Polycystic Ovary Syndrome, Obesity and Insulin Resistance

by

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Abstract

Polycystic ovary syndrome (PCOS) is a common and complex reproductive and metabolic condition with major health consequences across the lifespan. Insulin resistance is thought to be a key underlying feature of PCOS, but its role in metabolic and reproductive complications associated with the syndrome remains elusive. Therefore, the aim of the thesis was to comprehensively assess the role of IR in PCOS. I report that IR is definitively intrinsic to PCOS and exacerbated by BMI. But the effect of BMI on IR is more pronounced in PCOS than controls. Diagnostic criteria and age seem to have little effect on IR in PCOS. Furthermore, IR in PCOS seems to be negatively correlated with testosterone and positively correlated with sex hormone binding globulin. Gonadotropins seem to have little effect on IR. Various biomarkers associated with metabolic diseases appear more strongly associated with obesity rather than with PCOS status and Plasminogen activator inhibitor-1 may also be a novel independent biomarker with the ability to predict IR in women with and without PCOS. This intrinsic IR in PCOS was not attributed to mitochondrial dysfunction and was not related to the pathophysiology of reproductive dysfunction in PCOS as measured by Anti-Mullarian hormone (AMH). However, AMH was able to detect PCOS status and may also be useful in the diagnosis of PCOS. Collectively these chapters of related studies enhanced understanding of IR in PCOS, including the relationship between intrinsic and extrinsic factors and IR and provided information regarding potential markers to aid in diagnosing PCOS and IR.

Doctor of Philosophy by Publication Student Declaration

I, Samantha Cassar, declare that the PhD thesis by publication entitled ‘Polycystic ovary syndrome, obesity 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 27/11/2014

Thesis by Publication

All doctoral students at Victoria University are permitted to submit a thesis by publication. The thesis by publication is a thesis format that includes manuscripts that have been prepared or accepted for publication. These manuscripts may have more than one author in which signatures from co-authors are required. The papers do not have to be rewritten for the thesis; they can be inserted in their published format. The thesis must reflect a sustained and cohesive theme and framing or substantial linking text normally required in introducing the research and linking the chapter and manuscripts.

The work conducted in this thesis was part of a large National Health and Medical Research Council (NHMRC) funded project investigating IR in PCOS. I joined the research team at Monash Centre for Health Research and Implementation, Monash University in 2009. My PhD supervisors, Associate Professor Nigel Stepto and Professor Helena Teede, initially instigated this project, which focused on obese women with and without PCOS. I joined the team at the infancy stages of the second arm of study, at the beginning of my PhD, where my primary role was to investigate IR in lean women with and without PCOS. I established a new clinical room on the premises and I was responsible for ethics amendments, recruitment and screening of all participants and was the primary contact for the study. I also organised and accompanied each participant to their medical review and body composition appointments. I Organised and conducted fitness testing and assisted with euglycaemic-hyperinsunaemic clamp appointments, including setting up all consumables, cannulation of some participants, blood sampling, analysing blood glucose levels, centrifuging blood samples, assisting with muscle biopsies and preparing muscle samples for analysis and ensuring the health and welfare of all participants. I was also responsible for ensuring participant information was kept confidential and I created a database and ensured all data was kept currant and entered

correctly. I independently performed laboratory analysis and analysed and interpreted data collected.

My specific contributions are detailed at the beginning of each chapter. All of my work contributed to this thesis, which contains 7 chapters and 4 manuscripts in total. Of the 4 manuscripts, 3 have been published and the one remaining has been submitted for publication. One chapter remains unpublished but has been written in a manuscript format.

Details of Included Papers: Thesis by Publication

Item/ Chapter No.	Paper Title	Publication Status	Publication Title and Details
2	Insulin resistance in women with polycystic ovary syndrome: A systematic review and meta-analysis of euglycaemic	Currently under review	Human Reproduction Update Impact factor: 8.6 Quartile ranking: Q1
3	Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic–hyperinsulaemic clamp.	Published	Human Reproduction, Vol.28, No.3 pp. 777–784, 2013 Accepted 18 December 2012 Impact factor: 4.6 Quartile ranking: Q1 Scopus citation number: 45
4a	Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity.	Published	Clinical Endocrinology, DOI:10.1111/cen.12619 Accepted 22 September 2014 Impact Factor: 3.35 Quartile ranking: Q1

4b	Response to ‘insulin sensitivity and leptin in women with PCOS’	Published	Clinical Endocrinology, DOI:10.1111/cen.12678 Letter to the Editor Accepted: 17 November 2014 Impact Factor: 3.35 Quartile ranking: Q1
5	Mitochondrial dysfunction as a potential mechanism underlying intrinsic insulin resistance in Polycystic Ovary Syndrome	Not Submitted	
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Declaration by: **Signature:** **Date:**

Samantha Cassar  27/11/2014

Publications

Publications arising directly from this thesis:

*NK Stepto, *S Cassar, AE Joham, SK Hutchison, CL Harrison, RF Goldstein, HJ Teede, Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic–hyperinsulaemic clamp, Human Reproduction, Vol.28, No.3 pp. 777–784, 2013 (*Joint first authors)

S Cassar, HJ Teede, LJ Moran, AE Joham, CL Harrison, BJ Strauss, NK Stepto, Polycystic ovary syndrome and anti-Mullerian hormone: role of insulin resistance, androgens, obesity and gonadotrophins, Clinical Endocrinology, 2014 doi: 10.1111/cen.12557

S Cassar, HJ Teede, CL Harrison, AE Joham, LJ Moran, NK Stepto, Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity, Clinical Endocrinology, 2014 DOI 10.1111/CEN12619

S Cassar, HJ Teede, CL Harrison, AE Joham, LJ Moran, NK Stepto. Response to 'insulin sensitivity and leptin in women with PCOS, Clinical Endocrinology, 2014 DOI 10.1111/CEN.12678

Conference Presentations

Poster Presentations

S Cassar, NK Stepto, SK Hutchison, CL Harrison, HJ Teede, Exercise training enhances but does not restore insulin sensitivity and signalling in obese women with PCOS, 17th European College of Sport Science Congress 4-7 July 2012 Bruges, Belgium

S Cassar, NK Stepto, SK Hutchison, CL Harrison, HJ Teede, Mitochondrial function and insulin resistance in women with polycystic ovary syndrome, 15th International Biochemistry of Exercise Conference (IBEC) Congress 17-21 June 2012

Oral Presentations

S Cassar, NK Stepto, LJ Moran, SK Hutchison, CL Harrison, HJ Teede, Markers of Metabolic Disease Risk in Polycystic Ovary Syndrome, The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology 2013

S Cassar, HJ Teede, M Misso, WG Hopking, NK Stepto, Insulin sensitivity in women with a polycystic ovary syndrome: A systematic review and meta-analysis, The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology 2014

Additional Conference Presentations Arising from this Thesis

Anju Elizabeth Johan, Samantha Cassar, Nigel Stepto, Cheryce L Harrison, Samantha Kate Hutchison, Sunjeeva Ranasinha and Helena J Teede, Vitamin D in Polycystic Ovary Syndrome: Relationship to BMI and insulin sensitivity, Endocrine Society's 95th Annual Meeting and Expo, June 15–18, 2013 - San Francisco

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Abbreviations

AES	Androgen Excess Society
AMH	anti-mullerian Hormone
ASRM	American Society of Reproductive Medicine
ATP	adenosine triphosphate
AU	arbitrary units
BMI	body mass index
CI	confidence interval
CVD	cardiovascular disease
CYP-17	cytochrome P450c17
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DNA	deoxyribonucleic acid
ECT	electron transport chain
ESHRE	European Society of Human Reproduction and Embryology
FAI	free androgen index
FCCP	carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FFA	free fatty acids
FSH	follicle stimulating hormone
GDM	gestational diabetes mellitus
GIP	glucose-dependant insulinotropic polypeptide
GIR	glucose infusion rate
G/I ratio	fasting glucose to insulin ratio
GLP-1	glucagon-like peptide-1
GLUT4	glucose transporter 4

GnRH	gonadotropin releasing hormone
HbA1c	glycated haemoglobin
HOMA	homeostatic model of assessment
HPA	hypothalamic-pituitary axis
HRP	horseradish peroxidase
HRR	high resolution respirometry
IGT	impaired glucose tolerance
IKK-β	inhibitor of nuclear factor kappa-B kinase subunit beta
INSR	insulin receptor
INSR-1	insulin receptor substrate-1
INSR-1	insulin receptor substrate-2
IR	insulin resistance
IVGTT	intravenous glucose tolerance test
JNK	C-Jun N-terminal kinase
LH	luteinising hormone
MOOSE	Meta-analysis of Observational Studies in Epidemiology
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
NADH	nicotinamide adenine dinucleotide hydride
NAFLD	non-alcoholic fatty liver disease
NIH	National Institute of Health
NHMRC	National Health and Research Medical Council
OR	odds ratio
OXPHOS	Oxidative phosphorylation
PAI-1	plasminogen activator inhibitor-1

PCO	polycystic ovary
PCOM	polycystic ovary morphology
PCOS	polycystic ovary syndrome
PGC-1α	proliferator-activated receptor γ coactivator α
PKB	protein kinase B
PI3K	phosphoinositide 3-kinase
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-analyses
QUICKI	quantitative insulin sensitivity check index
QUOROM	Quality of Reporting of Meta-analyses
ROS	reactive oxygen species
ROX	residual oxygen consumption
SD	standard deviation
SHBG	sex hormone binding globulin
T2DM	type 2 diabetes mellitus
TGFβ	transforming growth factor β
VO_2max	Maximal oxygen consumption
WHO	World Health Organisation

Chapter 1 Review of Literature

1.0 Introduction and definition of polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a common metabolic and endocrine disorder affecting 6 to 21% of reproductive aged women depending on population, mean body mass index (BMI) and diagnostic criteria used (March et al., 2010, Boyle et al., 2012). High prevalence are rates seen in women whom are overweight or have an Indigenous or Asian background (Boyle et al., 2012, March et al., 2010). The features of PCOS, including menstrual dysfunction, infertility and hirsutism have been described in medical records for more than 2,000 years (Azziz et al., 2011). The syndrome was officially recognised in the 1930's by Stein and Leventhal who associated polycystic ovaries (PCO) to the clinical features of menstrual dysfunction, infertility, hirsutism and obesity (Stein and Leventhal, 1935). Since the 1980's, researchers expanded on these observations to report an association between hyperinsulinaemia and hyperandrogenism bringing to light possible aetiologies and a complicated metabolic and reproductive condition with psychosocial and economic consequences across the lifespan (Burghen et al., 1980, Dunaif, 1989, Shoupe et al., 1983, Teede et al., 2010). These ground-breaking studies also caused great debate as to whether insulin resistance (IR) is a unique feature of PCOS contributing to clinical features and health consequences.

During the past two decades skeletal muscle dysfunction has been associated with the pathogenesis of IR, including dysfunctional insulin signalling and mitochondria, and the release of cytokines. However, there is still little consensus regarding the underlying mechanisms of IR in PCOS. Therefore further investigation into the aetiology of PCOS and IR is vital for the adequate treatment of the syndrome.

The present chapter provides an overview of the background of this thesis by describing the literature regarding PCOS and proposed aetiology, which is followed by an explanation of IR and the current understanding of its role in the development of PCOS and associate reproductive consequences. It also concludes by highlighting gaps in our current knowledge.

1.1 Diagnostic Criteria of PCOS

The diagnostic criteria for the syndrome continue to evolve with advances in both the understanding of the condition and the precision of medical equipment and technology. Hyperandrogenism, menstrual irregularity, and PCO have been proposed as the diagnostic features of PCOS (Rotterdam, 2004). Hyperandrogenism is assessed by clinical and biochemical measurements. Clinical features of hyperandrogenism include hirsutism, acne, female-pattern alopecia (hair loss) and acanothosis nigricans, which occur in 73.9%, 53.3%, 34.8% and 5.3% of women with PCOS respectively (Ozdemir et al., 2010). These differ across ethnicities, are often subjective and altered by cosmetic treatment such as laser (McGill et al., 2007). This makes clinical features difficult to interpret when diagnosing PCOS. Biochemical hyperandrogenism is determined by free testosterone and/or free androgen index (FAI) and are composite measures that include assessments of circulating levels of serum testosterone and sex hormone binding globulin (SHBG) (Norman et al., 2007). However, it is unclear which androgens are best measured, what constitutes normal reference ranges in women and which analytical technique should be used, with recent publications questioning the specificity and accuracy of commonly used immunoassays (Barth et al., 2007, Handelsman and Wartofsky, 2013, O'Reilly et al., 2014)

The diagnosis of menstrual irregularity is based on identifying either oligoovulation; which is defined as less than eight periods per year or cycles which are longer than 35 days, and

anovulation; the absence of menstruation for more than three months without pregnancy (Norman et al., 2007). Approximately 90% of women with oligoovulation or anovulation are diagnosed with PCOS and up to 95% of women with PCOS present with these symptoms (Rotterdam, 2004). During adolescence, perimenopause and postmenopause, interpreting the cause of irregular menstrual cycles is challenging and makes diagnosis more difficult.

Polycystic ovarian morphology (PCOM) is most commonly measured using transvaginal ultrasound and is defined as the presence of 12 or more follicles in one or both ovaries, measuring 2-9 mm in diameter, and/or having an ovarian volume greater than 10 mL (Rotterdam, 2004). Over 90% of women with PCOS have PCOM and women with PCO can have up to six times more early developing antral follicles compared to the ovaries of healthy women. PCO remains non-specific for PCOS and prevalence rates of PCO are increasing because of more advanced imaging equipment (Leonhardt et al., 2014). Specificity of PCOM in PCOS diagnosis remains controversial. Anti-mullerian hormone (AMH) concentrations are reflective of the number of pre-antral and small antral follicles in the ovary and raised concentrations are evident in women with PCO and PCOS. This makes AMH concentrations a potential tool to aid in the diagnosis of PCOS (Dewailly et al., 2014, Homburg et al., 2013). Currently, IR is not included as a diagnostic criterion for PCOS, even though it is acknowledged to be a central feature of the condition (Teede et al., 2011). This is largely that IR cannot be simply and accurately measured in clinical practise, hence routine testing is not recommended in PCOS.

To date, there are three definitions or diagnostic criteria for PCOS, which can make the diagnosis experience confusing and lengthy for both the clinician and woman involved (Gibson-Helm et al., 2014). The original diagnostic criteria were formed in 1990 by the

National Institutes of Health (NIH) consensus group and were based on opinion rather than clinical evidence (Azziz et al., 2006). PCOS was defined as having clinical and/or biochemical signs of hyperandrogenism and oligoanovulation. In 2003, the second iteration of diagnostic criteria for PCOS was established by the European Society for Human Reproduction and Embryology and the American Society of Reproductive Medicine (ESHRE/ASRM) consensus group to include any two of the three criteria; clinical and/or biochemical signs of hyperandrogenism, oligo-anovulation and PCO on ultrasound. This set of criteria is referred to as the Rotterdam criteria and introduces different PCOS phenotypes; classic (hyperandrogenism and oligoanovulation), ovulatory (hyperandrogenism and PCO) and normoandrogenic (oligoanovulation and PCO) (Rotterdam, 2004). In 2006, the Androgen Excess Society (AES) published a position statement, based on an evidence based review that suggested hyperandrogenism should be the key component to diagnose PCOS together with oligoanovulation or PCO or both (Azziz et al., 2006, Azziz et al., 2009). All three definitions require the exclusion of other conditions that cause clinical features of PCOS. These conditions include congenital adrenal hyperplasia, Cushing's disease, thyroid dysfunction and hyperprolactinemia (Azziz et al., 2006, Azziz et al., 2009, Rotterdam, 2004).

The NIH and AES criteria tend to diagnose women with the more severe spectrum of the disease. However, the Rotterdam criteria are now internationally accepted by the NIH, Australian guidelines and European societies. Given that an estimated 70% of PCOS cases are undiagnosed in Australia (March et al., 2010), reducing confusion and promoting awareness of the endorsed diagnostic criteria is important (Rotterdam, 2004, Teede et al., 2011). The Rotterdam criteria are used to diagnose PCOS in this thesis as it has been endorsed by national and international bodies and are inclusive of the original NIH criteria.

1.2 Epidemiology

The lack of prior consensus on the definition and diagnosis of PCOS has undermined investigations attempting to accurately determine population-based prevalence rates for the condition (Hart et al., 2004, March et al., 2010). Despite PCOS being the most common endocrine disorders in reproductive aged women, prevalence estimates are highly variable ranging from 2-21% depending on the diagnostic criteria used, recruitment strategy, population studied (selected and unselected populations) and ethnicity (Asuncion et al., 2000, Boyle et al., 2012, Chen et al., 2008b, Diamanti-Kandarakis et al., 1999, Farah et al., 1999, Knochenhauer et al., 1998, Kumarapeli et al., 2008, March et al., 2010). The introduction of the Rotterdam criteria broadened the previous NIH definition by including PCO morphology and therefore two additional phenotypes and as a consequence prevalence rates rose from an estimated 6-8% to 12-20% (Boyle et al., 2012, March et al., 2010, Yildiz et al., 2012).

The only community based study in Australia to assess prevalence rates of PCOS using current international diagnostic criteria (Rotterdam) reported a prevalence of 11.9% in 728 women between 27-34 years of age (March et al., 2010). The prevalence increased to 17.8% using the Rotterdam criteria when imputed data was included for participants not consenting to ultrasounds (March et al., 2010). Furthermore, the prevalence of PCOS is higher in Australian Indigenous communities where it affects up to 21% of women (Davis et al., 2002, Boyle et al., 2012). A community based Iranian study investigated prevalence rates in 1126 women aged 18-45 years and found similar rates to that of the Australian study, 7.1%, using the NIH definition and 14.6% using the Rotterdam criteria (Tehrani et al., 2011). Prevalence rates in Southern China were 2.2% using the Rotterdam criteria (n = 915) in women recruited through their annual physical examination (Chen et al., 2008b); it was 4.0% in women living in South-eastern United States (NIH, n = 369) (Knochenhauer et al., 1998), 6.3% in a Sri

Lankan population (Rotterdam, n = 2,915) (Kumarapeli et al., 2008), 6.5% in women (NIH, n = 154) living in Spain volunteering to donate blood (Asuncion et al., 2000), 6.8% (n = 192) in a Greek population (Diamanti-Kandarakis et al., 1999) and 26% in the United Kingdom (n = 230) (Michelmore et al., 1999).

Additionally, the prevalence of PCOS increases by 9.2% for every single unit increment in body mass index (BMI) (Teede, 2013) and PCOS is five times higher in obese populations compared to women within a healthy weight range (Alvarez-Blasco et al., 2006). In contrast, reported prevalence rates of PCOS in underweight, normal-weight, overweight, obese and moderate-obese women to be 8.2%, 9.8%, 9.9%, 9.0% and 12.4% respectively, leaving authors to concluded that PCOS is likely due to intrinsic or inherited factors (Yildiz et al., 2008). Therefore, obesity may have a small effect on prevalence rates of PCOS, but it has profound effects on the presentation of clinical features and degree of IR in PCOS potentially due to the endocrine function of adipose tissue (Yildiz et al., 2008).

1.3 Significance and Economic Burden of PCOS

The short and long term health consequences associated with PCOS cause a large economic burden. Calculations of the health related economic burden are based on the estimates of prevalence rates, co-morbidities and the expense of diagnosing and treating the condition (Azziz et al., 2005). In 2005, the estimated economic burden of diagnosing and providing care for women with PCOS was $US4 billion annually in the United States (Azziz et al., 2005), equating to an estimated AU$800 million in Australia (Teede et al., 2011). PCOS therefore represents a major health and economic burden (Azziz et al., 2005). It is estimated that 40.3% of this economic burden is a result of diabetes associated with PCOS, 31.0% due to menstrual dysfunction and abnormal uterine bleeding, 14.2% treating hirsutism and 12.2%

infertility care (Azziz et al., 2005). Furthermore, PCOS is the most common cause of anovulatory infertility in women, with high costs in infertile obese Australian women (Clark et al., 1998). In the estimates detailed above, the NIH criteria were used to derive prevalence rates and the economic burden of PCOS. This may have underestimated the financial burden, as prevalence rates are two to three fold higher when Rotterdam criteria is used. Surprisingly, only 2.3% of the economic burden was attributed to diagnosis and evaluation of the condition (Azziz et al., 2005). It is now recognised that there is a need to increase awareness of PCOS and investment to aid in the early diagnosis and treatment preventing the onset of serious sequelae including infertility, IR, type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Azziz et al., 2005).

1.4 PCOS Aetiology

The aetiology of PCOS is complex and remains elusive. A combination of both genetic and environmental factors including ovarian dysfunction, hormonal disturbances, underlying hyperandroginism, IR, obesity and abnormalities in gonadotropin secretion have been implicated in the aetiology of PCOS.

1.4.1 Genetics

A family history of PCOS and genetics has been implicated in the aetiology of PCOS and variations in phenotypical expression. PCOS has high heritability with monozygotic twin sisters (tetrachoric correlation 0.71) being twice as likely to have PCOS compared to dizygotic twin sisters and other female siblings (tetrachoric correlation 0.38) (Vink et al., 2006). However, the mode of inheritance remains unclear with both autosomal dominant and multigenetic modes of inheritance being implicated (Vink et al., 2006). Typical reproductive and metabolic abnormalities in PCOS, including hyperandrogenemia and IR, are also

reported to cluster in families with PCOS. Mothers, sisters and brothers of women with PCOS are reported to have a defect in steriodogenesis leading to androgen excess compared to controls (Legro et al., 2002b, Yildiz et al., 2012). First-degree relatives of women with PCOS also have IGT or IR (Yildiz et al., 2012). Genome-wide association studies in Han Chinese women have reported 11 genetic loci linked to PCOS and these loci are found on regions that house genes responsible for gonadotropins, insulin signalling, reproductive hormones and T2DM (Chen et al., 2011, Shi et al., 2012). A replication study confirmed that some of these variations in loci were also evident in European women and may be important in the aetiology of PCOS independent of ethnicity (Goodarzi et al., 2012).

1.4.2 Prenatal Androgen Excess

Although beyond the scope of this thesis, the prenatal environment and in particular androgen excess intrauterine, is thought to play a pathophysiologic role in PCOS by contributing to reproductive and metabolic dysfunction in offspring (Abbott et al., 2005). Metabolic and reproductive dysfunctions including hyperandrogenism, PCO, elevated luteinising hormone (LH) concentrations, IR, hyperlipidemia, glucose intolerance, and increased risk of T2DM have been reported in a variety of pre-androgenised animal models (Abbott et al., 1998, Birch et al., 2003, Dumesic et al., 1997, Eisner et al., 2002, Manikkam et al., 2004, Recabarren et al., 2005). In rodent models the degree of reproductive or metabolic dysfunction was dose dependent upon testosterone exposure (Foecking et al., 2005, Wu et al., 2010). Women with PCOS are reported to have elevated androgen levels during gestation and have higher concentrations of enzymes that convert unconjugated steroids into androstenedione and subsequently testosterone in the placenta (Maliqueo et al., 2013, Sir-Petermann et al., 2002). Furthermore, testosterone levels are reported to be elevated to male levels in the umbilical vein of female foetuses born from mothers with PCOS (Barry et al., 2011). However, this

finding has not been replicated and is still a topic of debate (Anderson et al., 2010). Overall, the prenatal environment and excess androgen exposure intrauterine may play a role in the development of PCOS.

1.4.3 Hypothalamic-Pituitary Axis

The Hypothalamic-pituitary axis (HPA) is a complex feedback loop comprising of the hypothalamus (containing gonadotropin releasing hormone (GnRH) neurons), pituitary gland (responsible for the secretion of LH and follicle stimulating hormone [FSH]) and the ovary, which responds to changes in gonadotropin concentrations by follicular maturation and ovulation (Roland and Moenter, 2014). It is proposed that abnormalities in the HPA exist in women with PCOS. These abnormalities result in an increased GnRH pulse frequency and disruption in the release of LH and FSH leading to an increase in immature follicles on the ovary and menstrual dysfunction. Various studies have reported an increase in LH concentrations, LH/FSH ratio, and GnRH and LH pulse frequency and amplitude in women with PCOS (Taylor et al., 1997, Waldstreicher et al., 1988). Whether defects in gonadotropin release are inherent to PCOS or secondary to developing the condition is under debate. Insulin and hyperandrogenism are factors proposed to play a role in gonadotropin regulation. Hyperinsulinaemia causes an increase in LH receptor expression and premature release of the follicle, which combine to cause follicular arrest and subfertility or infertility (Diamanti-Kandarakis, 2008). Evidence for a regulatory role of androgens in altered gonadotropin secretion is supported by the finding that prenatally androgenised rhesus monkeys and sheep produce female offspring with LH hypersecretion, hypothesised to occur due to altered programming of the hypothalamus and an increased sensitivity of the pituitary gland to gonadotropin releasing hormones (Sarma et al., 2005). Elevated androgen levels can also

increase AMH through augmenting follicular growth and these high AMH concentrations may also negatively feedback on FSH production.

1.4.4 Obesity

Obesity is defined as excessive fat accumulation, which is commonly assessed using BMI. A BMI of 18.5 to <25 km/m^2 is considered to be healthy, whereas a BMI of 25 to 29 km/m^2 is classified as overweight and >30 km/m^2 as obese. Obesity may have a bidirectional relationship with PCOS; women with PCOS are predisposed to weight gain and excess weight gain appears to increase the prevalence of PCOS (Shorakae et al., 2014, Teede, 2013). Adipose tissue acts as an endocrine organ secreting proteins known as adipokines (including resistin, visfatin, and Plasminogen activator inhibitor-1 [PAI-1]), which play a role in energy metabolism through their interactions with the liver, skeletal muscle, brain, pancreas and reproductive system (Scherer, 2006). Obesity is strongly related to PCOS, with up to 61% of women with the condition being overweight or obese, although this varies with ethnicity and the cause and association are still being investigated (Lim et al., 2012). Obesity has been reported to precede oligoovulation and hyperandrogenism and has deleterious effects on metabolic features of PCOS, in particular insulin sensitivity (Gambineri et al., 2002).

Reproductive disturbances of oligoovulation and anovulatory infertility are more common in obese women compared to lean women irrespective of PCOS diagnosis (Teede, 2013). The risk of these reproductive disturbances worsens with increasing BMI (Teede, 2013). Obesity may also increase the risk of miscarriages and impair the outcome of assisted reproductive technologies (Pasquali et al., 2014). Furthermore, obese women with PCOS tend to have higher testosterone levels compared to lean PCOS women. Excess adipose tissue may contribute to the hyperandrogenism seen in PCOS by decreasing the levels of SHBG and

consequently increasing the bioavailability of sex steroids including testosterone (Holte, 1996).

Metabolically, IR is compromised by obesity in both the general population and in women with PCOS. The risk and degree of IR rises with increasing BMI; 27 kg/m^2 is the critical point where a marked decrease in insulin sensitivity is observed (Garcia-Estevez et al., 2004). Furthermore, obese women with PCOS display higher fasting and glucose-stimulated insulin concentrations and lower whole body insulin sensitivity compared to their non-PCOS counterparts (Dunaif et al., 1989). Although the degree of obesity is important in the development of IR, the distribution of this adipose tissue also seems to have an effect. Individuals with a high intra-abdominal fat distribution tend to be less insulin sensitive compared to individuals with a more subcutaneous fat distribution (Carey et al., 1996, Cnop et al., 2002, Fujimoto et al., 2000, Kahn, 2003).

