group settings. Exercise intensities will be prescribed and
888 monitored using heart rates (% of heart rate reserve [HRR]). Sessions will be conducted
889 at university fitness centres/exercise clinics under the supervision of accredited exercise
890 physiologists, who have trained many overweight women with and without PCOS
891 (Thomson, Buckley et al. 2008, Thomson, Buckley et al. 2010, Hutchison, Stepto et al.
892 2011, Harrison, Stepto et al. 2012, Albright, Steffen et al. 2014, Keating, Machan et al.
893 2014, Keating, Hackett et al. 2015). Adherence and compliance will be determined from
894 supervised exercise session attendance and completion of prescribed exercise (duration
895 and intensity [METs and %HRR]) respectively. Data from participants with less than

70% adherence will be included in the intention to treat analysis only. Any musculoskeletal injuries or changes in health status will be recorded via a provided web-based diary. Women will be supported throughout to building on the behaviour change intervention and self-select post intervention exercise engagement. They will be provided with home-based personalised exercise programs including HIIT if requested.

Preventing and managing injury: Absolute risk of cardio-vascular disease (CVD) in this young female population is low. However, participants will be screened with appropriate clinical monitoring during all exercise testing (ECG), and training sessions (heart rate monitor) with individualised training prescription and progression reducing the risks of injuries and adverse events (Thomson, Buckley et al. 2008, Thomson, Buckley et al. 2010, Hutchison, Stepto et al. 2011, Harrison, Stepto et al. 2012).

Volume matching and training progression: The SSE and HIIT intervention arms will be matched for training volume (MET.min/week) and progressed weekly by manipulating session time and intensity. Both SSE and HIIT will progress from 312 MET.min/week in week 1 to 530 MET.min/week in weeks 8-12, meeting exercise guidelines (Department of Health and Aging 2014). Exercise sessions will include warm-up and cool down protocols, and will be adjusted to individual capabilities and training adaptations (Tjønna, Lee et al. 2008, Harrison, Stepto et al. 2012).

7.5.9 Post-Intervention Assessment

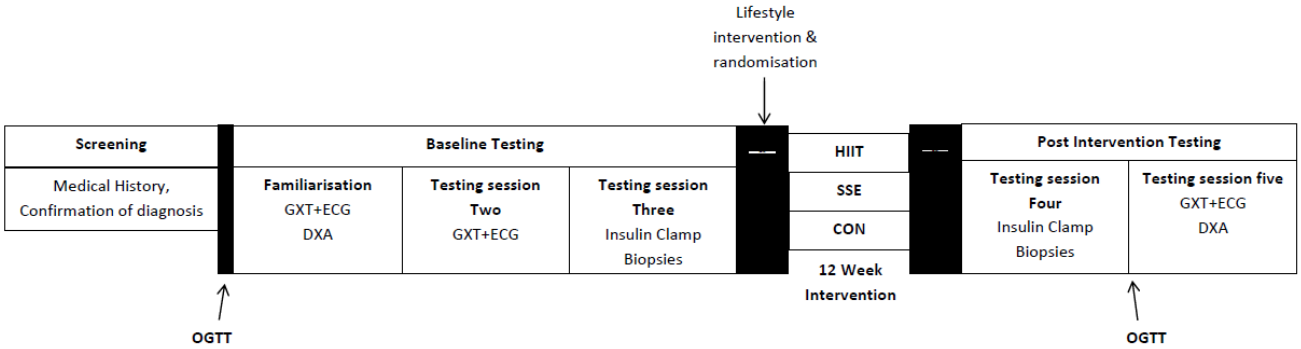
After the 12 weeks of training the hyperinsulinemic-euglycemic clamp, OGTT, questionnaires (SF-36, APAQ and DASS), muscle and fat biopsies, body composition measures (DXA, weight and hip waist circumference) and graded exercise tests will be repeated 2-4 days after last exercise session or after 12 weeks in the control group. Women will be asked to abstain from training or physical exercise during this period. A triaxial accelerometer will be given (or mailed if in the control group) 7 days before post-intervention testing to assess exercise in this period. They will again be asked to fill out a 3-day food dairy.

Follow-up: Twelve months after completion of the 12-week intervention phase, women will return to study centres for re-assessments of selected endpoints (OGTT, clinical and hormonal markers, body composition, lifestyle monitoring). Exercise behaviour change will be monitored from questionnaires and accelerometery (Actigraph™).

7.5.10 Outcome Measurements

Outcome measures will be taken pre-intervention and post-intervention. The structure of testing is shown in Figure 7.1. The primary outcome is the change in insulin sensitivity between the three interventions. Secondary outcomes include reproductive hormone profiles (AMH and steroid profiles), epigenetic profiling, and assessment of molecular pathways underlying insulin resistance in PCOS (fibrosis and TGFβ signalling), psychological status, body composition, physical activity behaviours, and other cardio-metabolic measurements such as improvement in fitness. Other outcome measures will include self-reported diet information and program enjoyment.

Figure 7.1: Schematic diagram of randomised control trial.



GXT, sign and symptom graded exercise test; ECG, electrocardiogram; Insulin clamp, hyperinsulinemic-euglycemic clamp; OGTT, oral glucose tolerance test; DXA, dual energy x-ray absorptiometry; HIIT, high intensity intermittent training; SSE, supervised standard exercise; Con, control.