It appears that obesity worsens the features of PCOS. Specifically, women diagnosed by the NIH criteria (menstrual dysfunction and hyperandrogenemia) were more obese compared to their sisters with the less severe phenotype of regular menstrual cycles and hyperandrogenemia. Healthy sisters unaffected by PCOS were reported to have a lower body mass compared to both PCOS groups (Kahn et al., 2006, Legro et al., 2002a). While obesity modifies the phenotype and/or severity of the features of PCOS, it is unclear whether it is a key player in the aetiology of the syndrome. To better understand the interaction between obesity and PCOS, this thesis will explore the impact of obesity on reproductive and metabolic disturbances in PCOS.

1.5 Health Complications of PCOS

PCOS is a lifelong condition associated with numerous clinical sequelae including reproductive, metabolic, cardiovascular, carcinogenic and psychosocial comorbidities (Teede et al., 2010). These comorbidities vary in severity across the lifespan with features of hyperandrogenism most prominent among adolescents, whereas fertility and reproductive issues are prominent among women in their 20's and 30's. Metabolic features, weight gain, and psychosocial challenges affect all ages, with metabolic features occurring earlier in individuals who are overweight (Teede et al., 2011).

1.5.1 Reproductive Consequences

Women with PCOS may be subfertile with ovulatory dysfunction exacerbated by hyperandrogenism and obesity associated with the syndrome (Group, 2012, Rotterdam, 2004). Subfertility refers to reduced fertility in couples unsuccessfully trying to conceive (Gnoth et al., 2005) and is most commonly a result of anovulation, which affects 55-91% of women with PCOS (Loumaye et al., 2003). In a large epidemiological study comprising 4,535 women from North Finland, women with self-reported PCOS symptoms suffered more frequently from subfertility (26% versus 17% in healthy controls) and took longer to conceive for the first time (Koivunen et al., 2008). Interestingly, the number of women conceiving and their family size (number of children) were similar between women with and without PCOS symptoms (Koivunen et al., 2008). It is feasible that women with PCOS took longer to conceive because of fewer ovulatory cycles (Liang et al., 2011). With time the overall fertility of women with symptoms of PCOS may not be greatly impaired. Furthermore, fertility is not necessarily impaired in all PCOS cases. Some women are able to conceive without medical intervention, depending on the severity of the condition (Shorakae et al., 2014).

IR and hyperandrogenism are two mechanisms proposed to contribute to PCOS consequences. Hyperinsulinaemia increases the availability of testosterone and other steroids by suppressing the production of SHBG. IR also augments LH and ovarian androgen production, leading to hyperandrogenism. This accelerates pre-antral and antral follicular growth in the ovary and elevated LH results in premature luteinisation causing follicular arrest and decreased oocyte quality (Piouka et al., 2009, Diamanti-Kandarakis and Dunaif, 2012). Obesity has an additional inhibitory effect on gonadotropin release due to an increase in aromatization of androgens in adipose tissue resulting in the suppression of LH and the consequent inhibition of the dominant follicle (Grossman et al., 2008). Treatment with insulin lowering medication induces regular menstrual cycles and improves pregnancy rates (Brettenthaler et al., 2004). The role of IR, androgens and gonadotropins in ovarian dysfunction will be further discussed in Chapter 7.

1.5.2 Pregnancy Risks

Along with subfertility, women with PCOS may also experience complications during pregnancy (Roos et al., 2011); increased risk of miscarriage during the first trimester (30-50% versus 10-15% in control women) (Jakubowicz et al., 2002) and higher risk of gestational diabetes mellitus (GDM; OR 2.9, 95% CI 1.7-5.1), pregnancy-induced hypertension (OR 3.7, 95% CI 1.9-6.8), pre-eclampsia (OR 3.47, 95% CI 1.9-6.2) and preterm birth (OR 1.75, 95% CI 1.2-2.6) (Boomsma et al., 2006). In utero, the embryo may be exposed to excess androgens and/or insulin that may have both short and long-term health effects on the child. Long-term epigenetic programming may be disrupted particularly in genes responsible for reproduction and metabolism (Hickey et al., 2006, Li and Huang, 2008). While short-term impacts may include the offspring of women with PCOS being at

increased risk of admission to a neonatal intensive care unit (OR 2.3, 95% CI 1.3-4.3) and have a higher perinatal mortality (OR 3.07, 95% CI 1.0-9.2) (Boomsma et al., 2006). This may be compounded by the impact of obesity, which is independently associated with spontaneous miscarriage, pre-eclampsia and GDM (Wax, 2009). The effect of PCOS and obesity alone or in combination can be detrimental to pregnancy outcomes.

1.5.3 Metabolic Complications

Women with PCOS are at an increased risk of developing impaired glucose tolerance (IGT; OR 2.5, 95% CI 1.6-3.8) and T2DM (OR 4.43, 95% CI 4.1-4.8) (Moran et al., 2010). Furthermore, women with PCOS are proposed to have a more rapid progression from IGT to T2DM prompting the International Diabetes Federation to identify PCOS as a significant non-modifiable risk factor for T2DM (Alberti et al., 2007, Norman et al., 2001). The increased risk of IGT and T2DM in PCOS may be attributed to a variety of factors including, adipose tissue distribution, IR, abnormal beta cell function, androgen excess, GDM and a family history of T2DM (Ciampelli et al., 1997, Dunaif et al., 1996, Dunaif et al., 1989, Legro et al., 1999, Holte et al., 1995, Vrbikova et al., 2004).In light of the increased risk of T2DM, evidence based guidelines for PCOS and the AES recommend annual to biannual 75g oral glucose tolerance tests for women with PCOS to enable early detection and treatment for IGT and T2DM (Azziz et al., 2009).

See section 1.7 for a more detailed discussion on IR.

1.5.4 Cardiovascular Complications

Cardiovascular disease (CVD) is one of the leading causes of death in Australian women (AIHW, 2010). Women with PCOS not only have increased early clinical markers of

atherosclerosis (endothelial dysfunction, impaired pulse wave velocity, increased carotid intima media wall thickness, carotid plaque and coronary artery calcification) but also increased prevalence of many cardiometabolic risk factors including hyperinsulinaemia, dyslipidaemia, hypertension, IR and diabetes, which are all made worse by the presence of obesity (Legro et al., 1999, Meyer et al., 2005a, Meyer et al., 2005b, Wild et al., 2011). Hyperandrogenism and low levels of SHBG have also been linked to increased CVD risk in both premenopausal and postmenopausal women (Sutton-Tyrrell et al., 2005). Although a large body of evidence reports an increased prevalence of the clustering of cardiovascular risk factors in PCOS, evidence that PCOS is associated with increased CVD is scarce and hampered by the lack of use of accepted diagnostic criteria, retrospective diagnosis of PCOS, and small sample size (Dokras, 2013).

1.5.5 Carcinogenic Complications

An association between PCOS and cancer was first reported almost 80 years ago. There are numerous potential risk factors that may mediate the development of cancer including chronic anovulation and hyperandrogenism with unopposed oestrogen action (Genazzani et al., 2001, Key and Pike, 1988), nulliparity, obesity and T2DM (Carmina and Lobo, 1999, ESHRE/ASRM, 2003, Chittenden et al., 2009). Women with PCOS are 3 times (OR 2.7, 95% CI 1.0-7.3) more likely to develop endometrial cancer and 2.5 times (OR 2.52, 95% CI 1.1-5.9) more likely to develop ovarian cancer compared to women without PCOS (Chittenden et al., 2009, Haoula et al., 2012, Schildkraut et al., 1996). Women with PCOS do not appear to be more likely to develop breast cancer (OR 0.9, 95% CI 0.4-1.8) and there are a lack of studies investigating a link between POCS and other cancers including cervical cancer. While an awareness of association between PCOS and various cancers is recommended, routine screening is not recommended in women with PCOS, unless risk factors or symptoms are

present .

1.5.6 Psychosocial Complications

Women with PCOS are also at increased risk of mental health disturbances including depression, anxiety, reduced quality of life, eating disorders, psychosexual dysfunction (Barnard et al., 2007, Barry et al., 2011, Deeks et al., 2011, Moran et al., 2012, Banting et al., 2014, Legro et al., 2013) and bipolar disorders (Klipstein and Goldberg, 2006, Rassi et al., 2010). It is difficult to identify the main cause of concern as PCOS involves several potentially distressing symptoms that vary in severity. Symptoms and co-morbidities associated with PCOS include coping with the condition itself, subfertility/infertility, loss of femininity and sexuality, body image dissatisfaction (weight, hirsutism and acne) and lower self-worth (Deeks et al., 2011, Kitzinger and Willmott, 2002). It has been suggested that younger women with PCOS are more likely to be affected by their appearance compared to older women with the syndrome (Farrell and Antoni, 2010). These negative feelings can interfere with emotional development and lead to social fears that limit interactions with friends, family and the community (Benson et al., 2009). Psychosocial consequences are highly relevant in clinical care as they can adversely affect lifestyle management, often considered to be first line treatment in PCOS.

1.6 Management Strategies in PCOS

Given that PCOS is a chronic and complex condition, a patient focused, self-management approach is encouraged, with emphasis on the short and long-term reproductive, metabolic and psychological features (Teede et al., 2011). Ongoing management is important and interdisciplinary care is often required for optimising lifestyle, including caloric restriction and exercise to prevent weight gain and encourage weight loss, which is considered the first

line of defense for PCOS.

A growing body of research has demonstrated that weight loss, achieved through lifestyle management, decreases abdominal fat, hyperandrogenism and IR, and improves lipid profiles, menstrual cyclicity, fertility, risk factors for T2DM and CVD and psychological health in women with PCOS who are overweight (Huber-Buchholz et al., 1999, van Hooff et al., 2000). There is currently insufficient data investigating the effects of lifestyle modification and weight loss in PCOS.

1.7 Insulin Resistance in PCOS

IR is a common metabolic disorder that underpins the pathophysiology of diabetes, metabolic syndrome, obesity and PCOS and their various health complications (Peppa et al., 2010). The mechanisms of IR are not fully elucidated and most commonly involve complex interactions between multiple intrinsic and extrinsic factors.

1.7.1 Definition of Insulin Resistance

Insulin acts to stimulate glucose uptake in peripheral tissues (primarily skeletal muscle) as well as to supress hepatic glucose production in order to maintain blood glucose homeostasis (Bergman, 2007, DeFronzo and Tripathy, 2009). A reduced ability of insulin to exert its physiological effects is termed IR. This can occur through impaired insulin-stimulated glucose uptake and glycogen synthesis at in skeletal muscle, adipose tissue and liver. Lower suppression of hepatic glucose output, increased adipose tissue lipolysis; and impaired mitogenic processes (alterations in growth, differentiation, DNA synthesis, regulation of gene transcription (Anonymous, 1998). As a consequence of IR, pancreatic β-cell insulin secretion is increased to provide sufficient concentrations of insulin to elicit action and achieve glucose homeostasis, resulting in compensatory hyperinsulinaemia commonly observed in people

with IR (Bergman et al., 1985, Kahn, 1985). The World Health Organisation (WHO) specifically defines IR as a glucose uptake (i.e. insulin sensitivity) below the lowest quartile for background population under hyperinsulinemic-euglycemic conditions (Grundy 2004).

1.7.2 Importance of Insulin Resistance in PCOS

IR is a common feature in women with PCOS and a compensatory increase in circulating insulin concentrations are required to maintain glucose homeostasis (Munir et al., 2004). It is hypothesized that this increase in insulin may contribute to hyperandrogenism, dysfunctional ovulatory cycles and altered follicular development in PCOS (Romualdi et al., 2011). Evidence in support of this hypothesis arises from studies investigating the role of insulin sensitizing medication. When women with PCOS are treated with metformin or troglitazone, an improvement in peripheral insulin sensitivity is reported as well as reductions in androgen concentrations and restoration of ovulatory cycles (Dunaif et al., 1996, Hasegawa et al., 1999, la Marca et al., 2000).

The mechanisms by which insulin mediates the production of androgens in the ovary are not completely understood. Alterations in LH receptor, insulin receptor (INSR), and cytochrome P450c17 (CYP-17) as a result of hyperinsulinaemia contribute to excess production of progesterone, 17α-hydroxyprogesterone and testosterone as compared to normal theca cells (Diamanti-Kandarakis et al., 2008, Diamanti-Kandarakis and Papavassiliou, 2006). The role of insulin in ovarian function becomes evident from the observations of severe ovarian hyperandrogenemia in women with syndromes of extreme IR (Poretsky et al., 1999). Furthermore, INSR are found in ovarian theca, granulosa and stomal cells identifying the ovary as a target for insulin activity (Dunaif et al., 2001). In theca cells, the binding of insulin to the INSR activates the phosphoinositide 3-Kinase (PI3K) signalling pathway and the

activity of 17α-hydroxylase, which is a key mediator of androgen production (Munir et al., 2004). Furthermore, decreased phosphorylation of MEK1/2 and MAPK-ERK1/2 in cultured theca cells was associated with increased P450c17 expression, which plays a key role in androgen synthesis (Nelson-Degrave et al., 2005). Therefore, insulin plays a role in the clinical features of PCOS and this concept will be explored in Chapter 6.

1.7.3 Prevalence of Insulin Resistance in PCOS

The prevalence of IR in PCOS is reported to be up to 70% (Moghetti et al., 2013). However, limited studies are available and prevalence rates are dependent on the population studied and methods used for assessment. Furthermore, many studies have not used weight, age or ethnicity-matched controls, factors that have been shown to affect IR and even fewer have assessed the prevalence of IR in PCOS using the gold standard euglycaemic-hyperinsulinaemic clamp. The different diagnostic criteria and phenotypic expressions also complicate research in this area.

IR prevalence was first identified in a small seminal study using weight-matched controls and the euglycaemic-hyperinsulinaemic clamp, where insulin stimulated glucose utilisation was below the lower range of weight-matched controls in 26% of obese and 60% of lean PCOS women (Dunaif et al., 1989). In another study of 40 obese PCOS women, 53% were insulin resistant when the frequently sampled intravenous glucose tolerance test (IVGTT) was used and IR was defined using the 10th percentile of the normal distribution in the age, weight and ethnicity matched controls as a cut-off value (Legro et al., 1998). In a larger study using controls that were not weight–matched, a higher prevalence of IR was reported in women with PCOS (Carmina and Lobo, 2004). IR was identified in 65% of PCOS women using glucose to insulin (G/I) ratios and 80% of women with homeostasis model assessment of IR

(HOMA) and qualitative insulin sensitivity check index (QUICKI) (Carmina and Lobo, 2004). Other studies using HOMA and QUICKI have reported lower prevalence rates of 18% (Fulghesu et al., 2006) and up to 51% (de Paula Martins et al., 2007) of IR in PCOS. In a study with unmatched controls but adjusting for age, ethnicity and BMI, IR was present in 64% of women with PCOS using HOMA with the upper 95th percentile of adjusted values for controls to establish the upper normal limit for HOMA-IR (DeUgarte et al., 2005). These published studies demonstrate high variability, making it difficult to gage the prevalence of IR in PCOS, due to different definitions, cut-off values, and measurement techniques of IR. Furthermore, confounding factors such as age and BMI are often overlooked. Given the important aetiological role of IR in PCOS, there exists a need for quality assessment of prevalence rates of IR using gold standard techniques and for evidence synthesis of studies using these methods in PCOS. This is addressed in a systematic review in Chapters 2 and in original research in Chapter 3.

1.7.4 Effect of Diagnostic Criteria on Prevalence of Insulin Resistance

Prevalence rates of IR in PCOS are also confounded by the transition of diagnostic criteria from the original NIH to Rotterdam. Women diagnosed with the classic phenotype of PCOS (hyperandrogenism and anovulation) tend to have higher prevalence rates of IR compared to those diagnosed with the ovulatory (Carmina et al., 2005a, Moghetti et al., 2013) and normoandrogenetic phenotypes (Broekmans et al., 2006, Goverde et al., 2009, Mehrabian et al., 2011). However, this is not always reported (Chae et al., 2008, Panidis et al., 2012, Shroff et al., 2007, Wang et al., 2010). In comparison to healthy women without PCOS acting as controls, the NIH phenotype was generally insulin resistant (Carmina et al., 2005a, Chae et al., 2008, Wang et al., 2010, Dewailly et al., 2006) but results were not clear in the ovulatory (Carmina et al., 2005a, Dewailly et al., 2006) and noromoandrogenic phenotypes (Chae et al.,

2008, Dewailly et al., 2006, Panidis et al., 2012). There are a number of limitations with the current studies making the interpretation of prevalence rates difficult. All, except one study (Moghetti et al., 2013), have used surrogate measures of IR, which may not be sensitive enough do detect IR in PCOS, with a need for more studies using the gold standard euglycaemic hyperinsulanaemic clamp (Buchanan et al., 2010). Furthermore, many studies did not take confounding factors, such as body composition, into account when assessing IR in different phenotypes. Therefore, to improve our knowledge in the area, Chapter 3 will investigate the prevalence of IR using gold standard clamps across in the NIH and Rotterdam diagnostic criteria for PCOS.

1.7.5 Insulin Resistance - Aetiology in PCOS

A decrease in insulin sensitivity has been reported in women with PCOS (Dunaif et al., 1989, Ciampelli et al., 1997, Diamanti-Kandarakis et al., 1998, Glintborg et al., 2006, Morin-Papunen et al., 2000). However, several studies have not supported this finding, especially in lean women with PCOS, when confounding factors are taken into account including BMI, ethnicity, fat distribution, family history and diagnostic criteria, making relationships less clear (Holte et al., 1994, Ovesen et al., 1993, Vrbikova et al., 2004). The prevalence of IR in PCOS is estimated to be up to 65% and occurs independently of obesity, but the effect of obesity on IR is additive to that of PCOS (Carmina and Lobo, 2004, Teede et al., 2011). The presence of IR is a precursor to the development of other metabolic complications including T2DM. Large population studies using weight, age and ethnicity-matched controls are required.

International PCOS research agendas highlight aetiology and therapies as key priority areas (Legro et al., 2006, Teede et al., 2011). IR underlies the reproductive and metabolic features

of PCOS (Azziz et al., 2006) but its aetiology remains unclear in PCOS as well as in other insulin resistant states. Current theories suggest intrinsic IR (genetic, inherent and unique to PCOS) and extrinsic factors (obesity/physical inactivity and adipokines/cytokines) work synergistically to promote an insulin resistant state (Corbould et al., 2005, Dunaif, 1997). Women with PCOS have hyperinsulinemia and decreased glucose-stimulated insulin secretion. Impaired muscle glucose uptake and whole body IR has been attributed to impaired insulin responsiveness of skeletal muscle and adipose tissue and specific abnormalities in insulin metabolism including basal hyperinsulinemia, reductions in glucose-stimulated insulin secretion (Dunaif, 1997, Dunaif et al., 1996), reduced hepatic glucose uptake, impaired suppression of hepatic gluconeogenesis (Dunaif et al., 1989) and abnormalities in insulin signalling in skeletal muscle (Dunaif, 1997, Corbould, 2008b, Corbould et al., 2005).

1.7.6 Possible Defects in Skeletal Muscle of Women with PCOS

Skeletal muscle accounts for up to 85% of whole body insulin-stimulated glucose uptake (DeFronzo and Tripathy, 2009). Insulin stimulates glucose uptake in skeletal muscle by increasing the translocation of glucose transporter 4 (GLUT4) from intracellular vesicles to the cell surface, mainly mediated through the activation of the PI3-K and AKT or protein kinase B (AKT/protein kinase B [PKB]) signalling pathways (Diamanti-Kandarakis and Papavassiliou, 2006). Women with PCOS have IR that is independent of obesity and this may be attributed to INSR and/or post-binding defect in the insulin signalling pathways (Dunaif, 1997, Dunaif et al., 1992, Dunaif et al., 2001). Muscle biopsies taken from women with PCOS during basal conditions have normal insulin receptor substrate 1 (IRS-1) and PI3K activity, however IRS-1-associated PI3-K activity was significantly reduced compared to age, weight and ethnicity matched control women during a euglycaemic hyperinsulinaemic clamp (Dunaif et al., 2001). Furthermore, there was no difference in the abundance of the INSR,

IRS-1, or the p85 regulatory subunit of PI3-K in women with PCOS, but there was an increased abundance of insulin receptor substrate 2 (IRS-2), suggesting a compensatory adjustment to help to maintain insulin mediated glucose uptake (Dunaif et al., 2001). Signalling abnormalities are also reported in the phosphorylation of AKT at Serine473 and Threonine308 sites and AKT's downstream target for GLUT4 translocation AS160, independently of obesity (Glintborg et al., 2008). Treatment with insulin sensitising medication (pioglitazone) improved insulin-stimulated glucose uptake, but not to normal control levels (Hojlund et al., 2008). IRS-1 phosphorylation on Serine312, a key site for inhibiting insulin-mediated IRS-1 tyrosine phosphorylation and activation, was also reported to be increased in PCOS (Corbould et al., 2005).

In contrast, others have failed to find changes in IRS-1-associated PI3-K activity and AKT/PKB activation in skeletal muscle biopsies in women with PCOS following insulin infusion, despite impaired rates on insulin mediated glucose disposal (Ciaraldi et al., 2009, Hojlund et al., 2008). The discrepancies between studies could be attributed to differences in the time course of muscle biopsies taken following insulin infusion (15 and 30 minutes post insulin infusion versus 3 hours) and the concentration of insulin achieved (physiological levels or supraphysiological levels) (Dunaif et al., 2001, Hojlund et al., 2008).

Insulin sensitivity in skeletal muscle may also be reduced due to dysfunction in mitogenic insulin signalling pathways. An attenuation in the insulin stimulated ERK mitogenic pathway and an increase in basal phosphorylation of ERK 1/2 were reported in a small group of women with PCOS (n=9) (Rajkhowa et al., 2009). The IRS/PKB pathway was similar in PCOS and controls. Together, this evidence highlights that impairments in the insulin signalling pathway occur in PCOS and may be responsible for the IR associated with PCOS.

However, further work is required to define the defects responsible for impairments in insulin-mediated glucose disposal present in PCOS.

Other possible mechanisms for the development of IR in skeletal muscle are adipokines and inflammatory markers. These markers have the ability to disrupt insulin signalling pathways either directly by inhibiting serine phosphorylation of the IRS or indirectly through inflammatory pathways including the c-Jun N-terminal kinase (JNK) and I-kappa B kinase β (IKKβ)/NFκB pathways (Tilg and Moschen, 2008). Some adipokines that may play a role in modulating IR include leptin, resistin, visfatin, and (PAI-1 (Makki et al., 2013). Little attention has been given to the interaction of these markers in the development of IR in PCOS; rather the majority of the research has focused on measuring these markers in isolation. Therefore, potential markers of IR in PCOS are further explored in Chapter 4.

1.7.7 Insulin Resistance and Mitochondrial Dysfunction in Skeletal Muscle

Another theory on the aetiology of IR became popular in the 1990's and implicates abnormalities in mitochondrial function in the development of IR (Morino et al., 2006). Mitochondria are surrounded by an outer and an inner bilipid membrane. The inner membrane consists of many folds that form cristae where the five oxidative phosphorylation enzymes are located. Nicotinamide adenine dinucleotide hydride (NADH) dehydrogenase (complex I) is the first enzyme in the electron transport chain (ETC) and catalyses the transfer of electrons from NADH molecules to coenzyme Q. Complex II (succinate dehydrogenase) transmits electrons from succinate to coenzyme Q and directly connects the citric acid cycle to the respiratory chain. From coenzyme Q, electrons can be transferred to complex III (cytochrome c reductase). Cytochrome c mediates electron transfer from cytochrome c reductase to cytochrome c oxidase (complex IV). Finally, electrons are

transferred to an oxygen molecule, which is reduced to water. ATP synthase, which is the prominent enzyme in complex V, is responsible for this proton gradient that drives ATP synthesis from ADP and phosphate (Dudkina et al., 2010).

The function of mitochondria is to produce energy, mainly ATP, by oxidative phosphorylation (OXPHOS) (Brand and Nicholls, 2011). Other functions include fatty acid oxidation, cell signalling, reactive oxygen species (ROS) production, mediating oxidative stress, regulation of apoptosis and cellular aging (Goodpaster, 2013, Holloszy, 1967, van Gurp et al., 2003). Metabolic homeostasis is tightly controlled by the mitochondria through the oxidisation of both carbohydrates and lipids and by transitioning between these substrates in response to insulin, substrate concentrations and the contractile status of the muscle (rest versus contraction) (Kelley et al., 1993). Abnormality in any of these processes can be termed mitochondrial dysfunction (Brand and Nicholls, 2011).

A role for mitochondria in IR emerged when researchers began to report defects in skeletal muscle mitochondria function in a range of different insulin-resistant populations; obese, T2DM and PCOS and in non-diabetic individuals with a family history of T2DM (Bullon et al., 2014, Kelley et al., 2002). Defects in mitochondria include a reduction in mitochondrial size, content (Kelley et al., 2002, Morino et al., 2005, Ritov et al., 2005) and oxidative enzyme activity (Heilbronn et al., 2007a, Ritov et al., 2005), decreased fat oxidation in skeletal muscle (Kelley et al., 1999, Kelley et al., 2002, Kim et al., 2000, Simoneau et al., 1999), down-regulation of genes involved in mitochondrial biogenesis and OXPHOS (Mootha et al., 2003, Patti et al., 2003), decreased messenger ribonucleic acid (mRNA) and/or protein expression of mitochondrial genes/proteins (Heilbronn et al., 2007a, Heilbronn et al., 2007b, Morino et al., 2005, Skov et al., 2007), reductions in mitochondrial DNA

(mtDNA) levels (Ritov et al., 2005) and decreases in mitochondrial oxidative capacity (Befroy et al., 2007, Mogensen et al., 2007, Petersen et al., 2004). Collectively, these studies support the role of mitochondrial dysfunction in the aetiology of IR. However, much debate exists in this area, as a large body of research also report no association between IR and mitochondrial function (De Feyter et al., 2008, Fisher-Wellman et al., 2014, Lefort et al., 2010, Rabol et al., 2011, Trenell et al., 2008) The mechanisms and associations of mitochondrial dysfunction and in IR in PCOS will be further explored in Chapter 5 of this thesis.

1.8 Summary and Research Gaps

PCOS is a very common condition with a significant reproductive, metabolic, psychological and economic burden. Given the role of IR in the pathophysiology and clinical consequences of PCOS, the syndrome appears to be an insulin resistant state and exploration of the aetiology of IR in PCOS is needed to better understand the condition and improve treatment options. Key research gaps remain in this area and include:

1. Evidence for the presence of intrinsic and extrinsic (obesity related) IR in PCOS using gold standard assessments of insulin sensitivity;
2. The need for accurate prevalence rates of IR in PCOS given the confounding factors such as age, BMI, diagnostic criteria, ethnicity and various methods used in measuring insulin sensitivity;
3. An understanding of factors that modulate IR in PCOS including sex steroids, inflammatory markers and adipokines;
4. Novel and optimal methods to measure IR in PCOS;
5. A better understanding of underlying mechanisms of IR in PCOS;
6. The role IR plays in clinical consequences of PCOS.

1.9 Aims of the Thesis

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

i) Systematically review and meta-analyse the literature to determine the impact of PCOS status on IR, age, BMI and PCOS diagnostic criteria and to investigate factors that potentially mediate IR in PCOS using a systematic approach (Chapter 2);

ii) Investigate prevalence of IR in PCOS in a cross-sectional study using gold standard euglycaemic hyperinsulinaemic clamp studies (Chapter 3);

iii) Explore other potential markers of IR in PCOS (Chapter 4);

iv) Explore the role of mitochondrial function in the underlying aetiology of intrinsic IR in PCOS (Chapter 5);

v) Explore role of IR in reproductive consequences, specifically ovarian dysfunction (Chapter 6).

1.10 Organisation of the Thesis

The following chapters explore IR in PCOS. An overall introduction in Chapter 1 is followed by Chapter 2, which is original research presented in the form of a systematic review and meta-analysis aimed at determining whether IR is intrinsic to PCOS and hormonal changes that may be associated with IR. Chapter 3 extends on the previous chapter by further exploring the intrinsic nature of IR in PCOS through a comprehensive cross-sectional study. In this chapter I also described the prevalence of IR in PCOS based on different diagnostic criteria. Chapter 4 details techniques to measure IR and proposes biomarkers, which may be useful in detecting IR in PCOS. Chapter 5 explores the role of mitochondrial dysfunction in IR and PCOS and finally Chapter 6 explores the relationship between of IR, ovarian function and reproductive consequences.

Chapter 2 Insulin Resistance in Women with Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis of Euglycaemic-Hyperinsulinaemic Clamp Studies

Declaration of Co-Authorship and Co-contribution: Papers incorporated in thesis by publication

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

Declaration by: **Signature:** **Date**

Samantha Cassar  27/11/2014

Paper Title:

Insulin resistance in women with polycystic ovary syndrome: A systematic review and meta-analysis of euglycaemic hyperinsulinaemic clamp studies.

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

Name	Contribution %	Nature of Contribution
Samantha Cassar	70	Designed the research, performed the literature search, independently reviewed the articles and assessed risk of bias, contributed to data analysis and interpretation, wrote the manuscript, constructed tables and figures, provided critical review of the manuscript and approved the final version for publication.
Marie L. Misso	5	Designed the research, performed the literature search, assisted with reviewing articles, undertook critical revision for important intellectual content and approved the final version for publication.
William G. Hopkins	5	Designed the meta-analysis models, performed statistical analysis and interpretation, wrote the manuscript, provided critical revision for important intellectual content and approved the final version for publication.

Christopher S. Shaw	2	Independently reviewed the articles, assisted in data analysis and interpretation, provided critical revision for important intellectual content and approved the final version for publication.
Nigel K. Stepto	9	Designed the research, assisted with data analysis and interpretation, provided critical revision for important intellectual content and approved the final version for publication.
Helena J. Teede	9	Designed the research, interpreted data, wrote the manuscript, provided critical revision for important intellectual content and approved the final version for publication.

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University

and will be held for at least five years from the date indicated below.

		Date
Samantha Cassar	[redacted]	27/11/2014
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2.0 Introduction

IR is central to the pathophysiology of PCOS (Teede et al., 2007), predisposes individuals to T2DM and is associated with metabolic and cardiovascular abnormalities including obesity, hypertension and coronary heart disease (Muniyappa et al., 2008). It is therefore important to accurately measure IR to identify individuals at increased risk of these conditions, determine aetiologies and appropriate therapeutic interventions. Currently, the assessment of IR can be performed by numerous direct and indirect methods, each having their own advantages and limitations, which will be discussed below. In general, invasive and detailed measures of insulin sensitivity using the euglycemic-hyperinsulinemic clamp are applied in smaller settings to determine IR (i.e. Low insulin sensitivity) where detailed mechanistic measures are required. Clinical or larger research settings rely on indirect methods as they are less time-consuming and more cost effective and feasible.

2.1 The Euglycemic-Hyperinsulinemic Clamp

The euglycemic-hyperinsulinemic clamp was developed in the 1970's and is considered to be the gold standard method in directly assessing insulin sensitivity in humans (DeFronzo et al., 1979). Following an overnight fast, insulin is infused intravenously at a constant rate of 40 mU/m^{-2} per minute for typically 120 minutes to create a steady state of hyperinsulinaemia. Simultaneously, glucose is intravenously infused at a variable rate to 'clamp' blood glucose concentration at euglycaemic levels, with blood glucose levels monitored every 5-10 minutes. It is assumed that during hyperinsulinaemic conditions hepatic glucose production is suppressed. Therefore the rate of glucose infusion during steady state conditions is equal to the rate of whole body glucose disposal, giving a direct estimate of insulin sensitivity/IR

(DeFronzo et al., 1979, Muniyappa et al., 2008). The glucose infusion rate or glucose disposal rate is often normalised to body weight or fat free mass to better estimate insulin sensitivity (Patarrao et al., 2014). As the primary glucose uptake tissue is muscle, the euglycemic-hyperinsulinemic clamp is effectively a measure of peripheral insulin sensitivity with lower glucose infusion rates denoting a reduction in glucose uptake and insulin sensitivity (Muniyappa et al., 2009, Patarrao et al., 2014). Therefore, lower insulin sensitivity is an indication of IR. In addition to directly assessing insulin sensitivity, the euglycemic-hyperinsulinemic clamp is regarded as a reference standard due to its ability to accurately (coefficient of variation 0.10) and reliably predict individual results (discriminant ratio of 6) (Mather et al., 2001). Although the insulin clamp is widely accepted as the reference method for insulin sensitivity, it is costly, labour intensive, invasive, time consuming, encumbrance for participants and requires an experienced operator. Another limitation with the insulin clamp is the assumption that the hyperinsulinemic conditions achieved during the clamp is sufficient to entirely supress hepatic glucose production. However, this is not always the case and stable isotopes of glucose can be used during the clamp to estimate hepatic glucose production so appropriate corrections can be made to glucose disposal rate. The insulin clamp also only measures insulin-stimulated glucose disposal rate at levels in the supraphysiological range for insulin, not at basal conditions (unless tracers are used), which is of clinical importance (Patarrao et al., 2014). This technique is therefore mainly used in metabolic and intervention research studies where IR is a primary outcome. It tends not to be feasible in large-scale epidemiological and clinical studies and in clinical practice (Mather et al., 2001).

2.2 Fasting Insulin

A practical approach to detect IR is considered to be measuring fasting circulating insulin levels, as it is inexpensive and simple (Muniyappa et al., 2009, Patarrao et al., 2014). However defining IR based on insulin concentrations alone is limited as a lack of standardisation, ill-defined cut-off values for diagnosis of IR and a marked inter-laboratory variance in the measurement (Wallace et al., 2004). Fasting insulin concentrations also vary widely among populations resulting in a considerable overlap between healthy individuals and those with insulin resistant states such as T2DM (Wallace et al., 2004, Muniyappa et al., 2008).

Fasting insulin can be combined with fasting glucose, termed fasting glucose to insulin ratio (G/I ratio), as an index of IR (Muniyappa et al., 2008). In healthy individuals there is tight regulation between glucose production and insulin secretion to maintain fasting glucose in homeostasis. An increase in fasting insulin levels while glucose remains within normal limits will result in a decrease in the ratio signifying IR. However, the ratio is unable to detect the physiological changes that underpin IR, which becomes problematic in individuals with impaired glucose tolerance and diabetes. In these insulin resistant states, insulin levels will typically be the same or slightly elevated and glucose levels are elevated indicating a high ratio value and therefore insulin sensitive. However, the opposite is true and the individual is actually insulin resistant. Given the disadvantages associated with fasting insulin and with the G/I ratio other indices of IR should be used.

2.3 Surrogate Indices for Insulin Resistance

The HOMA and QUICKI are commonly used surrogate markers of IR (Muniyappa et al., 2009, Patarrao et al., 2014). Both of these indices require the use of a mathematical equation that incorporates fasting insulin and glucose, enabling them to account for hyperglycaemia. The original HOMA equation is fasting glucose (mmol/L) x fasting insulin (mU/L)/ 22.5 (Matthews et al., 1985). The denominator of 22.5 acts as a normalising factor and is the product of normal fasting plasma insulin (5 U/ml) and normal fasting plasma glucose (4.5 mmol/l) of a healthy individual. Therefore, for an individual with 'normal' insulin sensitivity, HOMA = 1 (Muniyappa et al., 2009, Patarrao et al., 2014). HOMA-IR tends to correlate better with euglycaemic-hyperinsulinaemic clamp measures in insulin sensitivity once the large variation and skewed distribution is accounted for by logarithmic transformations. The equation for QUICKI takes this into account and is calculated as follows; QUICKI = 1/[log (fasting insulin, U/ml) + log (fasting glucose, mg/dl)]. In general populations, HOMA, log HOMA and in particular QUICKI correlate well with the gold standard insulin clamp (Katz et al., 2000). When measures of repeatability are performed, QUICKI and log HOMA report within-subject coefficients of variation of 5% and 55% respectively in a mixed lean and overweight population (Mather et al., 2001). Both surrogate indices depend on fasting insulin levels, which alone demonstrates poor test characteristics including repeatability with a coefficient of variation of 53% (Mather et al., 2001). Furthermore, insulin levels show biological variability due to short serum half-life and correlations between 1/(fasting insulin) and the insulin clamp are poor (Matthews et al., 1983, Muniyappa et al., 2008). Another major disadvantage of both the HOMA and QUICKI methods are their ability to only

provide information at the fasting state, ignoring peripheral glucose uptake, which is important when assessing IR (Patarrao et al., 2014).

2.4 Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (OGTT) can also be used to measure IR and is commonly used in clinical practice to diagnose IGT and T2DM by measuring the body's efficiency to utilise glucose. The test is administered after an overnight fast of 8 to 10 hours and blood glucose levels are sampled at 0, 30, 60, 90 and 120 minutes following the consumption of a standard glucose load (75 g). The advantage of this test is its ability to mimic normal physiological glucose and insulin flux more closely than the euglycaemic-hyperinsulinaemic clamp. However, glucose tolerance and insulin resistance/sensitivity are conceptually different. During an oral glucose load, suppression of hepatic glucose production is less effective than during the euglycaemic-hyperinsulinaemic clamp. As a result, OGTT derived IR reflects both the suppression of hepatic glucose production and peripheral glucose disposal (DeFronzo, 1992). Overall the OGTT has the advantages of enabling the fasting samples to be used for simpler basal calculations of IR as well as providing additional information about stimulated first phase insulin secretion. As with the other methods that rely on endogenous insulin production, the OGTT is only useful in subjects without Type 1 diabetes mellitus and is less useful in established T2DM especially those who are receiving insulin. The OGTT is more laborious and expensive than the basal fasting measurements because it involves at least 3 blood samples and can take 2 hours to complete, but appears to be a reasonable compromise given logistic concerns with the euglycaemic-hyperinsulinaemic clamp (Bergman et al., 1987).

2.5 Assessment of Insulin Resistance

There is currently no consensus on how best to measure IR in women with PCOS in a research setting. The euglycaemic-hyperinsulinaemic clamp, has been applied in limited PCOS studies to date to assess insulin sensitivity because of logistic challenges outlined above, however it remains the gold standard for the assessment of insulin sensitivity and in-turn IR. Therefore, the euglycaemic-hyperinsulinaemic clamp was selected to assess IR in this thesis as IR was a primary outcome of all studies and there was a strong focus on understanding mechanisms that underpin PCOS.

2.6 Mediators of Insulin Resistance

Given that PCOS is a complex endocrine disorder with strong associations between many of its clinical features and IR (Muniyappa et al., 2008), paucity of experimental data investigating potential mediators of IR as measured by the euglycaemic-hyperinsulinaemic clamp exists (Corbould, 2008a, Diamanti-Kandarakis and Dunaif, 2012). There has been increasing interest in the contribution of androgens (testosterone), SHBG and gonadotropins to IR in PCOS. Elevated levels of testosterone and low levels of SHBG have been associated with T2DM, IR and metabolic syndrome in women (Moran et al., 2010). IR is thought to play a role in the reproductive and endocrine features of PCOS by contributing to hyperandrogenism and causing a disruption in gonadotrophin secretion (Norman et al., 2007). Therefore alterations in sex steroid and gonadotropin physiology could interact with IR, which warrants further investigation.

2.6.1 Testosterone

In women, testosterone is primarily produced by the ovaries and to a lesser extent by adipose tissue through the conversion of dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) secreted by adrenal glands and androstenedione from the ovaries. In healthy women, only 1-2% of testosterone is biologically active, the remaining 66% of testosterone is bound to SHBG and 33% is bound to albumin (Rannevik et al., 1995). Therefore any alterations in SHBG concentrations will have a direct effect on the bioavailability of testosterone. Hyperinsulinaemia and upregulation of androgen production in the ovary and adrenal glands are responsible for increased androgen concentrations seen in PCOS (Moran and Azziz, 2001, Nelson et al., 1999, Nestler et al., 1998).

A link exists between high circulating concentrations of testosterone and the development of IR and T2DM in women (Legro et al., 1999, Rajkhowa et al., 1994), with the relationship documented to begin in early adolescence (McCartney et al., 2007). But this relationship is not always reported in PCOS (Banu et al., 2013). The extent to which hyperinsulinaemia causes hyperandrogenism or hyperandrogenism promotes IR is still currently unknown. Numerous studies have reported that testosterone or androgen-like progestin administration are capable of inducing IR in women (Diamond et al., 1998, Godsland et al., 1992). Furthermore, administration of testosterone to female transsexuals caused a reduction in glucose uptake (Polderman et al., 1994) and anabolic steroid abuse has also been observed to result in reduced insulin sensitivity in male power lifters (Cohen and Hickman, 1987). There is some evidence to support that decreasing androgen concentrations improves insulin sensitivity in obese women with PCOS (Diamanti-Kandarakis et al., 1995, Dunaif et

al., 1990). However, small improvements in insulin sensitivity have been reported in less insulin-resistant lean women with PCOS during androgen suppression or antiandrogen therapy (Elkind-Hirsch et al., 1993, Moghetti et al., 1996). Accordingly, it has been proposed that hyperinsulinemia causes hyperandrogenism rather than the reverse. Insulin may directly affect steroidogenesis by stimulating ovarian estrogen, androgen, and progesterone secretion (Nestler and Strauss, 1991). Circulating androgen levels tend to decrease when insulin concentrations are lowered either through a decrease in insulin secretion (diazoxide and somatostatin) or an improvement in insulin sensitivity (metformin and troglitazone) (Dunaif et al., 1996, Nestler et al., 1989, Prelevic et al., 1990, Velazquez et al., 1994).

2.6.2 Sex-Hormone Binding Globulin and Insulin Resistance

SHBG is mainly synthesised in the liver and regulates the bioavailability of androgens, including testosterone, through binding and transport of these sex steroids to target tissues (Wallace et al., 2013). Hepatic SHBG production is affected by numerous hormonal and metabolic factors including insulin, carbohydrate consumption and fasting glucose levels. Both cross-sectional and prospective studies support a relationship between lower SHBG levels and an increased risk of IR, metabolic syndrome and T2DM in women (Wallace et al., 2013, Le et al., 2012). An inverse relationship between SHBG and glycated haemoglobin (HbA1C) is also evident in non-diabetic postmenopausal women, suggesting a link between SHBG and glucose homeostasis before the development of IR and diabetes (Brand et al., 2011, Page-Wilson et al., 2009).

Although many studies report a relationship between IR and SHBG, little attention has specifically focused on the relationship between SHBG and IR in premenopausal women with PCOS utilising the euglycaemic hyperinsulinaemic clamp technique, generally accepted as the gold standard method. To date, only two studies have used the clamp method to assess this relationship in PCOS, both reporting an inverse relationship between IR and SHBG (Cibula et al., 2002b, Ducluzeau et al., 2003). The direction of the relationship between SHBG and IR is still unknown. Investigators report that hyperinsulinemia directly reduces serum SHBG levels in obese women with PCOS independently of any effect on serum sex steroids (Nestler et al., 1991). It is hypothesised that the SHBG and T2DM relationship is independent of SHBG's role in sex hormone bioavailability in postmenopausal women (Kalyani et al., 2009). In support, in women with PCOS exhibiting normal insulin levels and metabolic insulin sensitivity, reducing insulin secretion through the administration of diazoxide for 1 month increased SHBG levels, indicating that insulin may act directly on the liver to influence SHBG production (Baillargeon and Carpentier, 2007).

2.6.3 Gonadotropins

Serum LH concentrations and LH to FSH ratio are often elevated in women with PCOS (Rebar et al., 1976). In contrast, FSH levels are usually normal or slightly supressed in PCOS and are unable to reach the desired threshold to stimulate follicular maturation during the early follicular phase of the menstrual cycle (Hillier, 1994). BMI complicates the assessment of gonadotropins in PCOS as LH levels tend to be lower is obese women (Arroyo et al., 1997). Studies investigating the effect of insulin on gonadrotropin secretion are conflicting with some studies reporting no changes in gonadotropin levels during insulin infusion (Dunaif and Graf, 1989, Tosi

et al., 2012) while others reporting decreased GnRH sensitivity in pituitary glands (Lawson et al., 2008), thereby impacting on gonadotropin release. Elevated levels of LH cause theca cells in PCO to secrete more androgens (Gilling-Smith et al., 1994). Granulosa cells obtained from small antral follicles demonstrate an increased production of sex steroids in response to FSH (Mason et al., 1994). Therefore augmented gonadotropin concentrations may have an indirect role in IR through androgen stimulation.

2.7 Assessment of Literature

Given the importance of IR in PCOS and debate on the relative intrinsic and extrinsic IR in PCOS, a high level review of the current literature was warranted to comprehensively answer the question of whether IR is intrinsic to PCOS. Systematic reviews and meta-analyses are key research tools used to synthesise data and inform clinicians and policymakers to make evidence-based decisions regarding patient care and policy (Abuabara et al., 2012). The strengths of a systematic review and meta-analysis are their ability to provide a precise estimate of the effects of an intervention using all relevant literature as opposed to making inferences using individual studies alone and they allow investigation of consistencies and differences across studies (Higgins and Green, 2011).

Guidelines have been established to aid researchers develop, carry out, evaluate and report studies in systematic reviews and meta-analyses. The first set of guidelines referred to as the Quality of Reporting of Meta-analysis (QUOROM) originated in 1999, which further evolved into the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines in 2009. The PRISMA guidelines

consist of a 27-item checklist to aid researchers in planning and reporting studies and may also be used to evaluate a systematic review or meta-analysis (Liberati et al., 2009). In 2000, a Meta-analysis of Observational Studies in Epidemiology (MOOSE) checklist was established for specifically reporting meta-analyses containing observational studies (Stroup et al., 2000).

2.7 Summary

In summary, this chapter aims to investigate the effect of PCOS on IR, measured by the gold standard technique of the euglycaemic-hyperinsualinaemic clamp, using a high quality systematic review and meta-analysis techniques. Here I conducted a comprehensive evaluation of the literature focusing on intrinsic and extrinsic IR in PCOS, the impact of PCOS status, BMI and diagnostic PCOS criteria. A secondary aim of this chapter was to investigate the clinical relationships between IR and androgens, SHBG and gonadotropins in PCOS to improve our understanding of the mediating or moderating effects of these outcomes on IR.

2.8 My Role

Chapter 2 contains a manuscript of a systematic review and meta-analysis currently under review in Human Reproduction Update (Impact Factors: 8.6, Q1 Journal). As the first author of this manuscript, I contributed to the conception and design of the review, which aimed to investigate the effects of PCOS on IR in PCOS independent of age, BMI and diagnostic criteria as well as mediating or moderating effects of androgens, SHBG and gonadotropins on IR in PCOS. I independently liaised with members of the Australasian Cochrane Centre to develop a sound research question, optimise a search strategy and perform the literature search. I then independently

reviewed the articles resulting from the search to determine eligibility and assessed methodological quality and risk of bias. Following consultation I independently performed data extraction and contributed to data analysis and interpretation. I primarily constructed the figures and tables and was responsible for submission for publication. This manuscript addressed key gaps relating to the intrinsic nature of IR in PCOS and the effects of hormones on IR.

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Insulin resistance in polycystic ovary syndrome: A systematic review and meta-analysis of euglycaemic-hyperinsulinaemic clamp studies

Running Title: Insulin resistance and relationships of hormones in PCOS

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Abstract

Background: Polycystic ovary syndrome (PCOS) is prevalent, complex, and underpinned by insulin resistance (IR). However, controversies still exist as most studies do not apply gold standard methods to measure IR. Specifically, the degree of intrinsic IR in PCOS and the effect of BMI, and diagnostic criteria. IR is postulated to play a role in reproductive, metabolic and endocrine features of PCOS. Therefore key moderating relationships between IR, sex hormone binding globulin (SHBG) and reproductive hormones (testosterone, luteinizing hormone [LH] and follicle stimulating hormone [FSH]) in women with and without PCOS, independent of BMI are important to explore.

Methods: Medline and All EBM databases were searched for studies published up to September 2013. Studies were included if premenopausal women diagnosed with PCOS were compared to a control group for IR measured by a euglycaemic-hyperinsulinaemic clamp. Due to heterogeneity of clamp data, the difference in insulin sensitivity between the control and PCOS group was determined. A negative difference in insulin sensitivity between groups indicated lower insulin sensitivity in the PCOS group. The systematic review adheres to the principles of The Cochrane Collaboration and the guidelines for Meta-analysis and Systematic Reviews of Observational Studies (MOOSE). Meta-analyses were performed using mixed modelling and magnitude-based inferences.

Results: Overall insulin sensitivity (IS) was lower in women with PCOS compared to controls (mean effect -28%, 90% confidence limits ±3%; large, most likely lower). BMI exacerbated the reduction in IS by -15% (±6%; moderate, most likely lower) in

PCOS and increases in BMI had a greater impact in PCOS (-28, ±11%; large, most likely lower) compared to controls (-13, ±13%; moderate, most likely lower). The effect of age was negligible, after adjustment for BMI and diagnostic criteria. Women diagnosed by the original National Institute of Health Criteria (NIH) were 6% (±7.8%; small, possibly lower) less insulin sensitive compared to those diagnosed by the Rotterdam criteria. Low levels of SHBG we associated with reduced levels of IS (-12 ±7%, moderate, very likely negative), which was not confounded by BMI. Total testosterone had a positive association with IS (19 ±12%, moderate, very likely positive) when the confounding effects of BMI were taken into account. The relationship of LH with insulin sensitivity remained small and unclear even when BMI was included in the model (-9.5 ±14%). FSH had a trivial relationship with IR.

Conclusions: IR is intrinsic to PCOS and independently exacerbated by BMI. Importantly, we demonstrate that there are negligible differences in IR between the NIH and the more inclusive, Rotterdam diagnostic criteria. Furthermore, SHBG has a strong negative relationship with IR on euglycaemic-hyperinsulinaemic clamp studies and appears to be a useful marker of IR in PCOS. The relationship between testosterone and IR is complicated and future research is needed to explore this further.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disorder affecting up to 21% of premenopausal reproductive aged women, depending on the population studied and diagnostic criteria used (March et al., 2010, Boyle et al., 2012). The condition has substantial short and long term reproductive (infertility, miscarriage, pre-eclampsia, gestational diabetes), psychological (depression, anxiety, low self-esteem and reduced quality of life), and metabolic implications (obesity, insulin resistance [IR], metabolic syndrome, type 2 diabetes, cardiovascular risk factors) across the lifespan (Moran et al., 2011b, Teede et al., 2011, Boomsma et al., 2006, Moran et al., 2012). Due to the associated co-morbidities, PCOS represents a significant health and economic burden. The estimated annual cost for the condition in the US in 2004 was $(US) 4 billion, with 40% of the cost attributed to treating PCOS associated diabetes (Azziz et al., 2005). In this context, an early and accurate diagnosis of PCOS and recognition of IR and its metabolic complications are important in women with PCOS to optimise screening, prevention and management.

The pathophysiology of the syndrome is complex and remains elusive; however IR (i.e. reduced insulin sensitivity [IS]) and hyperandrogenism are thought to play key roles in the aetiology of PCOS (Teede et al., 2007). In a recent narrative review by Diamanti-Kandarakis and Dunaif (2012), the authors note it is generally accepted that obese women with PCOS are insulin resistant (Diamanti-Kandarakis and Dunaif, 2012). Obesity is known to increase androgen and insulin levels, may increase PCOS prevalence and exacerbate the clinical features of PCOS (Ciampelli and Lanzone, 1998, Teede et al., 2013). Diamanti-Kandarakis and Dunaif (2012) also note that consensus is yet to be achieved in lean women with PCOS with several studies failing

to demonstrate intrinsic IR in this group (Diamanti-Kandarakis and Dunaif, 2012). It has been hypothesised that IR in PCOS is unique or intrinsic to the condition, and is then further exacerbated by obesity related IR (Dunaif and Graf, 1989, Stepto et al., 2013). However, research is needed to finally establish if IR in PCOS independent of obesity.

Conflicting results are further confounded by the diagnostic criteria for PCOS, with the majority of studies using the original National Institute of Health (NIH) criteria, rather than the more recent and inclusive Rotterdam criteria, now endorsed by the NIH (Diamanti-Kandarakis and Dunaif, 2012). IR is reported to be more pronounced in the severe PCOS phenotype (original NIH criteria) of anovulation and hyperandrogenism, compared to women diagnosed with PCOS who have normal androgen levels or regular menstrual cycles included under Rotterdam. Further research is needed in this area (Barber et al., 2007, Panidis et al., 2012).

IR plays a role in the reproductive, metabolic and endocrine features of PCOS by contributing to hyperandrogenism and causing a disruption in gonadotrophin secretion (Norman et al., 2007). IR and associated hyperinsulinemia may have a direct role in stimulating the production of androgens by binding to insulin receptors located within the ovary and altering steroidogenesis, independently of any changes in gonadotropin concentration (Barbieri et al., 1986, Dunaif, 1997, Poretsky et al., 1984). Hyperinsulinaemia also plays an indirect role in hyperandrogenism by inhibiting SHBG production reducing testosterone binding and increasing free testosterone (Kiddy et al., 1989, Nestler et al., 1991). Furthermore, the majority of studies investigating sex steroids use immunoassays, which have been found to be

methodologically inadequate for high-quality clinical research (Handelsman and Wartofsky, 2013). Mass spectrometry is considered to be the gold standard in sex steroid measures (Handelsman and Wartofsky, 2013).

Studies investigating the effect of insulin on gonadrotropin secretion are more conflicting. Some studies report no immediate changes in gonadotropin levels during insulin infusion (Dunaif and Graf, 1989, Tosi et al., 2012), while others report that insulin decreases the pituitary glands sensitivity to gonadotropin releasing hormone (GnRH) (Lawson et al., 2008), thereby impacting on gonadotropin release. There is also conflicting information on the effect of insulin sensitisers with some reporting a reduction in luteinising hormone (LH) levels (Nestler and Jakubowicz, 1996), whilst others report an increase (Eagleson et al., 2003) or no change (Azziz et al., 2001) in gonadotropins. Hyperinsulinemia is known to decreases hepatic SHBG production and treatment with insulin-sensitising medication, including metformin and troglitazone, increase circulating SHBG (Ehrmann et al., 1997, Moghetti et al., 2000, Moran et al., 2013b). Low levels of SHBG are associated with metabolic dysfunction (Moran et al., 2013b), cardiovascular risk factors and type 2 diabetes in the general population and those with hyperinsulinemic states like PCOS (Chen et al., 2006, Haffner et al., 1989, Jayagopal et al., 2004, Lindstedt et al., 1991). However the strength of the relationship between IR and SHBG is unclear and its potential role as a marker of IR is yet to be determined.

IR can be measured using numerous direct and indirect techniques, including fasting insulin and glucose, homeostatic model assessment (HOMA), The quantitative *insulin* sensitivity check index (QUICKI) and oral glucose tolerance test (OGTT) related

measures comprising area under the curve for glucose and insulin (Muniyappa et al., 2008). These measures are all based on fasting or post glucose-load blood glucose and insulin levels, which are easily obtained and calculated but lack accuracy and are inadequate for mechanistic research where IR is a primary study outcome (Muniyappa et al., 2008). The euglycaemic-hyperinsulinaemic clamp is considered the reference standard to directly measure insulin sensitivity in research settings and can be used to define a specific cut-off level for IR (Muniyappa et al., 2008, Grundy et al., 2004). Only a limited number of studies on IR in PCOS have used euglycaemic-hyperinsulinaemic clamps.