7.6 Data collection and analysis

7.6.1 Anthropometric Assessment

Participants will be weighed lightly clothed and without shoes (HW-PW200, associated scales services). Height will be taken without shoes using a calibrated stadiometer (Proscale Inductive Series I, Accurate Technology Inc.). BMI will be calculated [weigh (kilograms)/height squared (squared metres)]. Waist and hip circumference measurements will be taken (Swain 2014). Waist to hip ratio is calculated as waist/hip circumference. Fat mass, abdominal fat mass and fat free mass will be measured by DXA and analysed by a qualified operator.

7.6.2 Fitness Parameters

Cardiorespiratory fitness via peak oxygen uptake (VO_{2peak}) will be assessed during a symptom-limited graded exercise protocol on a cycle-ergometer. The test will start after a 5-minute period at rest. The protocol will consist of three minutes stages at an intensity

of 25 watts (W), 50W, 75W respectively, and then an increase of 25W per minute. The test will be terminated objectively when:

- Participant can no longer sustain a pedal rate greater than 60rpm
- There is no longer a change in VO_2 with increasing work rate
- Respired expiratory rate (RER) reaches 1.1 or greater
- Patient wishes to stop
- Or clinical signs or symptoms of metabolic or cardiorespiratory abnormalities appeared.

Expired respiratory gases will be collected by the COSMED cardio pulmonary exercise test system breath-by-breath connected to automated gas analysers. The system will be calibrated before conducting each test using Hans Rudolph syringe and gases of known O_2 and CO_2 content (BOC gas). During this testing they will be monitored by 12-lead electrocardiography (ECG). This is a precautionary measure only and is not present as a diagnostic tool.

7.6.3 Physical activity and diet

Physical activity and exercise before baseline testing and before post intervention testing will be monitored by a triaxial accelerometer (Actigraph^{TM}). Average daily time spent in moderate to vigorous activity and METs will be calculated by the Freedson VM3 (2011) algorithms in Actlife software (Sasaki, John et al. 2011). During the 12-week intervention habitual physical activity will be objectively assessed using physical activity monitors (FitBit Flex^{TM}) and a smart phone application. Dietary habits will be assessed by 3-day food diary before baseline and post intervention testing. Food diaries

will be analysed by FoodWorks® (Xyris) for the major food groups (grains, fruit, vegetables, protein and dairy), total energy (macronutrients) and fat ratios.

7.6.4 Self-reported measures

Participants will monitor their menstrual cycles using a menstrual diary and which will be used to assess menstrual cyclicity throughout the study. Three questionnaires (DASS, SF-36 and APAQ) (Ware and Sherbourne 1992, Australian Institute of Health and Welfare 2003, Henry and Crawford 2005) will be completed prior to and after the intervention to establish psychosocial profile to identify any differences in mental health status after an exercise intervention.

7.6.5 Hyperinsulineamic euglycemic clamp

Participants will undergo a hyperinsulinemic-euglycemic clamp to measure insulin sensitivity (Hutchison, Teede et al. 2012, Stepto, Cassar et al. 2013, Levinger, Brennan-Speranza et al. 2016). Human insulin (therapeutic insulin used by patients with type 1 diabetes mellitus and T2DM [NovoNordisk ActRapid]) will be infused at a constant rate [40mU/min/m^2] while a variable rate glucose solution is infused to meet the target of 5mmol.L-1 blood glucose in the last 30 minutes of the clamp. During the clamp one hand will be warmed to arterialise the blood samples. Blood samples will be taken every 5 minutes to monitor glucose and an additional blood sample will be taken every 30 min during the clamp to analyse insulin. To reduce the risk of low potassium levels (hypokalaemia) participants will be asked to consume a single dose (600mg) of slow-release potassium.

7.6.6 Oral glucose tolerance test

On a separate day and after an overnight 8-hour fast a sterile polyethylene catheter will be inserted into the antecubital vein and baseline blood samples will be collected. Participants will then ingest a 75g glucose drink (within 5 minutes) and samples of blood will be collected at allocated time points (30, 60, 90 and 120 minutes). Serum will be snap frozen in liquid nitrogen and plasma will also be collected in Eppendorf tubes before being placed on ice and transferred to -80 C freezer for long-term storage.

7.6.7 Pathology analysis

Glucose will be measured by using an automated analyser (YSI 2300 STAT Plus). Insulin concentration will be determined by radioimmunoassay according to manufacturer instructions (HI-14K, EMD Millipore). Standard clinical pathology testing including; lipid profiles, haemoglobin A1c (HbA1c), anti-müllerian hormone (AMH) will be performed by a Health Pathology service. Serum steroid profiles including testosterone, dihydrotestosterone, 3α and 3β androstanediols, estradiol, estrone, dehydroepiandrosterone (DHEA), androstenedione and progesterone will be determined by LC-MS mass spectrometry. Enzyme-linked immunosorbent assay (ELISA) will be used to measure N-terminal pro-peptide of type I&III collagen (collagen synthesis biomarkers), and transforming growth factor 1/3 (TGFβ1/3). To minimize variability, samples will be stored at (-80 °C) and batch analysed by a single laboratory.

7.6.8 Tissue biopsies

Vastus lateralis muscle and peri-umbilical fat biopsies will be carried out under a local anaesthesia by a medical practitioner. After a local anaesthetic (1% Xylocaine) is injected under the skin, a small incision is made to access the thigh muscle or peri-umbilical fat tissue and a small amount of each tissue is extracted from a consistent depth using a Bergstrom biopsy needle with suction (Hutchison, Teede et al. 2012). Tissue samples will be used for the determination of DNA methylation profiles and protein levels of key tissue fibrosis molecules, insulin signalling proteins, and TGFβ ligands before and after the 12-week intervention.