PCOS is a common and complex condition underpinned by IR, yet the aetiology of PCOS and the underlying IR are not well understood. The key gaps in this area include the controversy surrounding whether IR is intrinsic to PCOS independent of BMI and the presence of IR across PCOS phenotype and diagnostic criteria. Furthermore, the relationship between IR and reproductive hormones remains unclear in PCOS. Methods used to test IR are also often inadequate. Therefore, we aimed to undertake a systematic review and meta-analysis to address the overarching question; is IR intrinsic to PCOS? Our secondary aim was to investigate key relationships between IR, SHBG and other reproductive hormones (testosterone, LH and follicle stimulating hormone [FSH]) in women with and without PCOS. We did this by analysing and synthesising data from studies in PCOS including insulin sensitivity measured with the gold standard euglycaemic-hyperinsulinaemic clamp and by comparing lean and overweight women with and without PCOS.

Methods

We conducted a systematic review according to the principles of The Cochrane Collaboration and a meta-analysis using mixed modelling and magnitude based inferences. The methodology used adheres to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines (Supplementary Table 1).

Data Sources and Searches

A systematic search of published literature was conducted in Medline and All EBM databases (including Cochrane Database of Systematic Reviews, ACP Journal Club Database of Abstracts of Reviews of Effects, Cochrane Central Register of Controlled Trials, Cochrane Methodology Register, Health Technology Assessment, NHS Economic Evaluation Database) using subject headings and key terms (Supplementary Table 2). The search strategy was limited to English language articles. Bibliographies of relevant studies identified by the search strategy and relevant reviews/meta-analyses were also searched for identification of additional studies. The search was conducted until September 2013.

Study Selection

To determine the literature to be assessed further, two reviewers who were not blinded to the names of investigators or sources of publication, scanned the titles, abstracts and keywords of every record retrieved to exclude any clearly irrelevant studies. Full texts of the remaining studies were then retrieved for further assessment if the information given suggested that the study met all the following selection criteria determined *a priori*: a) PCOS was diagnosed by the National Institute of Health (NIH) or the Rotterdam Criteria or equivalent (Group, 2004, Fauser et al.,

2012); b) comparisons were made between at least one PCOS and control group; c) participants were pre-menopausal; d); participants were 18-40 years of age; e) insulin sensitivity measured at baseline without any interventions, by a euglycaemic-hyperinsulinaemic clamp, which reflects IR. Studies were excluded if participants a) were taking medications or undergoing interventions that effect insulin sensitivity before baseline measures were assessed; b) were diagnosed with a confounding medical condition e.g. diabetes; c) smoked. Full text articles were independently assessed for inclusion by two reviewers (S.C, C.S) in consultation with a colleague (N.S). Disagreements were discussed and resolved by discussion and consensus with a third reviewer (N.S).

Data Extraction and Quality Assessment

Data were extracted from included studies using a specially developed data extraction form (Harris, 2010). Information was collected (if available) on general details (title, authors, reference/source, country, year of publication, setting, sample size), participants (age, inclusion/exclusion criteria, subgroups), results (measures of variability, number of participants) and validity of results. Authors were contacted by electronic mail (email) if essential data was not reported in a usable format (e.g., only plots were published) and if the study was published <10 years ago. This is because authors of more recent studies are more likely to be able to locate and provide data compared to authors of older studies (Selph et al., 2014). Each email request to authors was sent using an institutional email account and provided a brief description of the systematic review scope, complete with article citation, specific information needed and our contact information. If authors failed to respond within 2 weeks of the initial date of request then they were contacted again via email. If authors did not

respond to the second email, the study was not included in the meta-analysis. Data for IS and other subject characteristics were extracted and summarised as mean and standard deviation (SD); any standard errors of the mean (SE) were converted to SD using the formula $SD = SE\sqrt{n}$. To overcome the problem of different units of measurement for IS in different studies, the statistical effect of PCOS on IS was expressed as a factor by dividing the IS in the PCOS group by that in the respective control group (e.g., IS as a factor for lean PCOS with lean control and overweight PCOS and overweight control). We then log-transformed all measures for the meta-analysis and used back-transformation to estimate meta-analysed mean effects as percentages with 90% confidence limits (Snowling and Hopkins, 2006). Methodological quality and risk of bias of the included studies was assessed by a reviewer using criteria developed *a priori* (Harris, 2010). Individual quality items were investigated using a descriptive component approach that included items such as conflict of interest of authors, clearly focused research question, specified inclusion/exclusion criteria, groups selected from similar populations, outcomes measured in a standard, reliable and valid way and statistical issues such as sample size, power and methods of data analysis (Supplementary Table 3). Any disagreement or uncertainty was resolved by discussion to reach a consensus. Using this approach, each study was allocated a risk of bias rating.

Data Synthesis and Analysis

The log-transformed factor effects on IS were meta-analysed using the general linear mixed-model procedure (Proc Mixed) (Yang, 2003) in the Statistical Analysis System (Version 9.4, SAS Institute, Cary, NC). A series of meta-analyses was performed.

In the first meta-analysis, the overall relationship between PCOS and IS was analysed. A random effect representing the identity of each study-estimate was included to allow for true differences in the effect of PCOS between studies not accounted for by the other effects in the model. The weighting factor for each study-estimate was the inverse of the square of the standard error, derived as follows: the standard errors of the mean in the PCOS and control groups were expressed as coefficients of variation (CV), converted to factors (1+CV/100), log-transformed, squared, and added. The method of setting the residual variance to unity was used to apply the weighting (Yang, 2003).

In the second meta-analysis, the possible moderating effects of BMI, age and diagnostic criteria on the relationship between PCOS and IS were investigated separately. Fixed effects in the model were either linear numeric variables for BMI and age in the PCOS and control groups or a nominal variable for the diagnostic criteria used in the given study (NIH or Rotterdam). The model with BMI as a predictor was used to compare predicted differences in IS in the PCOS and control groups at the mean BMI of 22 kg/m^2 and 32 kg/m^2 respectively for lean and obese women (defined by a threshold BMI of 25 kg/m^2 -see below). The model for age as a predictor was used to compare differences in IS in the PCOS and control groups for younger and older women defined by the weighted mean of the means (27 y) and the weighted mean of the SDs (5 y) of the age of the women from each study. Younger women were aged 22 y (mean - SD) and 32 y (mean + SD).

The third meta-analysis was similar to the second meta-analysis, except that the possible mutual confounding effects of BMI, age and diagnostic criteria on the

difference in IS between PCOS and control groups were investigated by including all three moderators in the model. The effect of each moderator was therefore assessed while the other moderators were held constant.

The association between differences in key factors (testosterone, SHBG, LH and FSH) and differences in IS between PCOS and control groups were investigated in a fourth separate meta-analyses. In each analysis the relationship between IR and PCOS was assessed using a random effect model as per the first meta-analysis. A fixed-effect variable representing the difference in the log of the key factor between PCOS and control groups was then added to the model to determine the moderating effects of these key factors on IS. The difference in IS associated with each hormone was derived by multiplying the effect of the factor (represented by its coefficient in the model) by the difference in the mean concentration of the factor between PCOS and control women. The possible confounding effect of BMI on the association between differences in key factors and differences in IS of PCOS and control groups was investigated by including BMI in the model.

Publication bias and outliers

We examined a scatter plot of the t-statistic associated with each study-estimate value contributing to the study-estimate random effect versus the log of the standard error of the effect. This plot is superior to the usual funnel plot (Hopkins et al., 2009), because the t-value is effectively adjusted for uncertainty in the study estimates and for the contribution of study covariates. This approach identified one clear outlier (Micic et al., 2007). Upon investigation of the data, it was noted that for some measures mean and SD were reported instead of mean and SE as stated in the methods section. After

correction, this study was no longer an outlier, and the scatter plot revealed no evidence of the asymmetrical scatter associated with publication bias (Supplementary Figure 1). Therefore, no studies were excluded from the meta-analysis.

Inferential Statistics

Magnitudes of effects were evaluated via standardization. For this purpose, a between-subject standard deviation representing the typical variation in IS was derived from the square root of the weighted mean of the variances of the log transformed factor SD for the healthy lean women in a subset of studies. The subset was determined by plotting the log of the factor SD against the mean BMI from healthy women in all studies. The plot showed a reasonably clear increase in the SD in studies with mean BMI >25 kg/m^2. We therefore used all the studies with a mean BMI of <25 kg/m^2 as the subset. Sample-size bias in the standardized effects was negligible, owing to the large number of degrees of freedom in the estimate of the SD and was therefore not corrected.

The effects in log-transformed units were divided by this SD, and their magnitudes were interpreted with the following scale: <0.2, trivial; 0.2-0.6, small; 0.6-1.2, moderate; >1.2, large (Hopkins et al., 2009). In keeping with trends in inferential statistics (Sterne and Davey Smith, 2001, Snowling and Hopkins, 2006), we made magnitude-based inferences about true population values of effects by expressing the uncertainty in the effects as 90% confidence limits. Effects were deemed unclear if the confidence interval overlapped thresholds for substantial positive and negative values (i.e. ±0.2 standardized units); effects were otherwise deemed clear and reported as the magnitude of its observed effect (Hopkins et al., 2009). For an

improvement in IS, magnitude thresholds were 3.8, 12, 25 and 45%, representing small, moderate, large and very large respectively; for reduced IS corresponding magnitude thresholds were -3.7, -11, -20, and -31%. Magnitude-based inferences about effects were made more accurate and informative by qualifying the effects with probabilities that reflected the uncertainty in the magnitude of the true value (Hopkins et al., 2009); 25-75%, possibly; 75-95%, likely; 95-99.5%, very likely; >99.5%, most likely.

Results

The search returned 4,371 articles (Figure 1). Of these, 48 studies met the inclusion criteria but 16 studies did not report essential data in a usable format. Eight of the 16 studies studies were published >10 years ago, therefore authors were not contacted to obtain the missing outcome measure and data not included in the meta-analysis (Carmina et al., 1995, Ciampelli et al., 2002, Cibula et al., 2002a, Ducluzeau et al., 2003, Dunaif et al., 1992, Fox et al., 1993, Paradisi et al., 2003, Holte et al., 1995). Four authors of the 16 were contacted by email twice but did not provide the relevant data (de Boer et al., 2004, Ketel et al., 2011, Ketel et al., 2008, Rabol et al., 2011). Therefore, these studies were not included in the meta-analysis. Furthermore, 11 publications reported data that was previously published and already represented in the meta-analysis (Aroda et al., 2009, Dunaif et al., 1991, Harrison et al., 2012, Hutchison et al., 2012, Joham et al., 2012, Lawson et al., 2008, Moran et al., 2011a, Svendsen et al., 2010, Svendsen et al., 2009, Hutchison et al., 2011). The remaining 25 articles were included in the final meta-analysis (Aroda et al., 2008, Baillargeon and Carpentier, 2007, Ciaraldi et al., 2009, Eriksen et al., 2010, Eriksen et al., 2011, Kowalska et al., 2012, Kowalska et al., 2007, Lasco et al., 1995, Li and Li, 2012,

Moret et al., 2009, Nikolajuk et al., 2010, Oh et al., 2009, Ovesen et al., 1993, Park et al., 2007, Park et al., 2001, Patel et al., 2003, Svendsen et al., 2008, Tosi et al., 2009, Vrbikova et al., 2004, Yang et al., 2011, Dunaif et al., 1989, Micic et al., 2007, Manneras-Holm et al., 2011, Morin-Papunen et al., 2000b, Stepto et al., 2013). These articles provided a total of 34 study estimates as some studies reported effects for more than one PCOS and control group. The effects from each study estimates are summarised in Table 1 as percentages with 90% confidence limits calculated from means, SD and sample size in PCOS and control groups.

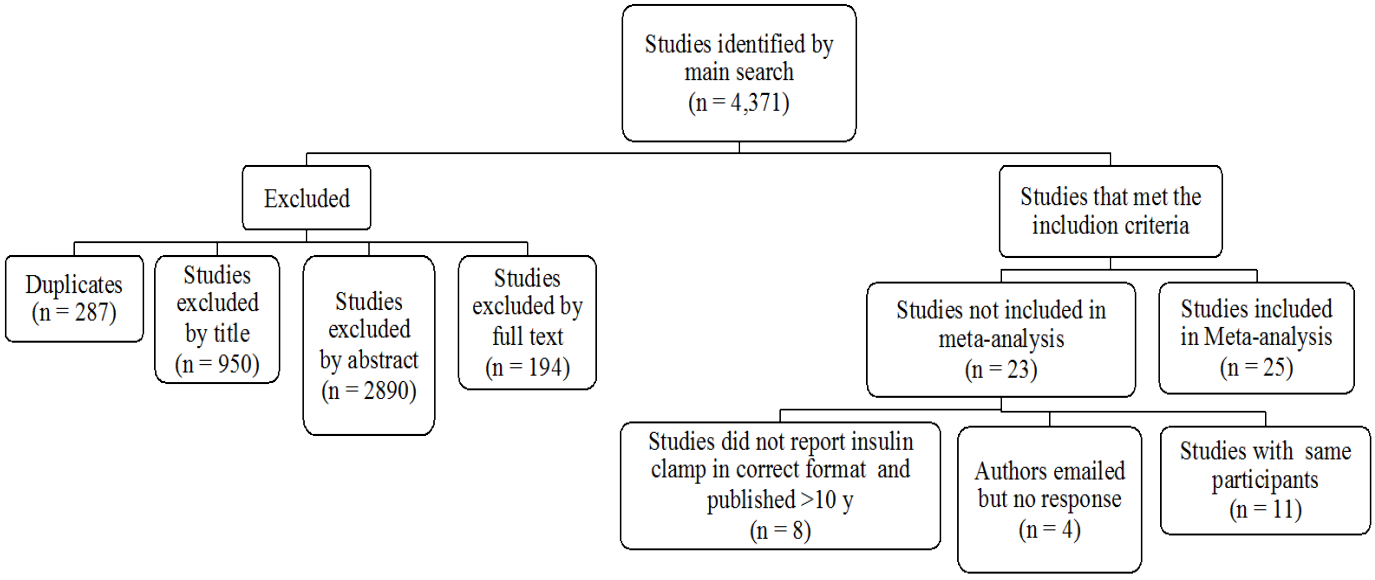

Figure 1 Flow diagram of the systematic review study selection process.

Table 1 Studies included in meta-analysis with age, BMI and insulin sensitivity in women with and without PCOS and the effect of PCOS on insulin sensitivity.

Study	Sample Size (Control, PCOS)	Diagnostic Criteria	Age (y) mean ± SD		BMI (kg.m^2) Mean ± SD		Insulin Sensitivity* Mean ± SD		Effect of PCOS on Insulin Sensitivity† (%) Mean (CI)
			Control	PCOS	Control	PCOS	Control	PCOS	
Aroda, 2008 (Aroda et al., 2008)	6, 31	NIH	32 ± 5	29 ± 6	36.2 ± 6.9	35.3 ± 7.4	8.8 ± 2.0	5.6 ± 2.9	-36.2 (-48.7 to -20.7)
Baillargeon, 2007 (Baillargeon and Carpentier, 2007)	17, 9	NIH	31 ± 7	24 ± 7	22.0 ± 2.1	22.6 ± 1.2	52.9 ± 19.0	48.5 ± 18.9	-8.3 (-29.2 to 18.8)
Ciaraldi, 2009 (Ciaraldi et al., 2009)	15, 42	NIH	32 ± 4	29 ± 7	33.9 ± 7	35.4 ± 7.1	9.7 ± 2.8	6.5 ± 2.2	-32.8 (-42.2 to 21.9)
Dunaif, 1989 (Dunaif et al., 1989)	8, 10	NIH	29 ± 6	27 ± 6	21.3 ± 1.1	22.3 ± 1.6	7.4 ± 1.1	4.8 ± 1.6	-35.9 (-47.8 to -21.6)
Dunaif, 1989 (Dunaif et al., 1989)	11, 17	NIH	30 ± 3	26 ± 5	33.3 ± 5.6	34.3 ± 4.6	3.8 ± 1.5	2.4 ± 0.7	-36.0 (-49.0 to -19.7)

Eriksen, 2010 (Eriksen et al., 2010)	14, 28	NIH	33 ± 8	31 ± 6	33.7 ± 6.4	33.2 ± 4.2	297 ± 86.1	150 ± 42.3	-49.5 (-56.8 to -41.0)
Eriksen, 2011 (Eriksen et al., 2011)	8, 8	NIH	32 ± 10	33 ± 3	35.1 ± 5.9	34.5 ± 4.0	271 ± 78.3	147 ± 23.1	-45.9 (-55.7 to -34.0)
Kowalska, 2007 (Kowalska et al., 2007)	25, 23	Rotterdam	26 ± 6	24 ± 4	21.8 ± 2	21.4 ± 2.1	11.6 ± 3.0	9.1 ± 3.5	-21.4 (-32.8 to -8.1)
Kowalska, 2007 (Kowalska et al., 2007)	20, 47	Rotterdam	28 ± 7	26 ± 6	31.0 ± 4.4	31.0 ± 4.0	9.38 ± 3.1	7.3 ± 3.1	-22.6 (-34.0 to -9.3)
Kowalska, 2012 (Kowalska et al., 2012)	28, 65	Rotterdam	27 ± 6	25 ± 6	28.2 ± 6.8	27.3 ± 7.2	58.1 ± 22.3	45.7 ± 18.7	-21.2 (-31.8 to -9.1)
Lasco, 1995 (Lasco et al., 1995)	6, 10	Rotterdam	28 ± 2	24 ± 2	36.4 ± 2.2	21.0 ± 0.8	2.8 ± 1.1	5.8 ± 2.4	-51.7 (-66.1 to -31.3)
Li, 2012 (Li and Li, 2012)	92, 78	NIH	26 ± 3	25 ± 5	20.5 ± 1.6	20.7 ± 1.8	12.4 ± 1.7	10.1 ± 2.5	-18.6 (-22.8 to -14.3)
Li, 2012 (Li and Li, 2012)	92, 33	NIH	26 ± 3	25 ± 5	20.5 ± 1.6	26.8 ± 2.4	12.4 ± 1.7	7.46 ± 1.8	-39.8 (-44.2 to -35.2)
Manneras-Holm, 2011 (Manneras-Holm et al.,	31, 31	Rotterdam	28 ± 4	29 ± 3	24.7 ± 4.9	24.8 ± 4.8	13.0 ± 4.1	11.0 ± 3.0	-15.38 (-25.1 to -4.41)

2011)									
Micic, 2007 (Micic et al., 2007)	8, 8	Rotterdam	25 ± 20	22 ± 8	20.2 ± 3.3	20.5 ± 3.7	7.8 ± 3.7	4.4 ± 2.2	-43.1 (-61.5 to -16.0)
Micic, 2007 (Micic et al., 2007)	8, 8	Rotterdam	29 ± 14	25 ± 18	31.0 ± 10.4	34.4 ± 18.5	3.92 ± 2.9	1.82 ± 1.8	-53.6 (-76.3 to -9.2)
Moret, 2009 (Moret et al., 2009)	5, 5	Rotterdam	21 ± 2	24 ± 4	21.3 ± 1.0	23.0 ± 4.3	9.8 ± 2.0	8.2 ± 2.7	-16.3 (-38.6 to 14.0)
Morin-Papunun, 2000 (Morin-Papunen et al., 2000b)	17, 15	Rotterdam	37 ± 3	29 ± 5	22.9 ± 1.2	22.7 ± 1.9	48.2 ± 9.9	41.1 ± 14.3	-14.7 (-28.1 to 1.1)
Morin-Papunun, 2000 (Morin-Papunen et al., 2000b)	17, 28	Rotterdam	35 ± 5	30 ± 5	31.8 ± 4.7	34.5 ± 5.3	31.6 ± 11.1	20.5 ± 7.9	-35.1 (-46.0 to -22.1)
Nikolajuk, 2010 (Nikolajuk et al., 2010)	18, 35	Rotterdam	26 ± 6	24 ± 4	22.2 ± 1.9	21.7 ± 1.8	8.9 ± 2.3	7.2 ± 2.9	-18.5 (-29.7 to -5.7)
Nikolajuk, 2010 (Nikolajuk et al., 2010)	16, 43	Rotterdam	27 ± 5	26 ± 6	30.7 ± 4.4	31.5 ± 4.3	5.9 ± 2.2	4.5 ± 2.4	-24.4 (-38.0 to -7.8)
Oh, 2009 (Oh et al.,	24, 39	NIH	26 ± 1	25 ± 1	19.9 ± 0.3	20.8 ± 0.2	6.3 ± 0.3	5.3 ± 1.4	-15.9 (-21.7 to -9.6)

2009)									
Ovesen, 1993 (Ovesen et al., 1993)	7, 7	NIH	26 ± 4	21 ± 5	21.3 ± 1.8	22.2 ± 2.1	3.8 ± 1.3	4.0 ± 1.1	5.3 (-20.4 to -39.2)
Park, 2001 (Park et al., 2001)	5, 9	NIH	31 ± 11	25 ± 12	25.6 ± 5.3	26.0 ± 9.3	9.4 ± 5.1	2.3 ± 0.9	-75.5 (-84.9 to -60.5)
Park, 2007 (Park et al., 2007)	34, 73	NIH	26 ± 3	25 ± 4	20.9 ± 3.2	20.4 ± 1.5	6.7 ± 1.6	5.3 ± 1.3	-20.9 (-27.0 to -14.3)
Patel, 2003 (Patel et al., 2003)	9, 11	NIH	26 ± 2	29 ± 2	27.4 ± 2.1	35.3 ± 2.7	8.3 ± 2.4	5.2 ± 3.3	-37.4 (-55.8 to -11.3)
Stepto, 2013 (Stepto et al., 2013)	19, 20	Rotterdam	28 ± 6	27 ± 4	22.0 ± 2.0	23.0 ± 2.0	339 ± 76	269 ± 66	-20.7 (-29.8 to -10.2)
Stepto, 2013 (Stepto et al., 2013)	14, 20	Rotterdam	35 ± 4	30 ± 6	35.0 ± 6	36.0 ± 7.0	2694 ± 66	175 ± 96	-33.7 (-47.1 to -17.0)
Svendsen, 2008 (Svendsen et al., 2008)	9, 17	Rotterdam	20 ± 4	28 ± 5	22.0 ± 1.4	23.0 ± 1.5	13.3 ± 4.1	10.4 ± 3	-21.8 (-32.4 to -9.6)
Svendsen, 2008 (Svendsen et al., 2008)	16, 18	Rotterdam	31 ± 5	29 ± 4	34.0 ± 3.2	33.0 ± 4.0	8.1 ± 2.8	6.9 ± 2.0	-14.8 (-28.9 to 2.0)
Tosi, 2009 (Tosi et al.,	35, 50	NIH	25 ± 5	22 ± 4	23.4 ± 5	24.0 ± 4	13.9 ± 2.0	10.3 ± 2.8	-25.9 (-31.2 to -20.2)

2009)									
Vrbikova, 2004 (Vrbikova et al., 2004)	15, 52	NIH	28 ± 6	24 ± 5	21.5 ± 2.0	21.5 ± 1.8	43.9 ± 11.1	41.7 ± 12.2	-4.9 (-16.3 to 8.1)
Vrbikova, 2004 (Vrbikova et al., 2004)	0, 30	NIH	28 ± 6	26 ± 5	21.5 ± 2.0	29.6 ± 3.7	43.9 ± 11.1	32.2 ± 10.0	-26.5 (-36.3 to -15.3)
Yang, 2011 (Yang et al., 2011)	116, 133	Rotterdam	26 ± 3	25 ± 4	21.0 ± 2.2	23.5 ± 3.5	12.1 ± 1.8	8.4 ± 2.8	-30.6 (-34.2 to -26.9)

*Units of measure for insulin sensitivity not included as units and calculations differed between studies.

†Difference in insulin sensitivity between PCOS and control women as a percent of control.

A total of 23 studies were included in the meta-analysis investigating the moderating effects of key hormones (Table 2); Eriksen et al. 2010 and Eriksen et al. 2011 were excluded as hormonal data were not reported.

Table 2 Studies included in the meta-analysis investigating moderating effects of key hormones.

Study	SHBG (nmol/L) Mean ± SD		Testosterone (nmol/L) Mean ± SD		LH (IU/L) Mean ± SD		FSH (IU/L) Mean ± SD	
	Control	PCOS	Control	PCOS	Control	PCOS	Control	PCOS
Aroda et al. 2008	22 ± 11.8	20 ± 15.6	0.7 ± 0.3	1.3 ± 4.9	--	--	--	--
Baillargeon et al. 2007	--	--	--	--			4.2 ± 1.2	4.7 ± 1.5
Ciaraldi et al. 2009	17.6 ± 13.9	17.5 ± 12.3	0.9 ± 0.5	1.9 ± 1.1	--	--	--	--
Dunaif et al. 1989	26.7 ± 10.8	28.5 ± 15.4	1.0 ± 0.4	1.6 ± 0.6	--	--	--	--
Dunaif et al. 1989	20.1 ± 8.7	15.6 ± 4.53	0.7 ± 0.2	1.80 ± 1.1	--	--	--	--
Kowalska et al. 2007	87.1 ± 47.5	69.3 ± 38.1	1.8 ± 0.5	2.7 ± 0.9	6.6 ± 4.5	11.7 ± 6.2	6.3 ± 1.5	5.9 ± 1.3
Kowalska et al. 2007	47.6 ± 28.7	41.8 ± 33.6	1.8 ± 0.3	2.8 ± 1.2	4.7 ± 2.2	8.2 ± 3.6	5.3 ± 1.9	5.6 ± 1.4
Kowalska et al. 2012	59.8 ± 48.8	41.5 ± 22.0	1.4 ± 0.3	2.1 ± 0.9	4.7 ± 2.1	7.9 ± 4.9	5.5 ± 1.6	6.0 ± 1.4
Lasco et al. 1995	--	--	2.9 ± 1.1	1.6 ± 0.7	7.9 ± 2.3	3.9 ± 1.1	4.8 ± 0.9	5.4 ± 1.1
Li et al. 2012	--	--	1.6 ± 0.6	1.9 ± 0.7	--	--	--	--
Li et al. 2012	--	--	1.6 ± 0.6	2.1 ± 0.9	--	--	--	--
Manneras-Holm et al. 2011	69.4 ± 30.4	49.0 ± 25.1	0.7 ± 0.3	1.5 ± 0.7	--	--	--	--
Micic et al. 2007	54.7 ± 48.9	26.2 ± 24.5	1.9 ± 2.0	3.6 ± 6.7	--	--	--	--

Micic et al. 2007	43.2 ± 40.6	15.8 ± 20.0	2.1 ± 1.5	4.9 ± 8.6	--	--	--	--
Moret et al. 2009	--	--	1.2 ± 0.1	1.8 ± 0.8	3.8 ± 1.8	13.6 ± 3.4	5.2 ± 0.8	6.1 ± 1.5
Morin-Papunun et al. 2000	60.5 ± 23.5	43.0 ± 16.7	1.3 ± 0.8	2.0 ± 0.8	4.7 ± 1.6	7.5 ± 2.7	6.2 ± 1.6	5.1 ± 1.9
Morin-Papunun et al. 2000	51.0 ± 27.6	30.8 ± 12.7	1.3 ± 0.4	2.3 ± 1.1	5.3 ± 3.3	7.0 ± 2.6	7.9 ± 5.8	6.4 ± 2.6
Nikolajuk, et al. 2010	89.5 ± 56.2	59.91 ± 46.7	1.8 ± 0.4	2.9 ± 1.1	6.9 ± 5.6	10.2 ± 5.3	6.0 ± 1.3	5.8 ± 1.5
Nikolajuk et al. 2010	41.5 ± 18.4	36.2 ± 18.0	1.8 ± 0.5	2.8 ± 1.1	4.3 ± 2.2	9.0 ± 4.0	5.6 ± 1.7	5.5 ± 1.4
Oh et al. 2009	56 .0 ± 7.0	43 ± 4	--	--	--	--	--	--
Ovesen et al. 1993	79.3 ± 12.2	59.9 ± 13.8	1.0 ± 0.3	2.9 ± 0.9	--	--	--	--
Park et al. 2001	89.1 ± 51.7	20.1 ± 37.5	1.1 ± 1.0	0.9 ± 0.5	12 ± 7.2	22.3 ± 16.6	9 ± 5.4	10 ± 9.6
Park et al. 2007	54.7 ± 25.7	50.5 ± 23.9	--	--	4.4 ± 4.3	10.2 ± 5.6	4.1 ± 2.2	5.5 ± 1.7
Patel et al. 2003	--	--	1.2 ± 0.3	2.6 ± 0.3	3.4 ± 1.5	7.9 ± 5.0	4.8 ± 1.5	4 ± 1.3
Stepto et al. 2013	79 ± 19	69 ± 34	1.5 ± 0.5	2.1 ± 0.8	--	--	--	--
Stepto et al. 2013	46 ± 29	32 ± 11	1.5 ± 0.8	2.6 ± 0.8	--	--	--	--
Svendsen et al. 2008	104 ± 33	67 ± 27	1.5 ± 0.3	2.1 ± 0.8	--	--	--	--
Svendsen et al. 2008	54 ± 21	57 ± 39	1.4 ± 0.4	2.4 ± 0.8	--	--	--	--
Tosi et al. 2009	72 ± 7	63 ± 6	2.1 ± 1.0	3.1 ± 1.5	--	--	--	--
Vrbikova et al. 2004	68.5 ± 21.3	48.6 ± 24.3	1.8 ± 0.6	3.4 ± 1.3	5.4 ± 2.1	7.6 ± 5.2	4.8 ± 1.8	--
Vrbikova et al. 2004	68.5 ± 21.3	36 ± 22.7	1.8 ± 0.6	3.3 ± 1.5	5.4 ± 2.1	5.9 ± 2.9	4.8 ± 1.8	--
Yang et al. 2011	--	--	1.45 ±0.7	2.0 ± 1.0	--	--	--	--

A moderate risk of bias was reported in the majority of studies included in the systematic review and meta-analysis following quality appraisal (Supplementary Table 3).

Overall relationship between insulin sensitivity and PCOS

In the first simple meta-analysis model, IS was lower in women with PCOS compared to controls (a large difference; Table 3). Unexplained variance between studies was estimated as a CV of 14.8% (90% confidence limits ±5.6%).

Table 3 Meta-analyzed overall mean effect of PCOS and insulin sensitivity, the predicted unadjusted and adjusted effects in study subgroups defined by BMI, age and diagnosis, and the effects of each of these moderators.

	Unadjusted Effects		Adjusted Effects	
	Effect, ±CL (%)	**Qualitative Inference**	**Effect, ±CL (%)**	**Qualitative Inference**
Overall Mean	-27.5, ±3.9	Large****	--	--
Moderation by mean BMI				
BMI=22 kg/m^2 (lean)	-19.9, ±4.2	Moderate****	-20.6, ±6.1†	Large****
BMI=32 kg/m^2 (overweight)	-32.1, ±4.3	Very large****	-31.3, ±6.5†	Very large****
Overweight PCOS vs lean PCOS	-15.2, ±6.4	Moderate****	-13.5, ±9.6†	Moderate**
Moderation by mean age			--	--
Age=22 y (younger)	-18.3, ±9.1	Moderate***	-23.5, ±10.5‡	Large****
Age=32 y (older)	-37.2, ±7.6	Very large****	-28.7, ±9.1‡	Large****
Older PCOS vs younger PCOS	-23.1, ±14.9	Large***	-6.8, ±21.6‡	Small (unclear)
Moderation by diagnosis				
Rotterdam	-24.9, ±5.9	Large****	-23.9, ±5.9§	Large****
NIH	-29.9, ±5.3	Large****	-28.3, ±5.5§	Large****
NIH vs Rotterdam	-6.4, ±10.2	Small (unclear)	-5.8, ±7.8§	Small*

Mean ±90% confidence limits

*****indicates likelihood that the true effect is substantial. A substantial reduction is reported as follows; *possibly, **likely, ***very likely, ****most likely

†Adjusted to mean age (27 y) and mean diagnosis ((Rotterdam+NIH)/2)

‡Adjusted to mean BMI (27 kg.m^{-2}) and mean diagnosis

§Adjusted to mean age and mean BMI

Moderating effects of BMI on insulin sensitivity

Compared to their respective controls, lean and overweight women with PCOS showed lower IS with large and very large magnitudes respectively (Table 3, Figure 2). Overweight women with PCOS had moderately lower IS compared to lean women with PCOS (Table 3). The residual between-study differences were 8.9% (±7.2%). Adjusting for differences in age and diagnostic criteria resulted in little change in the effect of BMI (Table 3) and little change in the between the residual between-study effect differences (9.6 ±7.2%).

This BMI only model was also able to produce separate effects of BMI on IS in PCOS and control women; a 10-unit higher BMI in women with PCOS was associated with a -26.6% (±11.1%; large, most likely) difference in IS. In control women, a 10-unit higher BMI resulted in a difference in IS of only -13.4% (±13.1%; moderate, likely lower).

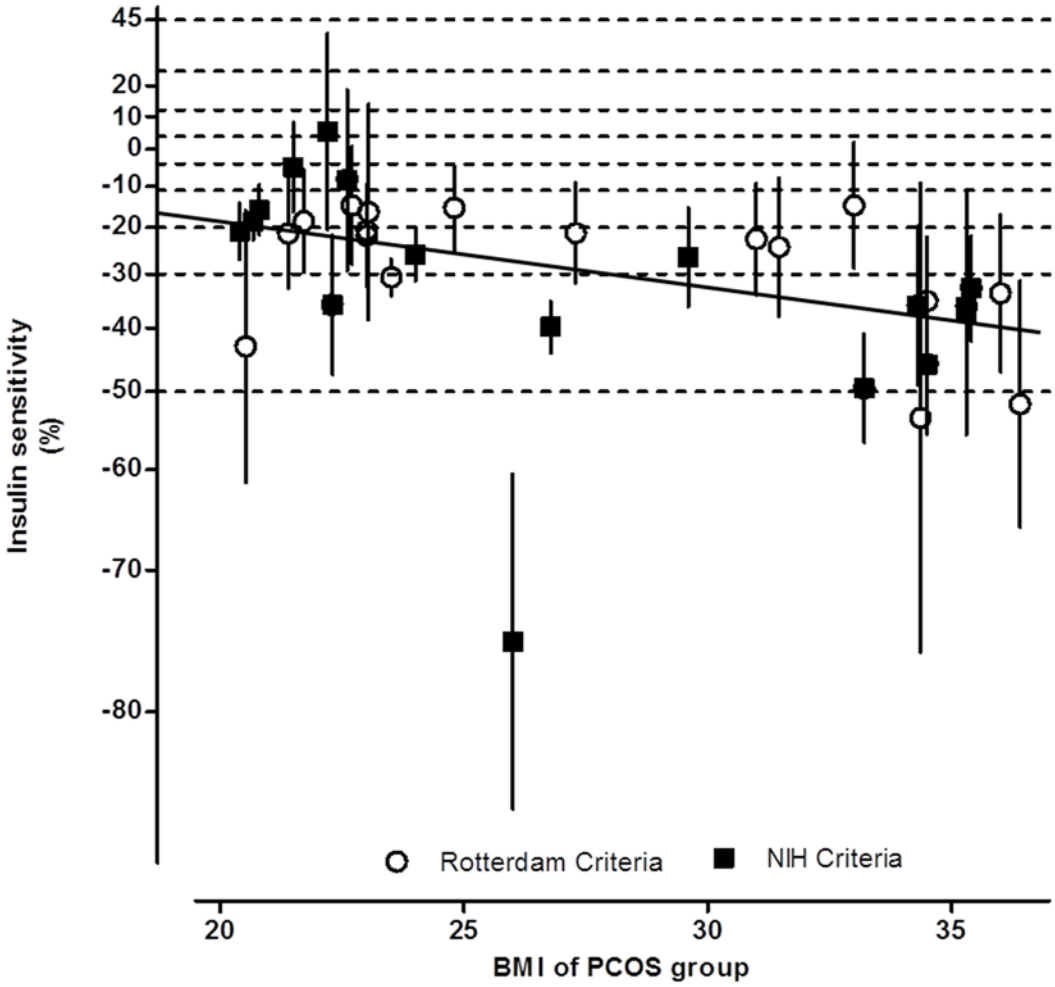

Figure 2 The effect of BMI on the difference in insulin sensitivity between PCOS and control groups. The dotted lines represent magnitude based thresholds. For an improvement in insulin sensitivity, magnitude thresholds were 3.8, 12, 25, and 120%, representing small, moderate, large, very large and extra large respectively; for reduced insulin sensitivity, corresponding thresholds were -3.7, -11, -20, -31 and -53%.

Moderating effects of age on insulin sensitivity

Compared to their respective controls, younger and older women with PCOS showed lower or much lower IS respectively (moderate and very large magnitudes). Older women diagnosed with PCOS had a lower IS compared to younger women with PCOS (a large magnitude), but this effect became trivial when BMI and diagnostic criteria were included in the model (Table 3). The residual between-study random effect differences were a CV of 12.9% (±5.7%).

As with BMI, the second model was also able to estimate separate effects for age on insulin sensitivity in PCOS and control women. A 10-year increase in age in women with PCOS was associated with a -24.7% (±21.3%; large, likely lower) difference in IS; in control women the difference in insulin sensitivity for women 10 years older was only -2.1% (±20.9%; unclear, trivial effect).

Moderating effects of diagnostic criteria as a surrogate for phenotype

The effect of PCOS on IS was large for both types of diagnostic criteria, but there was a clear small difference between them, whereby women diagnosed with PCOS by the original NIH criteria had lower IS (Table 3). The residual between-study differences in this analysis were a CV of 14.9% (±5.7%).

Insulin sensitivity, SHBG and hormone concentrations

Table 4 shows the uncorrected and BMI corrected associations of the differences in concentrations of SHBG, testosterone, LH and FSH with differences between IS comparing PCOS and control women. The mean lower concentration of SHBG in PCOS women compared to controls was associated with a moderately lower IS,

which was not confounded by BMI. Testosterone means were higher in PCOS and were associated with worse IS but the relationship indicates women with less testosterone are more insulin resistant at the same BMI. Mean higher concentration of testosterone in women with PCOS compared to controls was associated with moderately improved IS. Higher LH concentrations in women with PCOS tended (unclear effect) to be associated with lower IS, while FSH had a trivial relationship with only minor differences in FSH concentration between PCOS and control women.

Table 4 Associations of differences in hormone concentrations with the difference in insulin sensitivity between women with and without PCOS

Hormones	Difference in [Hormone][a] Mean ± SD	Unadjusted Effects		Adjusted Effects	
		Effect[b] (%) Mean ±CL	Qualitative Inference	Effect[c] (%) (Mean ±CL)	Qualitative Inference
SHBG (n=24)	-26% ± 20%	-12, ±7	Moderate ↓***	-12, ±7.1	Moderate ↓***
LH (n=14)	88% ± 62%	-4, ±25	Small (unclear)	-10, ±14	Small (unclear)
Testosterone (n=29)	75% ± 43%	9, ±16	Small (unclear)	19, ±12	Moderate ↑***
FSH (n=13)	1.0% ± 16%	0%, ±0.1	Trivial****	--	--

[a] Indicates the between study means and SD of the difference in the mean concentration of hormones in PCOS and control women.
[b] Indicates the difference in insulin sensitivity associated with the mean of the difference in the means (PCOS - control) of the hormone concentrations.
[c] Indicates the difference in insulin sensitivity associated with the mean of the difference in the means (PCOS - control) of the hormone concentrations when BMI is taken into account.
*** very likely
↓ Indicates lower insulin sensitivity
↑ Indicates higher insulin sensitivity

Discussion

In this novel and comprehensive systematic review and meta-analysis, we investigated whether lower IS, measured by euglycaemic-hyperinsulinaemic clamp, (the gold standard method to assess IR), is intrinsic to PCOS. We also explored potential moderators (BMI, age and diagnostic criteria) and mediators (SHBG, testosterone, LH and FSH) of IR in this condition. We report that overall women with PCOS were more insulin resistant than controls, independent of BMI, age or diagnostic criteria used. BMI not only independently exacerbated IR in PCOS, but we report, for the first time, that BMI had a greater impact on IR in PCOS than it did in controls. Increasing age adversely impacted on IR, yet the impact was trivial when adjusted for BMI and diagnostic criteria. Importantly, PCOS diagnostic criteria had only a small effect on IR, with women diagnosed with the original NIH criteria demonstrating higher IR, compared to women diagnosed with the more inclusive Rotterdam criteria. Furthermore, of the potential hormones and related binding proteins studied, SHBG had the strongest negative association with IR, with lower SHBG levels associated with higher IR, which was not confounded by BMI. Total testosterone had a negative association with IR, with higher testosterone levels associated with lesser degrees of IR, when the confounding effects of BMI were controlled. The relationship of LH with IR remained small and unclear when BMI was included in the model. FSH concentrations did not differ between PCOS and control women therefore we were unable to determine a relationship between FSH and IR in the meta-analysis model.

A key role of insulin is to regulate glucose homeostasis by stimulating glucose uptake in target tissues including skeletal muscle and adipocytes and by suppressing hepatic

glucose production (Cho et al., 2011, DeFronzo, 1988). IR can be defined as an impairment of insulin to mediate metabolism in skeletal muscle, adipocytes and liver. This includes glucose uptake, glycogen synthesis and inhibition of lipolysis, resulting in hyperinsulinaemia to achieve blood glucose homeostasis (Kahn and Flier, 2000, Groop et al., 1992). The gold standard for assessing IR *in vivo* is generally accepted to be the euglycaemic-hyperinsulinaemic clamp (DeFronzo et al., 1979). Using this technique, a number of authors have suggested that IR is intrinsic to PCOS (Dunaif et al., 1989, Stepto et al., 2013). However, the data is inconsistent, especially in lean women with PCOS (Moret et al., 2009, Ovesen et al., 1993). Challenges in existing literature have included small sample sizes and not accounting for variations in ethnicity, and clinical phenotype or diagnostic categories. Here we address these gaps and advance the field by definitively confirming in a robust systematic review and meta-analysis that PCOS is underpinned by intrinsic IR, independent of BMI and age, as well as across different diagnostic criteria and ethnic groups. This work highlights the clear need to recognise PCOS as an insulin resistant condition. It also highlights the need to be aware of metabolic risks driven by IR (Legro et al., 2013, Teede et al., 2011). Lifestyle modification, which improves IR, remains first line treatment in PCOS to prevent weight gain and to induce weight loss (Teede et al., 2011). This work also suggests that metformin, an insulin sensitiser should have a role in treatment of PCOS, however results have been inconsistent and the role of metformin in PCOS still requires further exploration (Legro et al., 2013, Teede et al., 2011). Use of other insulin sensitisers is limited by safety concerns. As we have confirmed intrinsic IR in PCOS, there remains a need to identify effective and safe insulin sensitisers for the treatment of PCOS.

Women with PCOS appear to have higher BMI, rates of weight gain and incidence of obesity, compared to women without PCOS, especially in Caucasian populations (Lim et al., 2013, Teede et al., 2013, Yildiz et al., 2012). The relationship between PCOS and weight appears bidirectional with PCOS predisposing women to obesity and obesity increasing the prevalence and severity of PCOS (Teede et al., 2013). Given the confounding impact of obesity on IR, this has long muddied the waters when assessing independent or intrinsic IR in PCOS (Teede et al., 2013, Lim et al., 2012). Here in this large systematic review and meta-analysis of gold standard euglycaemic-hyperinsulinaemic clamp studies, we clearly confirm that PCOS is underpinned by IR, independent of obesity and that BMI further exacerbates IR in PCOS. Hence IR is present in both lean and overweight women with PCOS. We also progress understanding in this area by demonstrating that increased BMI has a greater adverse impact on IR in PCOS, more so than in controls. This observation may be related to potentially greater upper body fat and visceral fat in PCOS (Kirchengast and Huber, 2001, Morin-Papunen et al., 2000b, Yucel et al., 2006). However, the relative distribution of visceral versus subcutaneous fat remains controversial in PCOS with visceral fat mass quantified by magnetic resonance imaging or by computerized tomography not differing between women with PCOS compared with BMI-matched controls (Manneras-Holm et al., 2011, Yalamanchi et al., 2012). Yet, data in women from Asian backgrounds suggests visceral fat distribution may differ in PCOS (Wulan et al., 2010, Lim et al., 2012). We have also shown that within women with PCOS, age had only a trivial effect on IR once BMI and diagnostic criteria were considered. Overall we have clearly demonstrated IR is intrinsic to PCOS, regardless of BMI status and that BMI further and disproportionately increases IR in PCOS. Further research is needed to determine the mechanisms by which BMI

disproportionately increases IR in PCOS and effective interventions are needed to target prevention and management of obesity in PCOS to ameliorate IR.

The recent NIH consensus workshop on PCOS endorsed the Rotterdam criteria as the definitive diagnostic criteria for PCOS (National Institutes of Health, 2012). The NIH also recommended that all PCOS studies include analyses by reproductive PCOS phenotype. Much of the prior PCOS literature has not reported by phenotype, therefore here we have completed an analysis by diagnostic criteria as a proxy. We report that woman with original NIH diagnosed PCOS (irregular cycles and hyperandrogenism) were 28% more insulin resistant compared to controls. Prior studies have suggested women with this phenotype were significantly more insulin resistant than other phenotypes included under the Rotterdam criteria. However most of these studies used less accurate measures of IR (Barber et al., 2007, Carmina et al., 2005a, Dewailly et al., 2006, Goverde et al., 2009, Panidis et al., 2012, Rizzo et al., 2009, Welt et al., 2006). In our current work we advance the field by showing that women diagnosed under the more inclusive or broader Rotterdam criteria (two of irregular cycles, PCO and hyperandrogenism) remained more insulin resistant compared to controls. We also show only marginally higher IR in studies using the original NIH criteria versus Rotterdam. In the majority of studies in this meta-analysis, 'Rotterdam' diagnosed women included those in the original NIH group, although this was not the case in all studies. Given that even young lean women diagnosed by Rotterdam criteria alone are insulin resistant (Stepto et al., 2013), this supports the recent NIH endorsement of the Rotterdam PCOS criteria. This systematic review also highlights the need for all future studies to phenotype the populations studied.

As PCOS is a complex endocrine disorder where strong associations between many of its clinical features (in addition to obesity) and IR have been documented (Diamanti-Kandarakis and Dunaif, 2012), our review and meta-analysis allowed us to explore some of the potential mediators or moderators of IR in PCOS by other factors such as SHBG, testosterone, LH and FSH.

We report in women with PCOS compared to controls, that SHBG had a strong inverse association with IR. We confirm previous reports in longitudinal and cross sectional studies of an association of SHBG and IR (Wallace et al., 2013). This would be expected on the basis of mechanistic studies demonstrating that hepatic SHBG production is suppressed as a result of IR and hyperinsulinemia (Le et al., 2012). Also treatment with insulin-sensitising medication, like metformin and troglitazone, increases circulating SHBG concentrations (Ehrmann et al., 1997, Moghetti et al., 2000). These reports have led to suggestions that SHBG could be used as a simple clinical marker of IR in women with PCOS. SHBG is not only lower, but also less variable in PCOS, compared to in healthy women (Jayagopal et al., 2003). Our current meta-analysis supports this recommendation, with more research with larger sample sizes now needed to derive specific cut-off ranges for SHBG to predict IR. Furthermore, low levels of SHBG are associated with adverse cardiovascular risk factors, diabetes and metabolic syndrome, independent of obesity, all important clinical implications of IR in PCOS, highlighting SHBG may also be a marker of metabolic risk in PCOS.

We confirm that LH levels are elevated in PCOS and FSH is relatively normal or slightly below normal levels (Katsikis et al., 2011). Overweight women, regardless of PCOS status, have been reported to have lower LH concentrations than lean women, but this is still controversial (Katsikis et al., 2011). Adipose tissue is thought to impact on LH levels as it contains aromatase, which converts androgens to estrogens. Consequently, elevated estrogen suppresses LH secretion and the elevated LH levels seen in lean PCOS women, are not necessarily observed in obese women with PCOS (Diamanti-Kandarakis and Dunaif, 2012). GnRH pulsatile secretion is more frequent in PCOS causing the activation of LH β-subunit gene and consequently increasing LH secretion (Katsikis et al., 2011). It has been reported that hyperinsulinaemia alters gonadotrophin secretion. However, this review supports other publications in providing evidence that IR is not directly related to gonadotropin levels in PCOS (Moran et al., 2003).

The primary observed androgenic abnormality in PCOS is elevated "free testosterone" or calculated free androgen index. These parameters largely rely on inclusion of SHBG into the equation introducing a confounder, which interferes in the analysis of the relationship between androgen levels and IR. Given that IR has a profound effect on SHBG, it is important that relationships between androgens and IR are studied independent of SHBG. Hence we focussed here on studies that reported total testosterone levels. In this context, in contrast to much of the existing literature, this meta-analysis suggests an inverse relationship with higher androgens associated with reduced IR, once corrected for BMI. The majority of the existing studies do not correct for BMI when reporting androgens. Many mechanisms underpin both IR and higher androgens including intrinsic PCOS drivers and increased BMI and central

adiposity. Whilst IR and hyperinsulinemia drive hyperandrogenism and insulin sensitisers appear to improve hyperandrogenism, less is known about the impact of androgens on IR (Diamanti-Kandarakis and Dunaif, 2012). Administering androgens to female-to-male transsexuals improves IR (Polderman et al., 1994), whilst blocking androgens through various treatments (combination of ethinyloestradiol, cyproterone acetate +/- antiandrogens) increases IR in women with PCOS, although the impact on IR may not be mediated by changes in androgens (Dahlgren et al., 1998, Meyer et al., 2007, Morin-Papunen et al., 2000a). These findings are inconsistently demonstrated and relationships remain unclear. The relationship between androgens and IR in this meta-analysis, which focused on total testosterone, only became significant when the confounding effects of BMI were taken into account. Testosterone administration in females appears to induce a male-like fat distribution and increase in muscle mass (Elbers et al., 1999, Elbers et al., 1997, Douchi et al., 2001, Notelovitz, 2002). Testosterone has an anabolic effect on muscle and elevated testosterone may act as a compensatory mechanism to counteract the intrinsic IR in PCOS as muscle is one of the main targets for the effects of insulin and accounts for the majority of glucose uptake (Corbould et al., 2006). Our novel data suggests that androgens may have a moderate effect on reducing IR in PCOS. However caution is needed when interpreting this data as immunoassays were predominately used for the measurement of androgens, which are less sensitive than mass spectrometry to detect androgen levels in women (Handelsman and Wartofsky, 2013). As we transition to more accurate methods, greater insights may be gained into the interrelationships between hyperandrogenism and IR in PCOS. In the interim, when evaluating relationships between IR and androgen status, measures of testosterone, measured via mass spectrometry, should be used.

The unexplained residual differences between studies, represented by the random effect, was large (~14%) but fell to moderate, when all predictors were included in the model (Hopkins et al., 2009). Therefore, the overall meta-analytic model did not account for all the between-study variation in the effect of PCOS on IR. Differences in study or subject characteristics including inherent problems in analyzing and reporting data from the euglycaemic-hyperinsulinaemic clamp, recruitment strategies, and ethnicity and lifestyle factors may account for some of the effects of PCOS on IR. Other key factors may also influence IR in PCOS, including low grade inflammation and sympathetic nervous system dysfunction (Diamanti-Kandarakis and Dunaif, 2012) and further research is needed to clarify key mechanisms underpinning IR in PCOS.

The strength of this meta-analysis is the extensive and comprehensive literature search and the focus on studies using gold standard methods to measure IR. However, as with many systematic reviews and meta-analyses, there are potential limitations of our findings that must be taken into consideration. Firstly, a meta-analysis cannot solve inherent confounding problems in the included studies, which may bias the results toward exaggeration or underestimation of risk estimates. There was a lack of available robust trials and challenges of reporting of data in the included studies as outlined. Not all identified studies were included with difficulties sourcing required data. The inability to acquire missing data from all eligible studies is not unexpected and deemed part of the meta-analysis process (Kelley et al., 2004). The inconsistent reporting of results made it necessary to express variables as factors by dividing the given variable in the PCOS group by that in the respective control group. Also

endpoints varied (including units and scales). The body of evidence was heterogeneous such that diagnostic criteria were not uniformly applied with many studies only including women with NIH-diagnosed PCOS, few reporting reproductive phenotypes and those that included Rotterdam-diagnosed PCOS often also included original NIH diagnosed cases. Small sample sizes and missing data limited evidence synthesis and meta-analysis. Study quality was also often poor with a moderate risk of bias found in most studies. Despite these limitations this body of work considerably advances the field by definitively demonstrating that IR is intrinsic to PCOS.

Conclusion

In this novel and comprehensive systematic review and meta-analysis of IR on euglycaemic-hyperinsulinaemic clamp studies in PCOS compared to controls we definitively report that IR is intrinsic to PCOS, independent of BMI and diagnostic phenotypes. We show that PCOS status has a large effect on IR. BMI independently exacerbates IR in PCOS and has a disproportionately greater impact than in controls. Age has limited effect on IR in PCOS, highlighting the need to be aware that it occurs even in lean and young women with PCOS. Importantly, we also demonstrate that expanding diagnostic criteria to more inclusive Rotterdam criteria appears to have negligible impact on prevalence of IR in PCOS. This work highlights the critical need for lifestyle management targeting both prevention of weight gain and management of excess weight in PCOS. This work also emphasises the need for effective pharmacological insulin sensitisers, structured exercise, and dietary and lifestyle interventions to assist in the management of excess BMI in PCOS. We confirm that SHBG has as strong relationship with IR in PCOS and may be a clinically useful marker of IR in PCOS. Finally, we suggest that androgens when studied as elevated

total testosterone, may actually ameliorate IR in PCOS and strongly suggest this is an area for future research.

Author's Roles

S.C designed the research, performed the literature search, independently reviewed the articles, performed data extraction, participated in data analysis and interpretation, wrote the manuscript, provided critical review of the draft and approved the final version. M.L.M designed the research, performed the literature search, assisted in reviewing articles and writing the manuscript, provided critical review of the draft and approved the final version. W.G.H designed the meta-analysis models, performed the data analysis and interpretation, wrote the manuscript, provided critical review of the draft and approved the final version. C.S.S independently reviewed the articles, assisted in data analysis and interpretation, assisted with the manuscript, provided critical review of the draft and approved the final version. N.K.S designed the research, assisted with independently reviewing the articles, assisted in data analysis and interpretation, assisted with the manuscript, provided critical review of the draft and approved the final version. H.J.T designed the research, provided data analysis interpretation, wrote the manuscript, provided critical review of the draft and approved the final version.

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Conflicts of interest

All authors declare no conflict of interests.