7.6.9 Western blotting

Protein levels of key tissue fibrosis molecules, insulin signalling proteins, and TGFβ ligands will be measured by western blotting. Protein extraction and western blotting will be performed as previously described in chapter 6.4.6- *Muscle Protein Extraction and Analyses (Western blots)*:.

7.6.10 Global methylation of peripheral blood mononuclear cells (PBMC) populations

Detailed methodology of PBMC isolation and quantification has previously been described in chapter 4.3.8- *PBMC isolation*. Briefly PBMC will be isolated and collected by centrifugation by Ficoll gradient, before being stained for flow cytometry. A specific gating strategy to analyse T helper cells, T cytotoxic cells, B cells and monocytes will be used to analyse the 5-methylcytosine quantity in each immune cell population.

7.6.11 Sorting of PBMC for genome-wide DNA methylation and transcriptome analysis

The PBMC will be sorted into four populations (monocytes, T helper cells, T cytotoxic cells and B cells) using the FACS-Aria (BD Biosciences). The detailed methods have previously been described in chapter 5.4.2-*Cell sorting*.

7.6.12 Genome-wide transcriptome and methylome analysis in PBMC and skeletal muscle

Total RNA and genomic DNA from the sorted PBMC populations, adipose tissue and skeletal muscle will be extracted using the Qiagen all prep DNA/RNA/miRNA universal kit (#80224) following manufacturer's instructions. RNA sequencing will be performed according to Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold protocol (Illumina). Detailed for these methods can be found in chapter 5.4.3- *RNA sequencing.* Reduced Bisulphite sequencing will be performed according Diagenode Premium RRBS Kit (#C02030033). Detailed for these methods can be found in chapter 5.4.4- *DNA methylation sequencing.*

7.7 Sample size

The sample size was determined by the primary outcome of between-group difference in change of glucose infusion rate (GIR) of 20mg/min/m^2 (an effect size of 0.35) in response to training (Harrison, Stepto et al. 2012). With a sample size of 20 per group, we are powered at 99% with α=0.05; allowing for a conservative 30% attrition, 42 participants will be powered at 82% (α=0.05). This sample size is sufficiently powered

for mechanistic investigations and will provide valuable pilot data on secondary outcomes should post hoc power analysis be insufficient.

7.8 Statistical Analysis

The primary endpoint and secondary endpoints will be assessed for skewness and transformed if required. The effects of the exercise interventions on endpoints will be assessed using two-way repeated measures ANOVA and generalised estimating equation models to evaluate if the biomarkers vary significantly over time among different groups. Bonferroni corrections will be applied to minimise type I error for multiple tests. Significance will be accepted when $p<0.05$.

7.9 Discussion

PCOS is an insulin resistant condition with previous studies showing that irrespective of BMI, 85% of women with PCOS have insulin resistance (Stepto, Cassar et al. 2013, Cassar, Misso et al. 2016). Insulin resistance is thought to underpin PCOS and to increase hyperandrogenism that drives inherent metabolic, reproductive, and psychological features of the condition (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Moran, Norman et al. 2015). However, the mechanisms of insulin resistance in the context of PCOS remain ill-defined, contributing to its exclusion from diagnostic criteria at present (Teede, Misso et al. 2011). To improve the metabolic and reproductive features of PCOS, insulin resistance is currently treated by weight loss or healthy weight maintenance (achieved through healthy diet and regular physical activity) and/or insulin-sensitising medications (Naderpoor, Shorakae et al. 2015). A

deeper understanding of the insulin resistance mechanisms in PCOS is required to improve treatment, with neither medications nor lifestyle completely normalising insulin resistance (Naderpoor, Shorakae et al. 2015). This paper describes the protocol of a study evaluating the effectiveness of HIIT or moderate intensity exercise on cardio-metabolic, reproductive and psychosocial health in overweight women with PCOS.

Compared to recommended lower-intensity regimes, more positive metabolic health outcomes are now being reported for HIIT including glycaemic control and cardio-respiratory fitness (Weston, Wisloff et al. 2013, Greenwood, Noel et al. 2016). To date its mechanisms of action and clinical efficacy for chronic illness have only been evaluated in small short-term (<2 week) RCTs with no gold-standard outcome measurements. Only one RCT has been conducted in women with PCOS to assess the benefits of HIIT to which they compared a resistance training program and a lifestyle intervention group (Almenning, Rieber-Mohn et al. 2015). They found positive outcomes after 10 weeks in insulin sensitivity, improvements in high density lipoprotein cholesterol and a decrease in fat percentage (Almenning, Rieber-Mohn et al. 2015). Altogether, HIIT in PCOS promises greater metabolic benefit with demonstrated physical and mental feasibility, tolerance, and safety (Greenwood, Noel et al. 2016). The proposed 12-week HIIT intervention has proved enjoyable for women with PCOS with significantly higher positive affective responses (feeling scale scores) reported for 1min HIIT vs. MOD (SSE) sessions in 8 women with PCOS from our pilot study Figure 7.2.