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Supplementary Table 1 MOOSE Checklist

Criteria		Brief description of how the criteria were handled in the meta-analysis
Reporting of background should include:		
✓	Problem definition	Polycystic ovary syndrome (PCOS) is a common condition underpinned by insulin resistance and exacerbated by obesity. We wanted to investigate whether insulin resistance is intrinsic to PCOS and determine the effect of BMI, age and diagnostic criteria on insulin resistance.
✓	Hypothesis statement	We propose that insulin resistance is intrinsic to PCOS and exacerbated by obesity.
✓	Description of study outcomes	Insulin sensitivity measured by euglycaemic hyperinsulinaemic clamp.
✓	Type of exposure or intervention used	Cases of PCOS were studied and observed characteristics recorded.
✓	Type of study designs used	We included case-control studies, prospective cohort studies, observational studies, cross-sectional studies, and comparisons of study populations.
✓	Study population	Individual studies were required to have participants with PCOS and a healthy control comparison group.
Reporting of search strategy should include:		
✓	Qualifications of searchers	• Dr Marie Misso is the Head of the Evidence Synthesis Program at Women's Public Health Research, Monash University and is highly experienced in systematic review and developing evidence based guidelines. • Professor Will Hopkins is head of Research Design and Statistics at Victoria University. • Professor Teede has a highly competitive clinical and public health research and translation track record with national/ international recognition and has published >160 articles.
✓	Search strategy, including time period included in the synthesis and keywords	• Medline and all of Evidence Based Medicine were searched in September 2013. • The search strategy was limited to English language articles.
✓	Databases and registries searched	Medline and Evidence Based Medicine
✓	Search software used, name and version, including special features	Medline and Evidence Based Medicine are available on the OVID SP platform. EndNote (version 20) was used to merge retrieved citations and eliminate duplications
✓	Use of hand searching	We hand-searched bibliographies of retrieved papers for additional references,

✓	List of citations located and those excluded, including justifications	Details of the literature search process are outlined in the flow chart and citation list is available as supplementary data.
✓	Method of addressing articles published in languages other than English	We placed restrictions on language and only searched for articles in English.
✓	Method of handling abstracts and unpublished studies	Abstracts and proceeding papers were assessed for eligibility according to the inclusion and exclusion criteria.
✓	Description of any contact with authors	Authors were contacted via electronic mail if essential data was not reported in a useable format. Details are provided in the Data Extraction section.
Reporting of methods should include:		
✓	Description of relevance or appropriateness of studies assembled for assessing the hypothesis to be tested	• Detailed inclusion and exclusion criteria are described in the methods section. • All studies provided euglycaemic-hyperinsulinaemic insulin clamp data.
✓	Rationale for the selection and coding of data	• Studies were included as per selection criteria. • Data extracted from each of the studies were relevant to the population characteristics, study design, exposure, outcome, and possible effect modifiers of the association. • Calculation of individual study estimates between women with PCOS and controls were undertaken as described in the methods.
✓	Assessment of confounding	Excluded studies if participants were treated with any therapy that may effect primary outcome of insulin sensitivity (including but not limited to; medication, exercise, smoking)
✓	Assessment of study quality, including blinding of quality assessors; stratification or regression on possible predictors of study results	Included studies were critically appraised using the Centre for Clinical Effectiveness assessment for case control studies.
✓	Assessment of heterogeneity	A mixed-model meta-analysis was used to assess the effect of PCOS on insulin sensitivity. True values of all effects were assumed to be heterogeneous and the analysis provides an estimate of the heterogeneity as a standard deviation representing unexplained typical true variation in the effect between studies. Inclusion of study and mean subject characteristics in the analysis as covariates may reduce heterogeneity and provide further useful information about the magnitude of the effect in different locations and with different subjects.
✓	Description of statistical methods in sufficient detail to be replicated	Description of methods of meta-analyses, and assessment of publication bias are detailed in the methods.
✓	Provision of appropriate tables and graphics	• We included a table detailing; • Terms used for database search

		• A flow chart of search and included/excluded studies • Table of included studies • Funnel like plot for publication bias • Table detailing association of insulin sensitivity with BMI, age and diagnostic criteria • Figure if relationship between BMI and insulin sensitivity in PCOS
Reporting of results should include:		
✓	Graph summarizing individual study estimates and overall estimate	Table 1, Table 2, Table 3 and Table 4.
✓	Table giving descriptive information for each study included	Table 1 and Table 2
✓	Results of sensitivity testing	Heterogeneity testing is included in the mixed model meta-analysis and also supplementary figure 1 which indicates publication bias.
✓	Indication of statistical uncertainty of findings	90% confidence limits were presented with all summary estimates. Inferential statistics were assessed using magnitudes of effects.
Reporting of discussion should include:		
✓	Quantitative assessment of bias	Risk of bias was assessed by producing a forest like plot of the t-statistic associated with each study estimate value contributing to the study-estimate random effect versus the log of the standard error of the effect.
✓	Justification for exclusion	Studies were excluded if they did not meet the inclusion criteria as detailed in the methods sections. Studies were also excluded if authors did not provide adequate information and did not reply when contacted.
✓	Assessment of quality of included studies	Quality appraisal of each study was performed using the Centre of Clinical Effectiveness appraisal templates and appraisal of each study is included as supplementary data.
Reporting of conclusions should include:		
✓	Consideration of alternative explanations for observed results	Potential confounders that may have caused residual confounding effects have been discussed.
✓	Generalization of the conclusions	The meta-analysis of the effect PCOS has on insulin resistance is reported in pre-menopausal women and BMI has a substantial impact on insulin resistance, especially in women with PCOS.
✓	Guidelines for future research	Future research focusing on lifestyle modification, (including structured exercise programs) and effective pharmacological interventions in the management of PCOS has been noted.
✓	Disclosure of funding source	Funding sources have been disclosed in the conflict of interest section of the manuscript.

Supplementary Table 2 Search strategy for systematic review[a]

1	exp[b] Polycystic Ovary Syndrome/
2	polycystic ovar$[c].mp[d].
3	poly-cystic ovar$.mp.
4	PCO$.mp.
5	(stein-leventhal or leventhal).mp.
6	Anovulation/
7	anovulat$.mp.
8	oligo-ovulat$.mp.
9	oligoovulat$.mp.
10	(ovar$ adj[e]5 (sclerocystic or polycystic or poly-cystic or degenerat$ or hyperandrogen$ or hyper-ndrogen$)).mp.
11	or/1-10
12	insulin resistan$.mp.
13	exp Insulin Resistance/
14	insulin resistance.mp.
15	insulin insensitiv$.mp.
16	insulin sensitiv$.mp.
17	exp Insulin/
18	insulin.mp.
19	exp Blood Glucose/
20	Blood Glucose.mp.
21	hyperinsulin$.mp.
22	glucose intolerance.mp.
23	euglycaemic-hyperinsulaemic clamp.mp.
24	euglycaemic hyperinsulaemic clamp.mp.
25	euglycemic-hyperinsulemic clamp.mp.
26	euglyc$ insulin clamp.mp.
27	insulin clamp.mp.
28	etiolog$.mp.
29	pathophysiol$.mp.
30	or/12-29
31	11 and 30
32	limit 31 to (English language and female and humans)

[a]Search was conducted for Medline with appropriate search terms utilised for other databases.
[b]exp = exploded.
[c]$ = any character.
[d]medical Subject Heading for Medline: mp, title, abstract, original title, name of substance word, subject heading word, keyword heading word.
[e]adj = adjacency

Supplemental Table 3 Quality appraisal of included studies

Study: Aroda V, Ciaraldi TP, Chang SA, Dahan MH, Chang RJ, Henry RR. Circulating and cellular adiponectin in polycystic ovary syndrome: relationship to glucose tolerance and insulin action, Fertility and Sterility. 2008;89(5):1200-1208.		
Description of study: Case control study. National Health and Medical research Council Level of Evidence III-3.		
Patient/population	Women with PCOS Age 29 ± 6 y, BMI 35.3 ± 7.4 Normal cycling and non-hirsute controls Age 32 ± 5 y, BMI 36.2 ± 6.9 Ethnicity not reported.	
N	PCOS n = 31 Control n = 6	
Setting	Special Diagnostic and Treatment Unit of the Veterans Affairs Medical Centre, San Diego, California, Uniter States of America.	
Intervention/exposure	Euglycaemic Hyperinsulinaemic Clamp for three hours. Glucose disposal rate was determined during the 140th and 160th minute and 160th and 180th minute.	
Comparison/control	Healthy women matched for age and BMI	
Outcomes	Anthropometric: BMI Diagnostic criteria: NIH Metabolic: Euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	PCOS diagnosis consistent with NIH criteria Control participants were screened by medical history, physical exam, laboratory evaluation and transvaginal ultrasound to confirm their healthy status.	
Exclusion Criteria	Medication of glucocorticoids, antiandrogens, or oral contraceptives within the previous 30 days and insulin sensitising medications the previous 60 days before study commencement. Pregnancy.	
Study Validity		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	The authors state their funding sources (p.1200)
Does the study have a clearly focused question?	Yes	All elements regarding participants, interventions, comparison groups and outcomes are described.
Is a case control study the appropriate method to answer this question?	Yes	A case control study is appropriate.
Does the study have specified inclusion/exclusion criteria?	Yes	Inclusion and exclusion criteria are described (p. 1201).

If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Authors have used relevant criteria for detecting PCOS and controls.
Were the cases and controls taken from comparable populations?	Not reported	Recruitment strategy or source population not reported.
Was case and control status established in a standard, valid and reliable way?	Yes	Investigators used the NIH criteria to determine PCOS status. Participants in the control group also underwent relevant tests to rule out PCOS (p. 1201).
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Glucosteroids, antianrogens, or oral contraceptive medications – 30 day washout period Ovulation induction agents, antiobesity medications, or insulin sensitizing agents – 60 day washout period. Effects of these mediations on insulin sensitivity may still be present following a 30-60 day washout period.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	A large difference in participation rate between the cases (PCOS) and control group exists.
Were the groups comparable with regards to key prognostic variables?	Yes	Groups were matched for age and BMI (p. 1201) and had similar waste to hip ratio (p. 1203).
Was there ≤20% drop-out?	Not reported	However, it seems that all participants that were recruited and completed all outcomes measured were included in the results and appropriately discussed (p.1201 and 1203).
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. A power calculation or statement is not provided.
Were all individuals included in the analysis?	Yes	All participants mentioned in the methods underwent analysis.

If statistical analysis was undertaken, was this appropriate?	Yes	The authors performed adequate statistical analysis for data collected (p.1202); however it is unclear whether analysis was planned a priori. Data was checked for normality and testosterone levels were log transformed to achieve normal distribution. The authors also presented point estimates measures of variability
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	Some of the criteria have been fulfilled and those criteria that have not been fulfilled may affect the conclusions of the study.

Results.
Anthropometric: There were no statistically differences in BMI between PCOS and controls groups (35.3 ± 7.4 vs. 36.2 ± 6.9kg/m^2) owing to the recruitment strategy of matching BMI between groups.
Metabolic: There was a significant difference in whole body insulin action (glucose disposal rate between PCOS and control groups (5.61 ± 2.90 vs. 8.79 ± 0.81 mg/kg/min; P<0.02), where women with PCOS were less insulin sensitive than the women in the control group.

Our Comments/Summary.
There is a moderate risk of bias for this study.
Control groups considerably less participants than PCOS group. Methods of recruitment not reported. Unknown is population recruited from individuals seeking treatment or community.

Study: Baillargeon JP, Carpentier A. Role of insulin in the hyperandrogenemia of lean women with polycystic ovary syndrome and normal insulin sensitivity, Fertility and Sterility. 2007;88(4):886-893.

Description of study: Case control study. National Health and Medical research Council Level of Evidence III-3.

Patient/population	Lean PCOS women; age 24.3 ± 2.3. BMI 22.6 ± 0.6 ≤25 kg/m^2 Ethnicity not specified but study was conducted in Canada.
N	Lean control n=17 Lean PCOS n=9
Setting	Metabolic Unit in Clinical research centre, Quebec, Canada.
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp
Comparison/control	Lean control participants were compared to lean PCOS participants
Outcomes	Anthropometric: BMI Diagnostic criteria: NIH Metabolic: Euglycaemic-Hyperinsulinaemic clamp

Inclusion Criteria	PCOS: absence of hypertension or acanthosis nigricans, normal fasting insulin levels, normal peak serum insulin levels during oral glucose tolerance test, and normal fasting serum to insulin ratio. Diagnoses consistent with NIH criteria. Control: Normal cycling women with normal weight and normal testosterone levels.	
Exclusion Criteria	Overweight or obese women (BMI >25 kg/m^2) Late-onset adrenal hyperplasia Medication that may affect insulin sensitivity including oral contraceptives and insulin-sensitising medication.	
Study Validity		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	It is unclear whether a conflict of interest exists, as a clean statement is not included in the publication. However, funding sources are reported and aren't deemed to provide a conflict.
Does the study have a clearly focused question?	Yes	Participants, interventions, comparison groups and outcomes are clearly described.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	Inclusion and exclusion criteria are defined but aren't entirely clear (p. 887)
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Authors aimed to increase the probability of enrolling PCOS women without insulin resistance through the use of inclusion and exclusion criteria. It is unclear whether the same inclusion/exclusion criteria were applied to control women, to reduce the probability of having insulin resistance.
Were the cases and controls taken from comparable populations?	Not reported	It is unclear how participants were recruited to participant in this study. Therefore the population studied may not be a true representative of the overall community population and selection bias may exist.
Was case and control status established in a standard, valid and reliable way?	Yes	Inclusion and exclusion criteria ensured that cases and controls were allocated to appropriate groups.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes measured are appropriate and important to answer study aims.

Was there sufficient duration of follow-up?	Partial	None of the participants ever used insulin-sensitising medication. It is also noted that none of the participants were using oral contraceptives or medications that may affect insulin sensitivity. However, the washout period for oral contraceptives or medications that may affect insulin sensitivity is not reported and it is unknown if the effect of these medications had sufficiently subsided.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who declined to participate and who did not complete the study was not reported. The PCOS group had less participants than the control group.
Were the groups comparable with regards to key prognostic variables?	No	The control group was older in age compared to the PCOS group and this was not accounted for in statistical analysis. Age may affect metabolic parameters and could be clinically important.
Was there ≤20% drop-out?	Not reported	Exclusion or dropout rates were not reported.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. A power calculation or statement is not provided when baseline data was analysed for key outcomes. However the authors do note that the small sample size reduces the power of the study (p. 892)
Were all individuals included in the analysis?	Not reported	The number of women who did not complete the study was not reported. No reference was made to missing data. However, all participants included in the methods section were included in statistical analysis.
If statistical analysis was undertaken, was this appropriate?	Partial	A t-test (p. 888) was used at baseline to compare key outcomes between the control and PCOS group. As mentioned above, age was statistically different between groups. Age may affect metabolic processes and therefore would have been best to be used as a covariate in the analysis when comparing key outcomes. Non-parametric statistical tests were used that are independent of the number of subjects (p. 892).
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	Some of the criteria have been fulfilled and those criteria that have not been fulfilled may affect the conclusions of the study.

Results. Mean M-value of lean PCOS women (48.5 ± 6.3 micromol/kg.min) was similar to lean control subjects (52.9 ± 4.6 micromol/kg.min). They also had comparable anthropometric measures, lipids, fibrinogen, and plasminogen activator inhibitor. The study also reported on the effect of reducing insulin or the secretion of LH on insulin sensitivity, however these results were not used in the systematic review or meta-analysis.
Author's Conclusions. In women with PCOS and normal insulin levels and metabolic insulin sensitivity, reducing insulin secretion significantly decreased androgen and increased SHBG levels. These results suggest that insulin contributes to hyperandrogenemia even in PCOS women with normal metabolic insulin sensitivity, which might be due to increased sensitivity of their androgenic insulin pathway
Our Comments/Summary. There is a moderate risk of bias for this study. Authors aimed to increase the probability of enrolling PCOS women without insulin resistance. It is unclear whether the same criteria were applied to the control group. It is also unclear how participants were recruited for the study and if women with PCOS and women allocated to the control were recruited from comparable populations. Age was different between PCOS and controls groups and this was not considered a confounding factor in statistical analysis. Age has an impact on insulin resistance.

Study: Ciaraldi TP, Aroda V, Mudaliar S, Chang RJ, Henry RR. Polycystic ovary syndrome in associated with tissue-specific differences in insulin resistance. 2008;94(1):157-163	
Description of study: Case control study. National Health and Medical research Council Level of Evidence III-3.	
Patient/population	Overweight women with NIH PCOS. Mean ± SE Age = 29 ± 1yr, BMI 35.4 ± 1.1
N	Control n=15 PCOS n=42
Setting	Not stated
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp
Comparison/control	Overweight, normal cycling women. Age 32 ± 1yr, BMI 33.9 ± 1.8
Outcomes	Anthropometric: BMI Non-fertility reproductive: Testosterone, SHBG Metabolic: Insulin sensitivity
Inclusion Criteria	Control: normal cycling, nondiabetic, nonhirsute PCOS: Nondiabetic, NIH criteria to define PCOS
Exclusion Criteria	Pregnancy, thyroid disease, prolactinoma, Cushing's syndrome and late-onset nonclassic congenital hyperplasia. Taking oral contraceptives, glucocorticoids, antiandrogens, ovulation-inducing agents, or antidiabetic or antiobesity medications within two months prior to screening.
Study Validity	

Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Author's state funding sources and provide affiliation and disclosure statements (p. 162).
Does the study have a clearly focused question?	Partial	Intrinsic insulin action was investigated, however the PCOS group were predominantly overweight or obese. Adiposity is considered to confound insulin action, therefore inferences made about intrinsic insulin action is limited. Analysis should have included a lean vs. overweight comparison or adiposity measures accounted for in statistical analysis.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	Diagnosis of PCOS was based on the NIH criteria.
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Inclusion and exclusion criteria are explained (p. 158), however it is not clear whether investigators specifically recruited overweight/obese women in the PCOS group or if this was by chance.
Were the cases and controls taken from comparable populations?	Not reported	It is unclear how participants were recruited; therefore the population studied may not be a true representative of the overall community population.
Was case and control status established in a standard, valid and reliable way?	Yes	
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes measured are appropriate and important to answer study aims.
Was there sufficient duration of follow-up?	Partial	Participants underwent a 2-month washout before screening, from medications that affect insulin sensitivity. This short period may not be sufficient for complete washout of effects of medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who were ineligible or did not complete the study was not reported. The PCOS group had more participants than the control group.

Were the groups comparable with regards to key prognostic variables?	Partial	The control group was older in age compared to the PCOS group and this was not accounted for in statistical analysis. Age may affect metabolic parameters and could be clinically important. The authors do note that 'there were no associations between age and metabolic endpoints measured in the study' (p. 159). Control participants were BMI-matched to the PCOS participants.
Was there ≤20% dropout?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. A power calculation or statement is not provided.
Were all individuals included in the analysis?	Not reported	Exclusion or dropout rates were not reported.
If statistical analysis was undertaken, was this appropriate?	Partial	An ANOVA (p. 158) was used at baseline to compare key outcomes between the control and PCOS group. As mentioned above, age was statistically different between groups. Age may affect metabolic processes; therefore an ANCOVA may have been a more appropriate test. However, authors reported no associations between age and endpoints measures.
Is the paper free of selective outcome reporting?	Yes	
Other		
What is the overall risk of bias?	Moderate	
Results. Non-fertility reproductive outcomes: There was a significant difference ($p < 0.05$) in testosterone between women with (0.55 ± 0.05 ng/ml) and without PCOS (0.25 ± 0.04 ng/ml). There was no differences between groups in SHGB concentration (17.5 ± 1.9 and 17.6 ± 3.6 nmol/L) Metabolic: Women with PCOS had impaired glucose tolerance and the lowest rate of maximal insulin-stimulated whole body glucose disposal compared to controls ($P < 0.01$).		
Our Comments/Summary. There is a moderate risk of bias for this study. Recruitment strategy for groups are not reported and confounding effects of age on insulin sensitivity was not taken into account.		

Quality appraisal of included studies

Study: Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes, 2007; 38(9): 1165-1174.			
Description of study: Case control study.			
Patient/population	Obese PCOS women (mean ± SE); age 26 ± 1, BMI 35.6 ± 1.3 kg/m^2 (two participants were diagnosed with diabetes and were removed from the analysis. Data for the meta-analysis was recalculated based on the raw data provided by authors). Lean PCOS women; (mean ± SE); age 27± 2. BMI 22.3 ± 0.5 kg/m^2 Ethnicity not specified but study was conducted in New York, United States of America.		
N	Obese PCOS n = 19 Obese control n = 11 Lean PCOS n = 10 Lean control n = 8		
Setting	Not reported		
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp		
Comparison/control	Lean control participants; 29 ± 2, BMI 21.3 ± 0.4 kg/m^2 Obese control participants; 30 ± 1, BMI 33.3 ± 1.7 kg/m^2		
Outcomes	Anthropometric: BMI Diagnostic criteria: NIH equivalent Metabolic: Euglycaemic-hyperinsulinaemic clamp to measure insulin sensitivity.		
Inclusion Criteria	PCOS diagnostic criteria are clearly reported and are equivalent to NIH criteria. Obesity was defined by a BMI of >27 kg/m^2 In good health		
Exclusion Criteria	Not specifically reported		
Study Validity.			
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Authors acknowledge funding sources on page 1173.	
Does the study have a clearly focused question?	Yes	The population, exposure/intervention, comparison control groups and outcomes were all detailed and appropriate to answer the research question.	
Is a case control study the appropriate method to answer this question?	Yes		
Does the study have specified inclusion/exclusion criteria?	Partial	Inclusion criteria are detailed on page 1166. However, specific exclusion criteria for the control group or whether criteria were determined *a priori* are not reported.	

If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Criteria used to diagnose PCOS were equivalent to NIH criteria. The inclusion/exclusion criteria for the control group are not entirely clear.
Were the cases and controls taken from comparable populations?	Not reported	Recruitment strategy or source population not reported.
Was case and control status established in a standard, valid and reliable way?	Partial	Investigators used equivalent criteria to the NIH criteria to determine PCOS status (p.1166). It is unclear how the control group were selected and PCOS ruled out. Furthermore, despite previous documentation of hyperandrogenism in all PCOS cases, androgen levels were deemed normal in two obese and three lean women with PCOS at the time of sampling. Furthermore, ovarian morphology was not used as a diagnostic criterion, as women with normal appearing ovaries can still be diagnosed with PCOS. Therefore, taking into account the variation in androgen levels, some women may have been incorrectly diagnosed with PCOS.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	BMI was assessed by the gold-standard method of hydrostatic weighing. All other outcomes were sufficiently measured.
Was there sufficient duration of follow-up?.	Partial	Medications know to affect gonadal function or carbohydrate metabolism were ceased for at least 1-month before the study began. It is contentious if a 1-month washout period is adequate to eliminate the effects of medication on gonadal function or carbohydrate metabolism. Oral contraceptive medication was ceased at least 3 months before study commencement. A 3-month washout period should be sufficient.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	

Were the groups comparable with regards to key prognostic variables?	Yes	Eleven obese and 8 lean women in the control group were matched to the women in the PCOS group on the basis of age, BMI and body composition.
Was there ≤20% drop-out?	Not reported	However, it seems that all patients who entered the trial were properly accounted for and attributed at the study's conclusion.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	
Were all individuals included in the analysis?	Yes	All participants mentioned in the methods underwent analysis.
If statistical analysis was undertaken, was this appropriate?	Yes	The authors performed adequate statistical analysis for data collected (p.1167-1168), however it is unclear whether analysis was planned a priori. Variation for the main outcomes of insulin sensitivity measured by the insulin clamp was maintained at 4 ± 0.3%. Data was checked for normality and log transformed when necessary to achieve homogeneity of variance. A two-way ANOVA was performed to compare each PCOS group to its body-composition-matched control group and the α-level was adjusted to 0.025. Linear regression to determine associations between insulin sensitivity and other variables was used.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported appropriately.
Other		
What is the overall risk of bias?	Moderate	

Results.
There were statistically significant interactions between obesity and PCOS in fasting glucose levels and basal hepatic glucose production ($P < 0.05$). Insulin-stimulated glucose utilization was significantly decreased in both PCOS groups whether expressed per kilogram total weight ($P < 0.001$) or per kilogram fat free mass ($P < 0.001$). The metabolic clearance rate of insulin did not differ in the four groups.

Author's Conclusions.
PCOS women have significant insulin resistance that is independent of obesity, changes in body composition, and impairment of glucose tolerance. PCOS and obesity have a synergistic deleterious effect on glucose tolerance. Hyperinsulinemia in PCO is not the result of decreased insulin clearance. PCOS is associated with a unique disorder of insulin action.

Our Comments/Summary.
The overall risk of bias is moderate. There may have been misclassification of participants in the PCOS groups based on data collected for PCOS diagnosis via NIH criteria. It is difficult to generalise results as recruitment strategy and population studied is not reported.

Study: Eriksen M, Pørneki AD, Skov V, Burns JS, Beck-Nielsen H, et al. (2010) Insulin Resistance Is Not Conserved in Myotubes Established from Women with PCOS. PLoS ONE 5(12): e14469.	
Description of study: Case control study.	
Patient/population	Overweight PCOS women (mean ± SEM); age 31.6 ± 1.2, BMI 33.2 ± 0.8 kg/m^2 Caucasian population
N	PCOS n = 14 Control n = 28
Setting	The Department of Endocrinology, Odense University Hospital, Odense, Denmark.
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp
Comparison/control	Healthy Caucasian premenopausal women acted as control participants; 33.8 ± 2.1, BMI 33.7 ± 1.7 kg/m^2
Outcomes	Anthropometric: BMI Diagnostic criteria: NIH equivalent Metabolic: Euglycaemic-hyperinsulinaemic clamp to measure insulin sensitivity.
Inclusion Criteria	PCOS diagnostic criteria are clearly reported and are equivalent to NIH criteria. Overweight defined as BMI ≥30 kg/m^2
Exclusion Criteria	Participants with diabetes (fasting plasma glucose ≥7.0 mmol/l), hypertension, elevated liver enzymes, s-prolactin or s-TSH outside reference interval, renal dysfunction, and congestive heart disease were excluded from the study.

Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Authors acknowledge funding sources and declare that no competing interests exist.
Does the study have a clearly focused question?	Yes	The population, exposure/intervention, comparison control groups and outcomes were all detailed and appropriate to answer the research question.
Is a case control study the appropriate method to answer this question?	Yes	

Does the study have specified inclusion/exclusion criteria?	Yes	
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Criteria used to diagnose PCOS were equivalent to NIH criteria. The inclusion/exclusion criteria for the PCOS and control group are stated.
Were the cases and controls taken from comparable populations?	Not reported	Recruitment strategy or source population not reported.
Was case and control status established in a standard, valid and reliable way?	Yes	Investigators used equivalent criteria to the NIH criteria to determine PCOS status and women without PCOS did not have hyperandrogenism or hirsuitism.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?.	Yes	Subjects refrained from oral contraceptive use for at least three months before evaluation, and no patient took medicine known to affect hormonal or metabolic parameters.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Yes	Eleven obese and 8 lean women in the control group were matched to the women in the PCOS group on the basis of age, BMI and body composition.
Was there ≤20% drop-out?	Yes	Two participants did not complete the study (one became pregnant and the other became ill in the medication intervention arm of the study).
Was the study sufficiently powered to detect any differences between the groups?	Not reported	
Were all individuals included in the analysis?	Yes	All participants mentioned in the methods underwent analysis.

If statistical analysis was undertaken, was this appropriate?	Yes	Statistical significance was evaluated using Student's t-test and calculated two tailed P values. Paired analysis was performed for comparison of acute and chronic insulin exposure in the same sets of myotubes.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported appropriately.
Other		
What is the overall risk of bias?	Low	

Results.
PCOS participants had increased fasting levels of serum insulin, free testosterone, and plasma triglycerides compared to controls. Insulin-stimulated glucose disposal (Rd) was 50% lower in PCOS subjects than controls ($P<0.001$), and this was primarily accounted for by a 60% reduction in non-oxidative glucose metabolism (NOGM), but also a 39% decrease in glucose oxidation. Treatment of PCOS subjects with pioglitazone significantly reduced fasting serum insulin and improved insulin-stimulated Rd, glucose oxidation, and NOGM. No significant changes were measured in fasting Rd and basal glucose metabolism (Rd, glucose oxidation and NOGM) between PCOS subjects and controls or during pioglitazone treatment (data not shown).