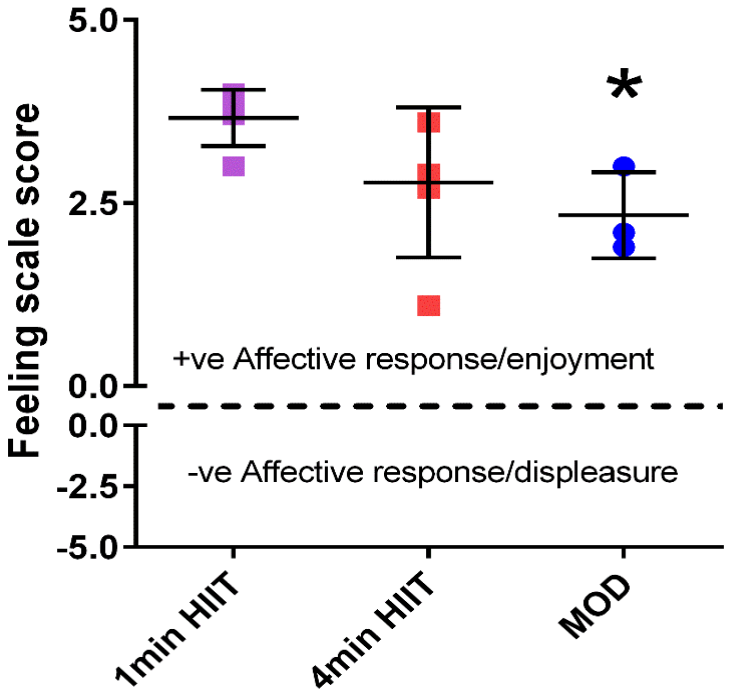


Figure 7.2: Average affective responses to 1min & 4min HIIT and moderate intensity exercise sessions.
Data from pilot iHIT-PCOS study showing an average feeling scale score for all sessions undertaken either in HIIT (n=5) or SSE (n=3; MOD) groups over 12 weeks. *-significantly different to 1min HIIT P<0.05.

This trial aims to demonstrate comparative efficacy of different exercise protocols to inform clinical practice in treatment of PCOS. It will advance the understanding of PCOS management by providing insights into the optimal exercise programme to improve insulin sensitivity. Finally, we will explore the impact of different exercise protocols on reproductive hormone profiles (anti-müllerian hormone and steroid profiles), psychosocial health and molecular mechanisms that affect insulin resistance.

7.10 Trial status

This RCT began recruiting in May 2016 with the first eligible participant enrolling in June 2016. The trial is expected to be completed in 2019.

CHAPTER 8. DISCUSSION AND FUTURE DIRECTIONS

8.1 Summary

PCOS has major metabolic (increased type 2 diabetes mellitus and cardiovascular risk factors) (Moran, Lombard et al. 2010), reproductive (leading cause of anovulatory infertility) (Teede, Misso et al. 2011) and psychological (increased symptoms of anxiety and depression) (Cooney, Lee et al. 2017) impacts, representing a substantial health burden. Unfortunately, it is under-recognised by health professionals leading to a delay in diagnosis (Gibson-Helm, Lucas et al. 2014, Gibson-Helm, Teede et al. 2017). This delay puts women with PCOS at an elevated risk of developing chronic conditions as many women are not receiving adequate treatment or may remain undiagnosed (Moran, Misso et al. 2010, Deeks, Gibson-Helm et al. 2010 , Moran, Strauss et al. 2011, Teede, Misso et al. 2011, Gibson-Helm, Teede et al. 2017). PCOS is incorrectly considered an uncommon condition and therefore has been neglected in research PCOS. As such optimal treatment is an issue, as the biological origins and pathophysiology of PCOS is poorly understood. Made more difficult by the fact that there is a complex interaction between genetics and environmental factors which are thought to play a role in driving both the metabolic and reproductive features of PCOS.

Insulin resistance a key metabolic factor has a central aetiological role in PCOS and may be involved in a complex interaction between perturbed GnRH pulsatility, hyperandrogenism and chronic low-grade inflammation (Nestler and Jakubowicz 1996, Diamanti-Kandarakis and Dunaif 2012, Shorakae, Teede et al. 2015, Cassar, Misso et al. 2016). Multi-disciplinary lifestyle interventions including diet, exercise and behaviour change is one of the first line treatments for women with PCOS (Teede, Misso et al. 2011) as it improves the metabolic and reproductive clinical features by

improving insulin sensitivity (Harrison, Stepto et al. 2012). However, gaps remain in the understanding of the molecular mechanisms of PCOS, PCOS-specific insulin resistance and exercise induced improvements in the pathophysiological features of PCOS.

In my thesis I explored in a series of independent but related studies the key basic science and clinical gaps that align with NHMRC-centre for research excellence and international evidence syntheses in PCOS. These included the biological origins of the syndrome by exploring candidate gene association (chapter 3), epigenetic modifications (specifically DNA methylation) in immune cells (chapters 4 & 5), molecular mechanisms of PCOS-specific insulin resistance and the impact of exercise therapy (chapter 6 and 7). The overall body of work aimed to address the following research questions on pathophysiology and lifestyle therapy in PCOS:

- What genes are implicated in PCOS, are these genes consistent across existing literature, and is there comparability between studies?
- How do DNA methylation in immune cells relate to biochemical and anthropometric markers in PCOS?
- What are the specific molecular mechanisms involved in insulin resistance in PCOS?
- What is the optimal exercise intervention for treating women with PCOS? How does exercise affect the molecular mechanisms of insulin resistance in PCOS?

I will now summarise and discuss the main findings in the context of the limitations and strengths of each study and how this thesis advances knowledge and potentially informs improvements in clinical practice.

8.2 Genetic associations in PCOS (Chapter 3)

Chapter 3 systematically explored the literature and synthesis of evidence using a novel approach (systematically reviewing systematic reviews) to understand the potential contributions of *a-priori* selected single candidate gene variants and their associations with PCOS. This work identified several candidate genes and their variants, but overall the data was underwhelming with no clear links between studied genes and biological origins and/or established pathophysiology of PCOS. However, this work identified the limitations and important methodological considerations that should inform and complement future genetic studies. Specifically, data obtained from genome-wide association studies will always require validation by candidate gene association studies and functional studies in PCOS.

In collaboration with Alba Moreno-Asso, Helena Teede, Joop Laven, Nigel Stepto, Lisa Moran and Melanie Gibson-Helm, I conducted a rigorous overview of systematic reviews regarding candidate gene association studies in PCOS following the PRISMA checklist Supplementary table 3.7 and AMSTAR tools (Shea, Grimshaw et al. 2007, Moher, Liberati et al. 2009). This novel approach has recently been introduced due to a large increase in publications of systematic reviews creating a need to assess the methodological quality on a given topic and the consistency of evidence contained within them (Silva, Grande et al. 2012). The single nucleotide polymorphisms (SNPs) identified from this overview were separated into three main categories metabolic dysfunction, imbalances in androgens, and gonadotrophins, and inflammation. Gene variants that are associated with metabolic dysfunction represented a majority of the

SNPs identified in in overview of systematic reviews. We found no consistent associations across ethnicities, genotyping methods or sources of recruitment in SNPs involved in metabolic function. This overall lack of evidence is mirrored by genome wide association studies which have not found any associations between PCOS and variants in obesity or T2DM genes (Hayes, Urbanek et al. 2015) including the gene variants documented in the systematic reviews assessed here (e.g.- insulin gene, insulin receptor). At this stage it is unclear what role metabolic gene variants play in the aetiology of PCOS. Quality of studies was an issue for SNPs in genes that affected androgens, and gonadotrophins due to combination of methodological flaws (i.e. not completing a comprehensive literature search or not assessing scientific quality). With the lack of high quality systematic reviews there is scope for further high quality, adequately powered systematic reviews to validate the androgens, and gonadotrophins gene loci findings of GWAS to establish associations and causality. Lastly, this overview could not confirm inflammatory gene variants associating with PCOS despite clinical evidence of chronic low-grade inflammation (Xiong, Liang et al. 2011). Only one of the included systematic reviews investigated the confounding influence of BMI and therefore are unable to decipher the influence of obesity on inflammation gene variants (Guo, Zheng et al. 2015). Obesity is known to exacerbate many of the symptoms of PCOS and it is crucial for future genetic association studies to consider the complexity of PCOS and explore both the PCOS-specific and obesity-related contributions. As summarised above due to various limitations we could not find candidate genes and their SNPs that were consistent across studies or consistent with GWAS findings.

This overview highlighted methodological flaws that should be addressed in future systematic reviews and primary genetic studies to improve the comparability of results when examining genetics in PCOS to prevent replication of studies that are of poor quality. This is of vital importance, particularly in genetics with the introduction of GWAS, where candidate gene studies will play an imperative role in validating and deciphering the functional gene variants and assist in determining the clinical relevance of GWAS findings (Wilkening, Chen et al. 2009, Vlahovich, Hughes et al. 2017, Williams, Williams et al. 2017). These recommendations will improve the quality of not only systematic reviews but also candidate gene studies and will allow the field of genetics in PCOS to evolve. Overall and despite limitations the data so far in genetics are limited suggesting that some of biological origins and pathophysiology may be driven by other factors along with genetics and genetic polymorphisms.

8.3 Epigenetics in PCOS (Chapters 4 and 5)

Investigations by genetic association studies to identify the SNPs have resulted in mixed outcomes, as highlighted in chapter 3. Additionally, gene risk loci identified by GWAS only account for only a small portion of the heritability providing evidence that there are other factors affecting heritability (Chen, Zhao et al. 2011, Shi, Zhao et al. 2012, Day, Hinds et al. 2015, Hayes, Urbanek et al. 2015). Exposure to environmental factors such as stress, poor nutrition, placental insufficiency and endocrine imbalances can alter the epigenome early-in-life, especially *in-utero*. This can lead to permanent phenotypic changes that can influence the health of an individual and increase the risk of metabolic disease later in life (Zheng, Xiao et al. 2014, Lillycrop and Burdge 2015). The mechanisms behind this re-programming has not been fully elucidated. However, from

animal models it has been established that perturbations to the epigenome through DNA methylation link the *in-utero* environment and the development disease and potentially of PCOS later in life (Zhu, Zhu et al. 2010, Xu, Kwon et al. 2011, Xu, Chua et al. 2014, Zhang, Cong et al. 2014). Therefore, in chapters 4 and 5 in a cross-sectional study I investigated whether women with PCOS displayed unique global or genome-wide DNA methylation patterns in immune cells compared to women without PCOS.

8.3.1 Global DNA methylation

In chapter four I used a novel flow cytometry technique to measure global DNA methylation in selected individual immune cell populations. During my candidature period I travelled to UNSW, Sydney to learn this technique under the direction of Dr David Simar. Global methylation is a non-sequence dependent measurement of methyl cytosine content and provides a marker of global methylation status (quantity) allowing one to explore its relationship with environmental (i.e. pathophysiological) stimuli. I found for the first time that women with PCOS display hypo-methylation in T helper cells, T cytotoxic cells, B cells and monocytes. Demonstrating that each immune cell population has been impacted by the prevailing PCOS-specific pathophysiology and altering the overall cytosine methylation in the DNA of these cell populations. This contrasts with other literature in the field (Xu, Azziz et al. 2010). This could be due methodologically different approaches to explore global DNA methylation in whole blood. This is important to consider in future epigenetics studies when assessing DNA methylation in blood samples as different approaches may hide subtle but important disease-specific DNA methylation changes in the immune cells of women with PCOS and chronic diseases more generally.