Author's Conclusions.
These results suggest that the mechanisms governing insulin resistance in skeletal muscle of PCOS patients in vivo are not primary, but rather adaptive.

Our Comments/Summary.
The overall risk of bias is low. Sound methodology used to answer research question.

Study: Eriksen M.B, Minet A.D, Glintborg D, and Gaster M (2011). Intact Primary Mitochondrial Function in Myotubes Established from Women with PCOS, The Journal of Clinical Endocrinology and Metabolism E1298–E1302.		
Description of study: Case control study.		
Patient/population	Overweight PCOS women (mean ± SEM); age 33.8 ± 1.2, BMI 34.5 ± 1.4 kg/m^2 Caucasian population	
N	PCOS n = 8 Control n = 8	
Setting	Not reported.	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	Healthy Caucasian premenopausal women acted as control participants; 32.2 ± 3.4, BMI 35.1 ± 2.1 kg/m^2	
Outcomes	Anthropometric: BMI Diagnostic criteria: NIH equivalent (reported in previous publication) Metabolic: Euglycaemic-hyperinsulinaemic clamp to measure insulin sensitivity.	
Inclusion Criteria	Elevated fasting insulin levels (>50 pmol/liter) in PCOS group Overweight defined as BMI ≥30 kg/m^2	
Exclusion Criteria	Participants with diabetes (fasting plasma glucose ≥7.0 mmol/l), hypertension, elevated liver enzymes, s-prolactin or s-TSH outside reference interval, renal dysfunction, and congestive heart disease were excluded from the study.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Authors acknowledge funding sources and declare that no competing interests exist.
Does the study have a clearly focused question?	Yes	The population, exposure/intervention, comparison control group and outcomes were described and appropriate to answer the research question.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	Criteria could be more detailed and specific. Criteria used to establish PCOS not reported but instead the reader is directed to another paper.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Criteria used to diagnose PCOS were equivalent to NIH criteria.

Were the cases and controls taken from comparable populations?	Not reported	Recruitment strategy or source population not reported. Participants were randomly chosen from a previous cohort. It is unclear what technique was used to randomly choose participants and how selection bias was avoided.
Was case and control status established in a standard, valid and reliable way?	Yes	Investigators used equivalent criteria to the NIH criteria to determine PCOS status.
Was case and control status established by assessors blind to the exposure?	Not reported	Participants were randomly chosen from a previous cohort. It is unclear whether researchers knew diagnosis (PCOS or control) or participant characterises before samples were randomly chosen for the experiment.
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?.	Yes	Subjects refrained from oral contraceptive use for at least three months before evaluation, and no patient took medicine known to affect hormonal or metabolic parameters.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Yes	Healthy Caucasian premenopausal women matched to PCOS subjects for BMI and age were studied as controls.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	
Were all individuals included in the analysis?	Yes	All participants mentioned in the methods underwent analysis.
If statistical analysis was undertaken, was this appropriate?	Yes	Statistical significance was evaluated using Student's *t* test and calculated two-tailed *P* values.
Is the paper free of selective outcome reporting?	Partial	Planned outcomes were measured and reported appropriately.
Other		

What is the overall risk of bias?	High
Results. Mitochondrial mass and measurable mitochondrial ATP synthesis, with and without ATP use, were not different between PCOS subjects and control subjects. PCOS subjects were insulin resistant and had significantly higher levels of testosterone, triglycerides, and insulin compared with controls subjects. Insulin-stimulated glucose disposal in PCOS patients was 54% lower than controls,	
Author's Conclusions. We found no evidence for a primary impaired mitochondrial function or content in myotubes established from PCOS subjects, and our results suggest that reduced expression of oxidative genes in PCOS subjects is an adaptive trait.	
Our Comments/Summary. The overall risk of bias is high. It is unclear how participants were selected for this experiment, which could bias results.	

Quality appraisal of included studies

Study: **Kowalska I, Adamska A, Maleckit MT, Karczewska-Kupczewska M, Nikolajuk A, Szopat M, Gorska M, Straczkowski M. Impact of the FTO gene variation on fat oxifdation and its potential influence on body weight in women with polycystic ovary syndrome, Clinical Endocrinology. 2012; 77: 120-125.**		
Description of study: Case control study. National Health and Medical research Council Level of Evidence III-3.		
Patient/population	Women with PCOS; Age 25 ± 6 y, BMI 27.9 ± 7.2	
N	PCOS n = 65 Control n = 28	
Setting	Not reported	
Intervention/exposure	Euglycaemic hyperinsulinaemic clamp.	
Comparison/control	Control women matched for age and BMI with PCOS group; Age 27 ± 6 y, BMI 28 ± 6.8	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity	
Inclusion Criteria	Not reported and previous publication not specifically referenced for inclusion criteria.	
Exclusion Criteria	Unclear. Not reported and previous publication not specifically reference for exclusion criteria.	
Study Validity		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Funding sources are reported and aren't deemed to provide a conflict. Authors also provide a statement noting that they have nothing to declare.
Does the study have a clearly focused question?	Yes	

Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	PCOS diagnosis is clearly explained. However, other inclusion or exclusion criteria are not reported and no specific reference to inclusion/exclusion criteria is made.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	The criteria reported were appropriate.
Were the cases and controls taken from comparable populations?	Not reported	Participants in the control group were mainly recruited from medical staff and students. Recruitment methods for the PCOS group are not provided.
Was case and control status established in a standard, valid and reliable way?	Yes	The Rotterdam criteria was used to discriminate between cases and controls.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes were measured appropriately.
Was there sufficient duration of follow-up?	Not reported	
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who were excluded or who did not complete the study was not reported.
Were the groups comparable with regards to key prognostic variables?	Yes	The control group and PCOS groups did not differ in age or BMI.
Was there ≤20% drop-out?	Not reported	The control group and PCOS groups did not differ in age or BMI.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. A power calculation or statement is not provided when analysing baseline data for key outcomes.
Were all individuals included in the analysis?	Not reported	
If statistical analysis was undertaken, was this appropriate?	Yes	Statistical analysis is reported on page 122 and seems appropriate.

Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	

Results.
Mean M-value of lean PCOS women (45.73 ± 18.67 micromol/kg/min) was significantly lower compared to control participants (58.06 ± 22.34 micromol/kg.min). Both groups had comparable anthropometric measures and lipids.

Author's Conclusions.
The FTO gene variation might influence the baseline lipid oxidation in PCOS patients. This might potentially be one of the mechanisms explaining the impact of the FTO gene on body weight in PCOS.

Our Comments/Summary.
There is a moderate risk of bias for this study.
It is unclear of the inclusion/exclusion criteria used in the study. It is not noted where women with PCOS were recruited. Small sample size.

Study: **Kowalska I, Straczkowski M, Nikolajuk A, Adamska A, Karczewska-Kupczewska M, Otziomek E, Wolczynski S, Gorska M,. Serum visfatin in relation to insulin resistance and markers of hyperandrogenism in lean and obese women with polycystic ovary syndrome. 2007; 22(7):1824-1829**

Description of study: Case control study. National Health and Medical research Council Level of Evidence III-3.

Patient/population	PCOS diagnosis according to Rotterdam Criteria.
N	Lean Control n = 25 Lean PCOS n = 23 Overweight Control n = 20 Overweight PCOS n = 47 (BMI <25 kg/m^2 used to define lean and BMI >25 kg/m^2 used to define overweight/obese)
Setting	Participants with PCOS and obese control participants were recruited from Outpatient Endocrinology and Gynaecology Clinics. Lean control participants were mainly medical students and staff.
Intervention/exposure	Euglycaemic Hyperinsulinaemic clamp.
Comparison/control	Control women matched for age and BMI with PCOS group.
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity, euglycaemic-Hyperinsulinaemic clamp
Inclusion Criteria	Controls: Normal menstruating Non-smokers, not taking any anti-inflammatory medication or medication know to affect carbohydrate and lipid metabolism (within 3 months before the study).

Exclusion Criteria	Morbidly obese (BMI ≥ 40 kg/m^2), diabetes, cardiovascular disease, hypertension, infections or other serious medical problems.	
Study Validity		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	It is unclear whether a conflict of interest exists as a clear statement is not included in the publication. However, funding sources are reported and aren't deemed to provide a conflict.
Does the study have a clearly focused question?	Yes	Information regarding participants, interventions, comparison group and outcomes are reported.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	
Were the cases and controls taken from comparable populations?	Partial	Women with PCOS and participants who were overweight/obese were recruited through Outpatient Endocrinology and Gynaecology Clinics. Bias may exist with this recruitment strategy as women seeking treatment may have more severe symptoms of PCOS and obesity.
Was case and control status established in a standard, valid and reliable way?	Yes	Inclusion and exclusion criteria ensured that cases and controls were allocated to appropriate groups.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes were measured appropriately and important to answer study aims. Serum LH (sensitivity 0.07 mIU/ml; intraassay CV 4.7%, interassay CV 6.3%), FSH (sensitivity 0.3 mIU/ml; intrassay CV 2.8%, interassay CV 4.6%), testosterone (sensitivity 0.35 nmol/l; intraassay CV 7.8%, interassay CV 10.1%) were measured by chemiluminescence method (ACS Chirone 180) and serum sex hormone-binding globulin was measured by immunoradiometric assay (ZenTech, Angleur, Belgium) with sensitivity below 0.3 nmol/l and intraassay and interassay CV 2.9%.

Was there sufficient duration of follow-up?	Yes	Participants were not taking any anti-inflammatory drugs within 3 months prior to the study or medication known to affect carbohydrate or lipid metabolism.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	No	The number of women who were excluded from the study or who did not complete the study was not reported. The obese PCOS group had nearly double the amount of participants compared to the other three groups.
Were the groups comparable with regards to key prognostic variables?	Yes	Groups were comparable in regards to age.
Was there ≤20% drop-out	Not reported	Drop-out rates were not reported.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. A power calculation or statement is not provided.
Were all individuals included in the analysis?	Not reported	
If statistical analysis was undertaken, was this appropriate?	Yes	Statistical analysis has been reported (p. 1826) and appropriately details tests performed. The variables that did not have normal distribution (*M* value, visfatin, fasting and postload insulin, TG, FFA, SHBG, free androgen index) were log-transformed prior to analyses. Differences between groups were evaluated using factorial analysis of variance, with PCOS status and obesity as categorical variables, followed by the post-hoc Fischer's protected least-significant difference test. The relationships between serum visfatin and other variables were assessed using the Pearson product–moment correlation analysis and multiple regression analysis.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	

Results. The PCOS group had lower insulin sensitivity (P=0.00049) in comparison to the control group. The decrease in insulin sensitivity was present in both the lean (P=0.019) and obese (P=0.0077) PCOS subjects, In the whole group, serum visfatin was negatively correlated with insulin sensitivity (r=-0.27, P=0.004). This relationship was also observed in the subgroup of lean (r=-0.30, P=0.038), but not obese women. Additionally, in lean women, visfatin was associated with serum testosterone (r=0.47, P=0.002) and free androgen index (r=0.48, P=0.002), independently of other potential confounding factors.
Author's Conclusions. Data indicates that visfatin is associated with insulin resistance and markers of hyperandrogenism in lean PCOS patients
Our Comments/Summary. There is a moderate risk of bias for this study. Recruitment of participants bias towards more severe PCOS and obesity symptoms.

Quality appraisal of included studies

Study: **Lasco A, Cucinotta D, Gigante A, Denuzzo G, Pedulla M, Trifiletti A, Frisina N. No changes of peripheral insulin resistance in polycystic ovary syndrome after long-term reduction of endogenous androgens with leuprolide, European Journal of Endocrinology. 1995; 133: 718-722**		
Description of study: Case Control.		
Patient/population	Women diagnosed with PCOS based on the Rotterdam criteria; (mean ± SD); age 28 ± 1.9, BMI 36.4 ± 2.2 kg/m^2	
N	PCOS n = 10 Obese women with hirsutism but no PCOS n = 6 (not used in meta-analysis) Lean control women n = 6	
Setting	Not reported.	
Intervention/exposure	Euglycaemic hyperinsulaemic clamp	
Comparison/control	Lean control women (mean ± SD); age 24 ± 2, BMI 21 ± 0.8 kg/m^2	
Outcomes	Anthropometric: BMI Metabolism: Euglycaemic-hyperinsulinaemic clamp to measure insulin sensitivity (M index).	
Inclusion Criteria	Normal glucose tolerance,	
Exclusion Criteria	Endocrine disorders (e.g., Cushing's syndrome, androgen secreting tumours or prolactinomas. Medications affecting glucose tolerance taken three months prior to the study.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	
Does the study have a clearly focused question?	Yes	Funding sources, affiliations and other conflicts of interests have not been reported.

Is a case control study the appropriate method to answer this question?	Yes	The study has a clear research question. A lean control group was used as a comparison to the overweight/obese PCOS group. Any differences between groups may have been as a result of obesity. An overweight control group may be more appropriate. The tests used to measure desired outcomes are appropriate.
Does the study have specified inclusion/exclusion criteria?	Yes	
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	A definition of PCOS diagnosis is stated. Clear inclusion/exclusion criteria are noted.
Were the cases and controls taken from comparable populations?	Not reported	
Was case and control status established in a standard, valid and reliable way?	Yes	Recruitment strategies were not reported in the study.
Was case and control status established by assessors blind to the exposure?	Not reported	The PCOS diagnostic criteria use was able to discriminate between women with PCOS and controls.
Were the outcomes measured appropriate?	Yes	Outcomes measured were appropriate to answer research questions.
Was there sufficient duration of follow-up?	N/A	Medications likely to affect glucose tolerance were ceased 3 months prior to the commencement of the study.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of participants who were excluded from the study or who did not complete the study has not been reported.
Were the groups comparable with regards to key prognostic variables?	Partial	The PCOS group and control group were of simular age (28 ± 1.9 vs 24 ± 2 respectively). However, the PCOS group was overweight/obese (BMI = 36.4 ± 2.2) compared to the control group (BMI 21 ± 0.8).
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	
Were all individuals included in the analysis?	Not reported	However, it seems that all participants mentioned in the methods were included in the analysis.

If statistical analysis was undertaken, was this appropriate?	No	Comparisons between groups were performed by Student's paired and unpaired tests (p. 791). Statistics should have allowed for assessment of confounding effects of BMI on outcome measure of insulin sensitivity.
Is the paper free of selective outcome reporting?	Yes	
Other		
What is the overall risk of bias?	Moderate	

Results.
Plasma LH, andostenedione, testosterone and fasting plasma insulin were higher in PCOS women compared to controls. M index has higher in controls when compared to women with PCOS.
Results were also reported for comparisons to obese women with hirsutism and following treatment with leuprolide. However, these results are not required for the systematic review and meta-analysis.

Author's Conclusions.
An acute increase in plasma insulin, as observed during an insulin clamp, does not affect androgen secretion and suppression of androgens concentrations do not modify insulin resistance.

Our Comments/Summary.
Small sample size. Recruitment strategy not reported. Statistics used did not account for confounding factors; differences in insulin sensitivity between PCOS and control groups could be exaggerated as obesity exacerbates insulin resistance alone.

Study: Li W and Li Q, Dysregulation of glucose metabolism even in Chinese PCOS women with normal glucose tolerance, Endocrine Journal. 2012; 59(9): 765-770.

Description of study: Cross-sectional study	
Patient/population	Women with PCOS Ethnicity: Chinese Women with PCOS were divided into Overweight/obese and lean groups, where overweight/obese was defined as BMI greater ≥24 kg/m^2.
N	PCOS n = 111 (lean n = 78 and overweight/obese n = 33) Control n = 92
Setting	Department of Endocrinology or the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Chongqing Medical University
Intervention/exposure	Euglycaemic hyperinsulinaemic clamp
Comparison/control	Healthy women, all within a healthy weight range <24 kg/m^2
Outcomes	Anthropometric measures: BMI Metabolic measures: Euglycaemic hyperinsulinaemic clamp.

Inclusion Criteria	PCOS diagnosed using the NIH criteria. Fasting plasma glucose (FPG)<6.1mmol/L and postprandial plasma glucose (PPG)<7.8mmol/L. Healthy control women had normal menstrual cycles and no signs of clinical and/or biochemical hyperandrogenism. All subjects were Han. No history of diabetes in first degree relatives.	
Exclusion Criteria	Congenital adrenal hyperplasia, androgen secreting tumours, Cushing's syndrome, and hyperprolactinemia. Use of hormonal medication within the last one month and the use of medication affecting insulin sensitivity in the last three months before the study began.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	The authors have reported that they have nothing to disclose or declare and acknowledge funding sources (p.769)
Does the study have a clearly focused question?	Yes	Aims are clearly stated and justified.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	The study reports detailed inclusion and exclusion criteria (p.766.)
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	All other inclusion/exclusion criteria are appropriate.
Were the cases and controls taken from comparable populations?	Unclear	It is clearly stated that the women with PCOS were recruited after visiting Department of Endocrinology or the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Chongqing Medical University. However, it is unclear whether the healthy control participants were recruited using the same strategy. Recruiting participants seeking treatment for endocrinological, obstetric or gynaecological conditions may results in unintentionally recruiting women with more sever forms of PCOS.
Was case and control status established in a standard, valid and reliable way?	Yes	

Was case and control status established by assessors blind to the exposure?	Not reported	The method of selection of PCOS women was determined through the use of the NIH criteria.
Were the outcomes measured appropriate?	Yes	Measurements seem to be standard, valid and reliable (p. 766). The authors report the intra-assay and inter-assay coefficients for testosterone (11.3 and 13.8% respectively).
Was there sufficient duration of follow-up?	N/A	One month may not be sufficient enough to wash out oral contraceptive medication and their effects on metabolism and insulin.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Yes	Age matched women were selected for the control group.
Was there ≤20% drop-out?	Partial	82% women with PCOS (91 out of 111 participants) and 36% of healthy control women (22 out of 92) completed the euglycaemic-hyperinsulinaemic clamp (p. 766). All other tests were completed.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was powered to detect differences between groups. The authors have not provided power calculations.
Were all individuals included in the analysis?	Yes	The number of individuals who did not complete the insulin clamp was noted in the study.
If statistical analysis was undertaken, was this appropriate?	Yes	Data was log transformed before analysis when it was not normally distributed (p. 767). Comparisons between more than two groups were assessed by a one-way ANOVA. Linear regression analysis was used to evaluate the relationships between outcomes.
Is the paper free of selective outcome reporting?	Yes	All planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	

Results. Compared with lean controls, lean PCOS women had lower M value (7.46±1.84 *vs.* 10.09±2.54 mg/ kg/min, P <0.05), higher HOMA-β index (6.40±0.59 *vs.* 6.05±0.96, P<0.05) but similar HOMA-β index (P >0.05). Overweight/obese PCOS women had both further lower levels of M value (7.46±1.84 *vs.* 10.09±2.54 *vs.* 12.40±1.68 mg/kg/min, P <0.05) and HOMA-β index (49.46±12.16 *vs*. 60.74±10.77 *vs.* 63.34±9.98, P <0.05).
Author's Conclusions Insulin resistance and dysregulation of glucose metabolism were common in Chinese PCOS women with normal glucose tolerance. BMI ≥ 25.5 kg/m^2 indicated impaired β cell function of PCOS women with normal glucose tolerance.
Our Comments/Summary. There is a medium risk of bias due to recruitment. Not all women completed insulin clamps.

Study: Mannerås-Holm L, Leonhardt H, Kullberg J, Jennische E, Odén A, Holm G, Hellström H, Lönn L, Olivecrona G, Stener-Victorin E, Malin Lönn,. Adipose Tissue Has Aberrant Morphology and Function in PCOS: Enlarged Adipocytes and Low Serum Adiponectin, But Not Circulating Sex Steroids, Are Strongly Associated with Insulin Resistance, Journal of Clinical Endocrinology and Metabolism. 2011; 96(2): E304-E311.	
Description of study: Case Control.	
Patient/population	Women with PCOS diagnosed by the Rotterdam criteria; (mean ± SD) age 28.5 ± 3.6, BMI 27.29 ± 7.18 kg/m^2
N	PCOS n = 74 (data provided for n = 31 as participants were matched for age and BMI with women in the control group). Controls n = 31
Setting	The study was conducted at Sahlgrenska Academy, University of Gothenburg.
Intervention/exposure	Euglycaemic hyperinsulainaemic clamp.
Comparison/control	The control participants were matched pairwise to 31 women with PCOS by age (±5 years) and BMI (±2 kg/m^2). The majority of the pairs were matched continuously during enrolment. Six pairs were matched after inclusion of all participants to meet the matching criteria.
Outcomes	Anthropometric: BMI Metabolism: Insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamp.
Inclusion Criteria	Inclusion criteria for women with PCOS were polycystic ovary morphology (12 or more 2- to 9-mm follicles or >10 ml in volume, in at least one ovary) and clinical signs of hyperandrogenism (hirsutism, acne) and/or oligo/amenorrhea. Hirsutism was defined as a Ferriman Gallwey score ≥8. Acne was determined by an affirmative answer to the question *Do you have excessive acne?* Oligomenorrhea was defined as an intermenstrual interval >35 days and <8 menstrual bleedings in the past year. Amenorrhea was defined as absent menstrual bleeding or none in the past 90 days.

Exclusion Criteria	Exclusion criteria for controls were evidence of polycystic ovary morphology, excessive acne or hirsutism, or menstrual irregularities (cycles >35 days). Exclusion criteria for all women were age <18 or >37 years, pharmacological treatment within 12 weeks (including hormonal contraceptives, naturopathic preparations, and homeopathic substances), breast feeding within 24 weeks, cardiovascular disease, diabetes mellitus, or other endocrine disorders (*e.g.*, congenital adrenal hyperplasia, Cushing's syndrome, or androgen-secreting tumours).	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	
Does the study have a clearly focused question?	Yes	Authors report that they have no conflict of interests and acknowledge funding sources (p. E310)
Is a case control study the appropriate method to answer this question?	Yes	The research question is clearly stated and focused.
Does the study have specified inclusion/exclusion criteria?	Yes	Inclusion and exclusion criteria are clearly defined (p. E305).
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	
Were the cases and controls taken from comparable populations?	Yes	Participants were recruited from the community through advertisements and frequent visits to various places. Participants were also matched for age and BMI.
Was case and control status established in a standard, valid and reliable way?	Yes	The authors have relevant criteria for detecting PCOS and ensuring the comparison group are healthy women with no signs or symptoms of PCOS. Potential participants were asked to describe their medical history and underwent a gynecological examination and two-dimensional transvaginal ultrasonography to investigate ovarian morphology. Women allocated to the control group did not have any signs and symptoms of PCOS.
Was case and control status established by assessors blind to the exposure?	Not reported	

Were the outcomes measured appropriate?	Yes	Outcome measurements were important to answer the research question and where standard, reliable and valid (p.E305-E306).
Was there sufficient duration of follow-up?	Yes	Washout period for medications were adequate to ensure endpoint measurements were not affected.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Yes	31 controls were matched pairwise to 31 women with PCOS. Matching was performed based on BMI and age.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Partial	The matching of groups based on age and BMI resulted in a nonnegative correlation between the value of the women with PCOS, on any variable, and the corresponding value of her control. Thus, the difference between women with PCOS and controls could be analyzed with a nonpaired instead of a paired test. A nonpaired test provides higher statistical power if the matching variables are of small importance (resulting in a low correlation between PCOS women and controls) and if the number of individuals missing her match is large enough. However, analysis of confidence intervals for the two options revealed that the paired test was more powerful in almost all cases. Thus, for pairwise comparisons between the BMI- and age- matched cases and controls, paired *t* tests were used (p. E306). However, sample size calculation and power calculations were not reported.
Were all individuals included in the analysis?	No	Only pairwise matched participants were included in characteristics analysis, therefore 43 women with PCOS were not included in this analysis. However, all women with PCOS were included in analysis investigating determinants of insulin sensitivity in this population. The authors report missing data for all outcomes measured. 32 of 74 women with PCOS (including four in matched pairs) and one control did not undergo clamp evaluations due to logistical reasons.

If statistical analysis was undertaken, was this appropriate?	Yes	Authors report in detail the analysis conducted and justifications for statistics chosen.
Is the paper free of selective outcome reporting?	Yes	All outcomes were measured and reported. Any missing data from control and PCOS groups was also noted.
Other		
What is the overall risk of bias?	Low	

Results.
Comparison of 31 pairs revealed lower insulin sensitivity, hyperandrogenemia, and higher free 17β-estradiol in PCOS. Abdominal adipose tissue volumes/distribution did not differ in the groups, but PCOS women had higher waist-to-hip ratio, enlarged adipocytes and reduced adiponectin. In regression analysis, adipocyte size, adiponectin, and waist circumference were the factors most strongly associated with insulin sensitivity in PCOS ($R^2 = 0.681$, $P < 0.001$).

Author's Conclusions.
In PCOS, adipose tissue has aberrant morphology/function. Increased waist-to-hip ratio indicates abdominal/visceral fat accumulation, but this is not supported by MRI. Enlarged adipocytes and reduced serum adiponectin, together with a large waistline, rather than androgen excess, may be central factors in the pathogenesis/maintenance of insulin resistance in PCOS.

Our Comments/Summary.
There is a low risk of bias in this study. Authors have used sound techniques to measure outcomes variables and have noted missing data points. A detailed explanation regarding recruitment, inclusion/exclusion criteria and statistical analysis was provided.

Study: Micic D, Sumarac-Dumanovic M, Kendereski A, Cvijovic G, Zoric S, Pejkovic D, Micic J, Milic N, Dieguez C Cananueva F.F.,Total Ghrelin levels during acute insulin infusion in patients with polycystic ovary syndrome, Journal of Endocrinological Investigation. 2007; 30 (10): 820-827.

Description of study: Case Control

Patient/population	Overweight (>25 kg/m^2) and lean (<25 kg/m2) women with PCOS, diagnosed based on the Rotterdam criteria.
N	Lean PCOS n=8 Lean Controls n=8 Overweight and obese PCOS n=8 Overweight and obese controls n=8
Setting	Not reported.
Intervention/exposure	N/A
Comparison/control	Overweight (>25 kg/m^2) and lean (<25 kg/m2) healthy women acted as controls.
Outcomes	Anthropometric: BMI Metabolism: Euglycaemic-hyperinsulinaemic clamp.
Inclusion Criteria	Control participants had regular menstrual cycles and showed no signs and symptoms of hyperandrogenism.

Exclusion Criteria	Thyroid, renal, liver, or cardiovascular dysfunctions, previous gastrointestinal surgery and type 2 diabetes, Cushing's syndrome and congenital adrenal hyperplasia. Taking medication 3 months prior to the study.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	Authors acknowledge support received (p. 826) but do not disclose any conflicts of interest.
Does the study have a clearly focused question?	Yes	Research questions and rational are clearly stated.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	Inclusion/exclusion criteria are stated for both the PCOS and control group (p. 821).
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Eligibility criteria used to specify women with PCOS and controls were appropriate.
Were the cases and controls taken from comparable populations?	Not reported	
Was case and control status established in a standard, valid and reliable way?	Yes	Women with PCOS were identified through the use of the Rotterdam criteria. Healthy control women did not present with any of the symptoms relating to PCOS – hyperandrogenism and irregular cycles.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes measured were appropriate.
Was there sufficient duration of follow-up?	Yes	All participants were without medication for at least 3 months before the study. Participants were not on calorie restriction diets or undertaking regular exercise.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	

Were the groups comparable with regards to key prognostic variables?	Yes	Groups were similar in regards to key characteristic criteria.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size and power calculations were not reported.
Were all individuals included in the analysis?	Not reported	The number of participants who were excluded from the study, refused to participant, or did not complete the study was not reported.
If statistical analysis was undertaken, was this appropriate?	Yes	Data was assessed for normality by the Kolmogorov-Smirnov test. Relationships between variables were assessed by Pearsons' and Spearman's correlations tests. ANOVA was used to assess differences in means.
Is the paper free of selective outcome reporting?	Yes	All planned outcomes were measured and reported.
Other	During the data extraction process it was noted that some outcomes were reported as means and standard deviation instead of means and standard error of means as indicated in the methods section.	
What is the overall risk of bias?	Moderate	

Results.
Fasting ghrelin was significantly lower in non-obese PCOS than in controls (64.74+/-25.69 vs 108.36+/-52.60; $p<0.05$) as well as in overweight and obese PCOS in comparison with controls (38.71+/-14.18 vs 98.77+/-40.49; $p<0.05$). Insulin infusion significantly suppressed ghrelin in all subgroups of investigated women. Analysis of variance for repeatable measures confirmed that there was no significant difference in pattern of response between PCOS and controls.