I then explored whether there were any relationships between established clinical/pathophysiological measures and global DNA methylation. Circulating AMH was negatively associated with global hypo-methylation in T cells (T helper and T cytotoxic cells) but not B cells or monocytes. Interestingly, when a subset analysis was conducted in the T helper cells, the association between global DNA hypo-methylation and AMH was only significant in women with PCOS, suggesting this may be intrinsic effect of PCOS. AMH is a negative regulator of follicle growth and is involved in follicular arrest of the pre-antral and antral follicles in the ovaries where it is predominantly expressed (Dewailly, Andersen et al. 2014). However, this data highlights a potential association between global DNA methylation in T cells and circulating AMH. AMH is a member of the TGF-β superfamily, which is known to communicate with the epigenome and control a variety regulatory epigenetic signals such as chromatin remodelling, histone modification and DNA methylation (Bai and Xi 2017). Further studies are warranted to explore this association to establish causal links. Namely, are elevated circulating levels of AMH providing an environmental cue for alterations to the level of global DNA methylation in T cells?

8.3.2 Genome-wide DNA methylation and transcriptome analysis in T helper cells

Chapter 4 highlighted that PCOS resulted in an overall reduction in global DNA methylation in a number of PBMC populations. In chapter 5, I therefore expanded the prior analysis to explore genome-wide methylation patterns and its perturbations in immune cell function in women with and without PCOS. Due to financial and time constraints associated with my candidature timeframe I focused on investigating the

genome-wide transcriptome and methylome in T helper cells of women with and without PCOS. T helper cells are crucial to the coordination of the adaptive immune response by regulating macrophages, B cells and T cytotoxic and have been implicated in cardiometabolic and inflammatory diseases (Patel, Buras et al. 2013, Hirahara and Nakayama 2016). Genome-wide DNA methylation and transcriptomic analysis establishes differentially methylated/expressed loci across a whole genome and can elucidate molecular pathways of a condition. This is the first study to analyse the transcriptome and DNA methylome profile in one specific population of immune cells (T helper cells).

In the transcriptomic (RNASeq) analysis, BMP signalling was enriched and upregulated. Interestingly, AMH and BMP are both members of the TGF-β superfamily (Akhurst and Hata 2012). In chapter 4 it was found that circulating AMH was associated with the global hypo-methylation in T cells (T helper and T cytotoxic cells). While AMH's own specific receptors are only expressed in ovarian tissue, it can signal through other BMP receptors (Josso and Clemente 2003). Both AMH and BMPs activate the same downstream signalling molecules (Smad 1/5/8) that triggers a signalling cascade (Josso and Clemente 2003). It is possible that the circulating AMH, which is elevated in women with PCOS, may be activating and upregulating the BMP signalling in T helper cells. Therefore, it is possible to link AMH with determining epigenetic markers and regulation of T helper cells.

cGMP signalling was down-regulated and is an important intracellular secondary messenger that has a diverse array of protein targets (Denninger and Marletta 1999).

This includes the cGK pathway that signals IL-2 production which also regulates T cell activation and prevents hyperactivity of active T cells (Fischer, Palmetshofer et al. 2001, Ahluwalia, Foster et al. 2004). In theory, the upregulation of BMP signalling and downregulation of cGMP signalling indicates a possible dysfunction in the activity of T cells. IL-2 regulates the T cells by either suppressing or activating T cells dependent on environmental milieu therefore loss of regulation may be associated with chronic inflammation.

Lastly the transcriptomic analysis found several genes that are non-coding RNAs such as lncRNAs. LncRNAs and DNA methylation are believed to be linked and observed in inflammatory processes and can co-ordinate features of the immune system including cell differentiation, function and recruitment (Atianand and Fitzgerald 2014). LncRNAs can act as a molecular scaffold for methylation and in theory may be linked to some of the differentially methylated CpGs found from the genome wide methylation analysis (Rinn and Chang 2012). Interestingly, 3 genes and 5 promoters were differentially methylated lncRNAs, functional analysis of downstream implications of these findings are required to investigate whether is there is a link between the transcriptome and DNA methylome in PCOS.

In total the genome-wide methylome analysis found 5 581 CpGs, 5 genes and 8 promoters that were differentially methylated in T helper cells between women with and without PCOS. Interestingly, the gene body and promoter regions of secretoglobin family 1D member 4 (SCGB1D4) was differentially methylated between women with and without PCOS which according to GO ontology has a functional role in chemotaxis

and cell-cell signalling. Cytochrome C Oxidase Subunit 6C Pseudogene 15 (COX6CP15) is a pseudogene that was also differentially methylated in both the gene body and promoter. Pseudogenes are ubiquitous and can be transcribed into RNA to regulate expression of their functional gene transcript (Tutar 2012, Johnsson, Ackley et al. 2013). These data provide a novel pathway to explore the functional implications of these differentially methylated genes and promoters in T helper cell function.

GO pathway enrichment analysis showed the highest enrichment in differentially methylated genes were related to T cell proliferation, or function, immune response, cytokine production, response to prolactin, responses to steroid hormones and inflammatory response. We found hyper-methylation of the CpGs near genes related to prolactin consistent with other studies (Li, Zhu et al. 2016). Women with PCOS have lower levels of circulating prolactin (independent of BMI) and these are associated with adverse metabolic profile (Glintborg, Altinok et al. 2014). Further, prolactin is responsible for proliferation, differentiation and maintenance of immune cells numbers providing further evidence that prolactin may associated with PCOS. (Yu-Lee 2002). Hypo-methylated CpGs was found near promoters were related to pathways in lipid transport, and metabolism, and regulation of T cell proliferation. Altered lipid homeostasis in T helper cell is known to promote a pro-inflammatory environment providing an interesting pathway to investigate in PCOS (Hubler and Kennedy 2016). Hyper-methylation of CpGs was found near promoters associated with genes in vesicle trafficking which in theory means a down-regulation of these genes. Vesicle trafficking is involved in regulating chemotaxis, which is an essential process that directs the immune cells and inflammatory processes (Colvin and Luster 2011). Therefore, I

speculate that PCOS is linked with alterations in vesicle trafficking and its downstream effects would alter immune and inflammatory processes.

We have shown that PCOS and its associated hormonal and metabolic milieu impact the global DNA methylation in selected immune cells and the transcriptome and methylome of T-helper cells. The findings of both the global DNA methylation (chapter 4) and genome-wide DNA methylation (chapter 5) provide initial evidence that the immune system is altered epigenetically in PCOS and further work is needed to expand these studies findings to better understand the implications of and contribution to the aetiology of PCOS.

8.4 Pathophysiology of insulin resistance in PCOS and the role of exercise therapy

In the previous chapters 4 and 5 I explored the association of genetics and DNA methylation to the pathophysiology to PCOS. While these provide partial explanation and are indeed involved, insulin resistance appears to play a key role in the complex aetiology of PCOS. Specifically, it is thought that the intrinsic insulin resistance (obesity-independent insulin resistance) compounded by extrinsic insulin resistance (BMI-related insulin resistance) contributes to the aetiology of PCOS (Dunaif, Segal et al. 1989, Diamanti-Kandarakis and Dunaif 2012, Stepto, Cassar et al. 2013, Moran, Norman et al. 2015, Cassar, Misso et al. 2016). Previous studies have implicated impaired insulin signalling within the adipose tissue, fibroblasts and skeletal muscle of women with PCOS. However, additional *in vivo* and *in vitro* techniques are required to

better understand the molecular mechanisms of insulin resistance in a variety of tissues in PCOS. In chapter 6 I therefore explored in a prospective cohort study and a randomised control trial the molecular mechanisms of PCOS, the role of insulin resistance in skeletal muscle and the impact of obesity and exercise. I provide new insights into PCOS-specific insulin resistance and the associated early signalling events in skeletal muscle. I could not confirm the previously postulated aberrant signalling at IR or IRS1/2 in PCOS-specific insulin resistance, at least in skeletal muscle (Corbould, Kim et al. 2005, Corbould, Zhao et al. 2006). However, I did find aberrant insulin signalling in AS160, mTOR and PKC λ/ξ in PCOS-specific insulin resistance. I also considered other possible processes that may impact insulin signalling like TGFβ ligand signalling and extracellular matrix deposition (Raja-Khan, Urbanek et al. 2014). Implicating dysfunctional signalling in the TGFβ family that are regulated by fibrillin's and latent TGFβ binding proteins and this may lead to increased fibrosis predisposing women with PCOS to insulin resistance (Raja-Khan, Urbanek et al. 2014). The gene expression data of elevated collagen, extra-cellular matrix deposition enzymes and TGFβ2R gene expression (pro-fibrotic gene profile), elevated ligands (AMH and TGFβ1) provides initial evidence for this link between the dysfunctional insulin signalling and TGFβ ligands, signalling networks and extracellular matrix deposition.

Exercise is one of first therapies to target improvements in insulin sensitivity. However, like other studies, exercise improved but did not normalise insulin sensitivity (Hutchison, Stepto et al. 2011). This may in part be to be improved but not normalised insulin stimulated signalling at Akt, AS160, and mTOR proteins in women with PCOS. Further, gene expression data showed that 12-weeks of exercise training differentially

regulated 4 genes (COL1A2, COL3A1, DCN and LOX) that may be linked to extracellular matrix deposition or fibrosis after the exercise training intervention. Together aberrant insulin signalling and fibrosis may be associated with the resistance to improved but not normalised insulin sensitivity in women with PCOS. This study highlighted new areas for further research in understanding the mechanisms of PCOS-specific insulin resistance and impact of exercise therapy that is one of the first lines of treatment for overweight women with PCOS.

Based on findings in chapters 4, 5 and 6 I designed a RCT in chapter 7 to understand the molecular mechanisms of insulin resistance in PCOS. Specifically, the RCT is designed to yield further novel human data to provide insights in the molecular response to insulin sensitising therapies like exercise to inform clinical practice. And potentially novel targets for the future that are specific to PCOS. More importantly it addresses the key clinical gaps in PCOS therapy of what is the best exercise dose for treating PCOS.

8.5 Limitations

In my thesis I was able to advance knowledge in the pathophysiology and molecular basis in the PCOS however limitations must be considered. In chapter 3 I conducted an overview of systematic reviews. While I followed gold-standard systematic review methods (PRISMA (Moher, Liberati et al. 2009) and AMSTAR tools (Shea, Grimshaw et al. 2007)) this is a novel concept and therefore this style of systematic review has yet to validated and there are no specific guidelines for conducting an overview of systematic reviews. However, this overview brings attention to a new way to analyse genetic studies. This may help overcome the common methodological flaws such as

sample size that generally hamper genetic studies to progress our knowledge in the genetic contributions to the aetiology of PCOS.

In chapter 4 I conducted regression analysis which cannot distinguish the causality of the hypo-methylation in cell subsets. This limits the capacity to clearly conclude whether methylation changes have any causal impacts on molecular pathways. I also may have been hampered by our sample size, and therefore could not identify all confounding variables in order not to violate the assumptions of the statistical model. However, my study did allow me to discover that each immune cell type has unique global methylation pattern and this improves evidence that immune cells are epigenetically regulated. These findings provide valuable data to warrant larger scale study into DNA methylation and PCOS. The results from chapter 5 provided novel pathways to investigate however much of the interpretation at this stage is speculation. Functional analysis is required to validate the proposed pathways from the transcriptome analysis and DNA methylome analysis. Again, I was hampered by small sample size in the transcriptome analysis of T helper cells. This was due to technical issues as isolating single populations in PBMC meant that I started with smaller sample input which required optimising existing protocols for RNA sequencing and some samples needed to be excluded from analysis at this point. There were thresholds set for quality control and therefore a number of samples needed to be excluded from the analysis due not to meeting the thresholds. This meant that the samples included have met the quality control threshold and therefore have confidence in the findings. In this context this is the first study to analyse the transcriptome and DNA methylome profile in one specific population of immune cells providing valuable data to warrant a larger

scale and more targeted studies in epigenetics. I have provided novel evidence of a link between the T helper cell methylome, and transcriptome and PCOS. I also provided additional new pathways to explore in the role of inflammation in PCOS.

In chapter 6, sample size was limited due to the invasive nature of the procedures used. However, this study utilised gold-standard methods (hyperinsulinemic-euglycemic insulin clamps) to assess insulin sensitivity in a community-recruited, well-characterised population of lean and overweight women with or without PCOS. These findings provide valuable data highlighting new molecular targets for additional larger scale studies to investigate dysfunctional insulin signalling defects, the role of TGFβ ligands and extracellular matrix deposition in intrinsic insulin resistance and the potential impact on exercise improvements.

8.6 Overall conclusions

While I acknowledge my research has a number of limitations, this thesis contributed to advancing knowledge to start answering pertinent questions around the molecular pathways involved in the pathophysiology of PCOS. Chapter three, summarised the limitations and important methodological considerations that need to be considered for all future genetic studies including GWAS or candidate gene polymorphism studies, in PCOS and complex diseases more generally. Chapter four used a novel flow cytometry technique in immune cells that found that each type of immune cell has a unique global DNA in women with PCOS. Complementing existing data that epigenetically regulated immune cells may have further implications in PCOS (Shen, Qiu et al. 2013, Li, Zhu et al. 2016). Chapter five was the first study to analyse the transcriptome and DNA

methylome profile in one population of immune cells (T helper cells). The findings provided novel evidence of epigenetic modifications of T helper cells in PCOS and some support for a role of inflammation in PCOS. Chapter six identified dysfunctional insulin signalling defects distal to IRS1/2 at AS160, mTOR and atypical PKC proteins. We also provided novel evidence for the role of TGFβ ligands and extracellular matrix deposition in intrinsic insulin resistance and the potential impact on exercise improvements. In chapter 7, I designed a gold standard randomised control trial (RCT) aimed to build on novel data found in chapter 4, 5 and 6. This RCT will improve the understanding of the molecular responses to exercise, which will yield human data to advance understanding of PCOS aetiology, provide insights into the mechanisms of insulin resistance in PCOS and demonstrate comparative efficacy of different exercise regimes informing clinical practice.

Unexpectedly from chapter 4, 5 and 6, members of the TGFβ superfamily seemed to have independent but associated roles in inflammation and insulin resistance and as previously known in reproductive dysfunction (Cassar, Teede et al. 2014). The TGFβ superfamily are a group of structurally related regulatory proteins with diverse biological functions including reproduction, extracellular matrix formation, inflammation, metabolism, and development of bone, skeletal muscle, and fat (Diamanti-Kandarakis and Dunaif 2012). We found AMH to be elevated in the circulatory system and negatively associated with global hypo-methylation in T cells (T helper and T cytotoxic cells). BMP signalling was upregulated in T helper cells and is known to affect immune cell function based on other chronic inflammatory conditions. Additionally, there is aberrant gene expression in TGFβ ligands in skeletal

muscle. This family of proteins provides an interesting pathway that warrant further exploration.

Overall this thesis has advanced knowledge in the molecular components of the pathophysiology of PCOS and raised further research questions to be investigated in the future.

8.7 Future directions

The work in this thesis raised the following questions that I believe are pertinent in advancing our knowledge, and clinical practices in PCOS.

- Systematically review current GWAS to form a reliable list of candidate genes that can be validated and assist in determining the clinical relevance using the recommendations found in chapter 3.

- We established novel pathways, and identified candidate genes in the genome-wide transcriptomic and epigenomic analysis. These now need to be validated by measuring gene expression and proteins to clarify how these gene/pathways may be causal in the pathophysiology of PCOS.

- Further characterisation of the non-coding RNAs identified from the transcriptomics analysis to clarify the potential down-stream effects which would providing additional clues as to the molecular mechanisms of PCOS.

- To investigate genome-wide DNA methylation in monocytes, T cytotoxic and B cells as these were also hypo-methylated according to our global methylation

analysis. Additionally, examine different tissue types such as adipose tissue and skeletal muscle to investigate epigenetic cross-talk across differing tissue types.

- Identify epigenomic markers conserved across different tissue to start exploring the in-utero programming of PCOS.
- Longitudinal studies to investigate in-utero programming and the evolution of the PCOS phenotype later in life.

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