Author's Conclusions.
Women with PCOS had lower fasting ghrelin and decreased insulin sensitivity independently of their BMI, compared to the controls. In addition, there were no differences between fasting ghrelin levels among non-obese, overweight, and obese women with PCOS. During euglycemic hyperinsulinemic clamp, ghrelin decreased in all studied groups to a similar extent, implying that, compared to chronic hyperinsulinemia, acute hyperinsulinemia reduces ghrelin levels independently of the degree of insulin resistance.

Our Comments/Summary.
There is a moderate risk of bias. We believe an error was made when data for the insulin clamp was reported – Mean and SEM are noted in the methods however, it seems that SD's were reported instead for M index, which is a key outcome.

Study:	Moret M, Stettler R, Rodieux F, Gaillard R.C, · Waeber G, Wirthner D. Giusti V, Tappy L, Pralong F.P., Insulin Modulation of Luteinizing Hormone Secretion in Normal Female Volunteers and Lean Polycystic Ovary Syndrome Patients, Neuroendocrinology. 2009; 89: 131-139.

Description of study:	
Patient/population	Women with PCOS (BMI <30 kg/m^2) diagnosed using the Rotterdam criteria; (mean ± SEM) age 24.4 ± 1.86, BMI 23.04 ± 1.92.
N	Lean women with PCOS n = 5 Lean healthy Control women n = 5
Setting	Outpatient gynaecological endocrinology clinic of the Lausanne University Hospital.
Intervention/exposure	Euglycaemic hyperinsulanaemic clamp
Comparison/control	Lean healthy control women; age 21.2 ± 0.86, BMI 21.28 ± 0.43.
Outcomes	Anthropomertric: BMI Metabolic: Insulin sensitivity
Inclusion Criteria	Women diagnosed with PCOS based on the Rotterdam criteria.
Exclusion Criteria	Control women; irregular menstrual cycles, personal or family history of endocrine pathology or taking oral contraceptive, hormonal or non-hormonal medication.

Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	The authors acknowledge funding support from the Swiss National Science Foundation. No conflicts of interest were declared.
Does the study have a clearly focused question?	Yes	A clear research question was reported and the population studied, outcomes measured and comparisons between groups were appropriate.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	Inclusion/exclusion criteria have been reported and are able to discriminate between PCOS and controls.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Inclusion/exclusion criteria have been reported, however it is unclear whether women in the PCOS group were excluded if they were taking medication that affected outcome measures (e.g., metabolism). It is also unclear if women in the control group were recruited with a BMI <30 kg/m^2.

Were the cases and controls taken from comparable populations?	Partial	Women with PCOS were recruited from a gynaecological endocrinological outpatient clinic. This may unintentionally result in recruiting women with more severe symptoms of PCOS, which may confound results and be unrepresentative of the whole population. It is unclear how the control group was recruited.
Was case and control status established in a standard, valid and reliable way?	Yes	The Rotterdam criteria were used to discriminate between PCOS and control women.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes measured were appropriate to answer the research question.
Was there sufficient duration of follow-up?	Not reported	It is stated that participants were not taking medication that could affect key outcomes. However, it is not reported if participants stopped taking medication in order to participate in the study and if they underwent a washout period.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who were excluded from or who refused to participate in the study was not reported.
Were the groups comparable with regards to key prognostic variables?	Yes	Participants had comparable characteristic criteria.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Adequate sample size calculations and power analysis for key outcomes were not calculated.
Were all individuals included in the analysis?	Yes	All individuals reported in the study were included in statistical analysis.
If statistical analysis was undertaken, was this appropriate?	Yes	All data was log transformed prior to statistical analysis using Student's t tests.
Is the paper free of selective outcome reporting?	Yes	All outcomes measured have been reported.
Other		

What is the overall risk of bias?	Moderate	

Results.
Baseline LH secretion in PCOS subjects was significantly different from controls: they had higher LH levels, higher LH/FSH ratios as well as a faster LH pulse frequency than normal women. Insulin administration did not affect the pattern of LH secretion of PCOS patients, whereas it significantly increased the LH pulse frequency while decreasing the LH interpulse intervals in the controls.

Author's Conclusions.
These data confirm that an abnormal pattern of LH secretion characteristic of PCOS can be observed in lean patients, and appears independent of peripheral insulin levels. Furthermore, our results in lean controls provide the first direct evidence that peripheral insulin can modulate the activity of hypothalamic gonadotropin-releasing hormone (GnRH) neurons in the human.

Our Comments/Summary. Difficult to make conclusions without caution due to the limited sample size. Women with PCOS were recruited from an outpatient clinic seeking treatment for endocrinological or gynaecological concerns. This may indicate selection bias as more severe symptoms of PCOS may be represented in this population.

Study: Laure C. Morin-Papunen, Ilkka Vauhkonen, Riitta M. Koivunen, Aimo Ruokonen and Juha S. Tapanainen,. Insulin sensitivity, insulin secretion, and metabolic and hormonal parameters in healthy women and women with polycystic ovarian syndrome Human Reproduction. 2000; 15(6): 1266-1274.

Description of study:	
Patient/population	Women with PCOS diagnosed by criteria equivalent to the Rotterdam criteria where divided into groups based on BMI status (obesity defined as BMI >27 kg/m^2). Obese PCOS; (Mean ± SE), age 30.1 ± 0.9 years, BMI 34.5 ± 1.03 kg/m^2. Lean PCOS; age 28.9 ± 1.2 years, BMI 22.9 ± 0.3 kg/m^2
N	Lean Control n= 17 Lean PCOS n= 15 Overweight Control n= 17 Overweight PCOS n= 28
Setting	Women with PCOS participated in the study following referral to the Reproductive Endocrinology Unit, University Hospital of Oulu.
Intervention/exposure	Euglycaemic hyperinsulinaemic clamp
Comparison/control	Lean control women; age 37.1 ± 0.8 years, BMI 22.7 ± 0.5 kg/m^2 Obese control women; age 35.1 ± 1.2 years, BMI 31.8 ± 1.15 kg/m^2
Outcomes	Anthropometric: BMI Metabolic: insulin sensitivity
Inclusion Criteria	Control women with normal menstrual cyclicity (27–34 days) and normal ovaries as observed in transvaginal ultrasonography. PCOS women were included in the study if they met criteria equivalent to the Rotterdam criteria.

Exclusion Criteria	Diabetics, smokers, alcohol users and those using sex hormones or other medication known to affect lipoprotein metabolism during the 2 months preceding the study were excluded. Late onset adrenal hyperplasia in PCOS subjects was excluded on the basis of a normal serum 17-hydroxyprogesterone concentration (17-OHP <9 nmol/l).	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Partial	Conflicts of interest have not been reported, however funding sources have been acknowledged.
Does the study have a clearly focused question?	Yes	A clear research question with appropriate population sampled and outcomes measured have been used in this study.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	Inclusion/exclusion criteria are clearly stated and are able to discriminate between PCOS women and controls (p. 1266-1267).
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Inclusion/exclusion criteria are appropriate.
Were the cases and controls taken from comparable populations?	Partial	Control women were contacted through an advertisement in a local newspaper and recruited after a phone conversation. Women with PCOS were recruited following referral to the Reproductive Endocrinology Unit, University Hospital of Oulu. Women seeking treatment for reproductive and endocrinological concerns may have the more sever phenotype of PCOS. Some of the participants had participated in other studies on glucose metabolism, and their data were analysed retrospectively.
Was case and control status established in a standard, valid and reliable way?	Yes	The inclusion/exclusion criteria were able to distinguish between PCOS and control women.
Was case and control status established by assessors blind to the exposure?	Not reported	

Were the outcomes measured appropriate?	Yes	Outcomes measured were appropriate to answer the research question. Intra and inter-assay coefficients of variation were 1.3 and 1.5% for SHBG respectively, 4.9 and 6.5% for LH, 3.8 and 4.3% for FSH and 5.6% for testosterone (p. 1268).
Was there sufficient duration of follow-up?	Not reported	It is noted that participants did not use any medication. However, whether participants were previously taking mediation that affects endpoint measures is unclear.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who refused to participate in the study not reported.
Were the groups comparable with regards to key prognostic variables?	Yes	Key baseline characteristics were comparable between groups.
Was there ≤20% drop-out?	Not reported	Dropout rates are not reported.
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size and power calculations for important outcome variables have not been reported.
Were all individuals included in the analysis?	Not reported	The number of missing data points or any participants not included in the analysis has not been reported. However, the table of results indicates that all participants reported in the study were included in analysis.
If statistical analysis was undertaken, was this appropriate?	Yes	Authors report that student's two-tailed t-test was used for comparison of normally distributed variables, with or without log transformation. The Mann–Whitney U-test was used for variables with a persisting skewed distribution after log transformation. A linear regression method was used to identify the influence of age on variables in the control and PCOS groups. If the level of significance was <0.05, covariance analysis was carried out to evaluate the impact of this variable on the results (p. 1268).
Is the paper free of selective outcome reporting?	Yes	All planned and measured outcomes have been reported.
Other		
What is the overall risk of bias?	Moderate	

Results.
The women with PCOS had a higher mean WHR than the controls. A trend towards hyperinsulinaemia and impairment of insulin sensitivity was observed in lean women with PCOS, but only in obese PCOS participants were these changes significant. Early phase insulin secretion but not the early phase C-peptide secretion was increased in women with PCOS compared to controls, suggesting that peripheral hyperinsulinaemia in PCOS women was mainly due to the observed lowered hepatic insulin extraction and insulin resistance in skeletal muscle. Moreover, the presence of a family history of type 2 diabetes did not affect early phase insulin or C-peptide secretion in the PCOS group.

Author's Conclusions.
Authors conclude that a marked impairment of insulin sensitivity in obese women with PCOS was observed. There was also a tendency towards decreased insulin sensitivity in lean women with PCOS subjects, but only in obese women did these changes become statistically significant, suggesting that obesity, and particularly abdominal obesity, is an important contributor to the development of insulin resistance in PCOS. Whether the hyperinsulinaemia of these patients is secondary to a primary impairment of insulin action, to primarily increased abdominal obesity, or to an initial defect in β-cell function, could not be solved by this study and needs further investigation.

Our Comments/Summary.
There appears to be selection bias in the PCOS group.

Study: Nikolajuk A, Kowalska I, Karczewska-Kupczewska M, Adamska A, Otziomek E, Wolczynski S, Kinalska I, Gorska M, Straczkowski M., Serum soluble glycoprotein 130 concentration is inversely related to insulin sensitivity in women with polycystic ovary syndrome, Diabetes. 2010; 59: 1026-1029.	
Description of study:	
Patient/population	Lean PCOS; (mean ± SD) age 24.11 ± 3.94 years, BMI 21.71 ± 1.81 kg/m^2 Overweight PCOS; age 25.6 ± 5.57 years, BMI 31.46 ± 4.34 kg/m^2 Obesity defined by BMI >25 kg/m^2 PCOS diagnosed using the Rotterdam criteria.
N	Lean Control n = 18 Lean PCOS n = 35 Overweight control n = 16 Overweight/Obese PCOS n = 43
Setting	Participants with PCOS and obese control participants were recruited from Outpatient Endocrinology and Gynaecology Clinics. Lean control participants were mainly medical students and staff (reported elsewhere).
Intervention/exposure	Euglycaemic hyperinsulinaemic clamp
Comparison/control	Lean women; age 26.33 ± 5.56 years, BMI 22.19 ± 1.92 Overweight women; age 27.44 ± 5.27 years, BMI 30.66 ± 4.37
Outcomes	Anthropometry: BMI Metabolism: Euglycaemic-Hyperinsuliaemic clamp.
Inclusion Criteria	Controls were healthy with regular menstrual cycles.
Exclusion Criteria	Morbid Obesity (BMI >40 kg/m^2). Cardiovascular disease, hypertension, infections or other serious medical problems, smoking and taking anti-inflammatory medication or medication know to affect glucose and lipid metabolism.

Study Validity.

Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Authors acknowledge funding sources and 'no potential conflicts of interest relevant to this article were reported' (p. 1029).
Does the study have a clearly focused question?	Yes	The research questions in clearly stated and appropriate populations and outcomes were studied.
Is a case control study the appropriate method to answer this question?	Yes	

Does the study have specified inclusion/exclusion criteria?	Partial	It is reported that 'none of the women had morbid obesity (BMI >40 kg/m^2), cardiovascular disease, hypertension, infections, or other serious medical problems; all were non-smokers, and they were not taking any anti-inflammatory drugs and drugs known to affect glucose and lipid metabolism' (p. 1027). It is unclear whether inclusion/exclusion criteria were determined *a priori*.
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Inclusion/exclusion criteria used were appropriate to answer the research question and to discriminate between women with PCOS and controls.
Were the cases and controls taken from comparable populations?	Partial	Women with PCOS and participants who were overweight/obese were recruited through Outpatient Endocrinology and Gynaecology Clinics. Bias may exist with this recruitment strategy as women seeking treatment may have more severe symptoms of PCOS and obesity.
Was case and control status established in a standard, valid and reliable way?	Yes	Inclusion and exclusion criteria ensured that cases and controls were allocated to appropriate groups during the screening process.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes were measured appropriately and important to answer study aims
Was there sufficient duration of follow-up?	Partial	Participants were not taking anti-inflammatory drugs or drugs known to affect glucose and lipid metabolism. It is unclear whether participants ceased medication prior to commencing the study.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who were excluded from the study or who did not complete the study was not reported
Were the groups comparable with regards to key prognostic variables?	Yes	The groups were comparable for age and divided based on PCOS status and BMI.
Was there ≤20% drop-out?	Not reported	

Was the study sufficiently powered to detect any differences between the groups?	Not reported	It is unclear whether the study was sufficiently powered to detect differences between groups. Sample size and power calculations were not reported.
Were all individuals included in the analysis?	Not reported	Exclusion or dropout rates have not been reported.
If statistical analysis was undertaken, was this appropriate?	Yes	Statistical analysis performed is deemed appropriate. Variables were log-transformed if they were not normally distributed. The differences between groups were estimated with factorial ANOVA, with PCOS status and obesity as categorical factors and the studied parameters as dependent variables. The relationships between variables were studied with the Pearson product-moment correlation analysis and with multiple regression analysis (p. 1826).
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
What is the overall risk of bias?	Moderate	

Results.
Both obesity and PCOS were characterized by an increased sgp130 (P <0.0001 and P = 0.0002, respectively). sIL-6R concentration was lower (P = 0.0036) in women with PCOS independently of obesity. Serum sgp130 was negatively correlated with insulin sensitivity when control and PCOS women were analysed together (r = −0.36, P <0.0001) and in the PCOS subjects separately (r = −0.34, P = 0.002). In multiple regression analysis, this correlation was significant after adjustment for BMI, waist, percent of body fat, postload glucose and insulin, triglycerides, and high-sensitive C-reactive protein.

Author's Conclusions.
Serum sgp130 is inversely and independently associated with insulin sensitivity in women with PCOS. An increased serum sgp130 in obesity and PCOS suggests an inhibition of intracellular gp130 signaling in insulin-resistant conditions.

Our Comments/Summary.
There is a medium risk of bias due to recruitment method. It is unclear whether inclusion/exclusion criteria a priori decision.

Study: **Oh JY, Lee JA, Lee H, Oh JE, Sung YA and Chung H., Serum C-reative protein levels in normal-weight polycystic ovary syndrome, Korean J International Medicine. 2009; 24:350-355.**	
Description of study:	
Patient/population	Lean women with PCOS; (mean ± SD) age 25 ± 1 years, BMI 20.8 ± 0.2 kg/m^2 Lean defined as a BMI <23 kg/m^2 PCOS diagnosed by the NIH criteria.

N	Lean PCOS n = 39 Healthy Controls n = 24	
Setting	Not reported.	
Intervention/exposure	Euglycaemic hyperinsuliaemic clamp	
Comparison/control	Lean women; age 26 ± 1 years, BMI 19.9 ± 0.3	
Outcomes	Anthropometry: BMI Metabolic: Insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamp.	
Inclusion Criteria	Lean women, BMI <23 kg/m^2). The degree of obesity was classified according to the Asia-Pacific perspective. Women diagnosed with PCOS using the NIH criteria.	
Exclusion Criteria	Control women with a family history of diabetes or PCOS. Specific disorders such as adult-onset congenital adrenal hyperplasia, hyperprolactinemia, and androgen-secreting neoplasia.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	A funding source for the study has been reported (p. 354), however, no conflict of interest statement is provided.
Does the study have a clearly focused question?	Yes	The study has a clear research question and has included appropriate participants and outcome measures.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	The study details criteria relating to BMI and PCOS diagnosis, however it is unclear if they were an *a priori* decision.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Inclusion/exclusion criteria detailed in the study were appropriate to answer key research questions. However, authors have not reported whether participants ceased medication effecting important outcomes measures.
Were the cases and controls taken from comparable populations?	Not reported	The method of recruitment is not reported. It is unclear if participants were selected from comparable populations.
Was case and control status established in a standard, valid and reliable way?	Yes	The NIH criteria were used to establish women with PCOS.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	Outcomes measured were appropriate.

Was there sufficient duration of follow-up?	Not reported	It is not known if participants were taking medication that may affect key endpoint measures.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of women who declined to participate in the study has not been reported.
Were the groups comparable with regards to key prognostic variables?	Partial	Groups were comparable in age but the PCOS group has a higher BMI compared to the control group.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size and power calculations have not been provided.
Were all individuals included in the analysis?	Not reported	Missing data points or excluded participants were not reported. However, it seems likely that all recruited participants were included in statistical analysis.
If statistical analysis was undertaken, was this appropriate?	Yes	Data showing a skewed distribution were logarithmically transformed prior to statistical analysis. Data are expressed as the mean± standard deviation, unless otherwise stated. An unpaired *t*-test was used for comparison of continuous variables between PCOS and control groups. A multivariate general linear model was applied for comparisons between two groups after adjusting for specific variables. Linear correlations were examined using Pearson's correlation. A multiple regression analysis was performed to determine which variables predict serum hsCRP levels (p. 351-352).
Is the paper free of selective outcome reporting?	Partial	It is reported in the methods section that LH and FSH were analysed, however these outcomes are not reported in the results section either in text or tabulated.
Other		
What is the overall risk of bias?	High	

Results. Serum hsCRP concentrations were higher in women with PCOS than in healthy controls. However, this difference was no longer significant after adjusting for body mass index (BMI). hsCRP levels were correlated with waist circumference (r=0.46, p<0.01), BMI (r=0.46, p<0.01), visceral fat area (r=0.45, p<0.01), and systolic (r=0.42, p<0.05) and diastolic blood pressure (r=0.39, p<0.05). hsCRP also tended to be negatively associated with insulin-mediated glucose uptake (IMGU) (r=-0.31, p=0.07). A multiple regression analysis revealed that BMI (β=0.29, p<0.05), systolic blood pressure (β=0.39, p<0.01), and IMGU (β=-0.31, p<0.05) predicted serum hsCRP levels in women with PCOS.
Author's Conclusions. PCOS by itself does not seem to be associated with increased hsCRP levels, whereas known CVD risk factors affect serum hsCRP levels in PCOS.
Our Comments/Summary. There is a high risk of bias. Recruitment strategy is not described and selective outcome reporting exists.

Study: Ovesen P, Moller J, Ingerslev H J, Jørgensen J O, Mengel A, Schmitz O, Alberti KG and Moller N,. Normal basal and insulin-stimulated fuel metabolism in lean women with the polycystic ovary syndrome, Journal of Clinical Endocrinology and Metabolism. 1993; 77: 1636-1640.		
Description of study:		
Patient/population	Lean women with PCOS; age, 27.1 ± 2.0 years, BMI 22.2 ± 0.78 kg/m^2 PCOS diagnosed by criteria equivalent to the Rotterdam criteria.	
N	PCOS n = 7 Control n = 7	
Setting	Not reported.	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	Lean healthy women; age 25.7 ± 1.4 years; BMI 21.3 ± 0.69 kg/m^2	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Control participants with regular menses and normal androgen levels.	
Exclusion Criteria	Taking hormonal medications at least 6 weeks prior to the study.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	It is unclear whether there are any conflicts of interest as they are not reported.
Does the study have a clearly focused question?	Yes	The research question has been reported and the population and outcomes studied are appropriate to answer the research question.

Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	The study details inclusion/exclusion criteria but it is unclear whether they were defined *a priori*.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Participants were not taking hormonal medication for at least 6 weeks prior to the study. However, 6 weeks may not be sufficient time to eliminate the effects of medication on key outcomes measures including insulin sensitivity.
Were the cases and controls taken from comparable populations?	Not reported	Recruitment methods were not reported; therefore conclusions regarding population sampled cannot be established. It is reported in the acknowledgements that some participants were enrolled in the study based on referral from doctors. These women may have a more severe form of PCOS as they are seeking treatment.
Was case and control status established in a standard, valid and reliable way?	Yes	Criteria equivalent to the Rotterdam criteria were used to diagnosed women with PCOS. These criteria are sufficient to distinguish between women with PCOS and controls.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	The outcomes measured are appropriate to answer the research question.
Was there sufficient duration of follow-up?	No	Participants ceased medication that may affect key endpoints 6 weeks before study commencement. This may not be sufficient time for the body to return to normal homeostatic conditions.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	No	
Were the groups comparable with regards to key prognostic variables?	Yes	The groups were comparable for age, BMI and free fat mass.
Was there ≤20% drop-out?	Not reported	

Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size and power calculations were not provided. These calculations would be beneficial due to the small sample size.
Were all individuals included in the analysis?	Not reported	
If statistical analysis was undertaken, was this appropriate?	Yes	An unpaired t-test was used to compare group means (p. 1637).
Is the paper free of selective outcome reporting?	Yes	All planned outcomes have been measured and reported.
Other		
What is the overall risk of bias?	High	

Results.
In the basal state, comparable metabolic indices were recorded: serum insulin, 35.9 ± 7.7 (PCOS) vs. 37.3 ± 2.87 pmol/L (controls); plasma C-peptide, 364.1 ± 66.2 vs. 397.2 ± 66.2 pmol/L; plasma glucose, 4.95 ± 0.09 vs. 4.77 ± 0.09 mmol/L; forearm arterio-venous difference in glucose, 0.17 ± 0.04 vs. 0.15 ± 0.07 mmol/L; isotopically determined endogenous glucose production, 1.9 ± 0.1 vs. 2.0 ± 0.1 mg/kg.min; and serum nonesterified fatty acids, 545 +/- 40 vs. 617 ± 54 mumol/L (all P > 0.05). During the clamp endogenous glucose production was similar (-0.9 ± 0.1 vs. -0.5 ± 0.2 mg/kg.min; amount of exogenous glucose necessary to maintain euglycemia, 4.0 ± 0.4 vs. 3.8 ± 0.5 mg/kg.min.
Author's Conclusions.
By showing normal basal and insulin-stimulated substrate metabolism in lean hyperandrogenemic PCOS patients, these data suggest that insulin resistance may be an epiphenomenon, rather than a primary feature of PCOS.
Our Comments/Summary
There is a high risk of bias due to small sample size, insufficient washout period for medications and recruitment strategy not reported.

Study: Park KH, Kim JY, Ahn CW, Song YD, Lim SK, Lee HC ,. Polycystic ovary syndrome (PCOS) and insulin resistance, International Journal of Gynaecology and Obstetrics. 2001; 74: 261-267.	
Description of study:	
Patient/population	Women with PCOS; (mean ± SEM) age 25.0 ± 4.1 years, BMI 26.0 ± 3.1 kg/m^2 PCOS diagnosed by the NIH criteria.
N	PCOS n = 9 Obese Type 2 Diabetes n = 6 (not used in meta-analysis). Control n = 5
Setting	Clinic of the Severance Hospital.
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp

Comparison/control	Control group; age 31.0 ± 5.1 years, BMI 25.6 ± 2.40 kg/m^2
Outcomes	Anthropometric: BMI Nonfertility: Testosterone, LH, FSH, SHGB Metabolic: insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamp
Inclusion Criteria	PCOS, without glucose intolerance or diabetes confirmed by the 75-g oral glucose tolerance test (OGTT)
Exclusion Criteria	Control: Diabetic

Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	It is unclear whether a conflict of interest is present. Funding sources and affiliations are not reported.
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcome measures have been included.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	The study reports inclusion/exclusion criteria rating to PCOS diagnosis, however criteria are not specific in other areas; for example the age and BMI range is not reported and whether participants were taking mediation that affects insulin sensitivity during the study.
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	The inclusion/exclusion criteria were appropriate to discriminate between control and PCOS groups, however reporting of criteria needed to be more specific and detailed.
Were the cases and controls taken from comparable populations?	Not reported	Recruitment strategies are not reported.
Was case and control status established in a standard, valid and reliable way?	Partial	The NIH criteria were used to discriminate between women with PCOS and controls. An oral glucose tolerance test or fasting glucose sample was used to assess glucose intolerance/diabetes. The oral glucose tolerance test was only used in the PCOS group.
Was case and control status established by assessors blind to the exposure?	Not reported	

Were the outcomes measured appropriate?	Yes	The outcomes measured were appropriate to answer the research questions. The insulin clamp is the gold standard method for assessing insulin sensitivity. The coefficient of variation (CV) of blood glucose during the last 60 min was less than 3% in all cases.
Was there sufficient duration of follow-up?	Not reported	
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Partially	The insulin sensitivity of the body fat matched controls, obese type 2 diabetes, and the PCOS group were compared (p. 262). The PCOS group was significantly older than the other groups.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power analysis calculations have not been provided. The small sample size may bias results.
Were all individuals included in the analysis?	Not reported	It is unclear if there were any missing data points or if participants did not complete the study.
If statistical analysis was undertaken, was this appropriate?	No	A t-test or X^2 test was used to compare variables between the three groups. An ANOVA or ANCOVA (age) may have been more appropriate to compare three groups as it will minimise type 1 errors. Covarying for age would also account for the significant different in age between groups.
Is the paper free of selective outcome reporting?	Yes	All outcomes that were mentioned in the methods were analysed and reported in the results.
Other		
What is the overall risk of bias?	High	

Results.
Results are reported on page 263-265. Women with PCOS showed significantly elevated insulin responses during OGTT, but their blood glucose levels were comparable with the controls. The subjects with PCOS had more insulin resistance than the other groups. There was no difference among the groups in terms of clinical characteristics and metabolic profiles, except age, LH, testosterone, and SHBG.

Author's Conclusions. Authors conclude that PCOS women have significant insulin resistance which is independent of adiposity.
Our Comments/Summary. Moderate to high. Small sample size, t test instead of ANOVA or ANCOVA. Insufficient information about recruitment and specific inclusion and exclusion criteria.

Study: Park KH, Choi Y, Lee HJ, Oh JY, Hong YS, Sung YA., Phenotypic characteristics according to insulin sensitivity in non-obese Korean women with polycystic ovary syndrome, Diabetes Research and Clinical Practice. 2007; 77s: s233-s237.		
Description of study:		
Patient/population	Lean women with PCOS; age 24.7 ± 3.9 y, BMI 20.4 ± 1.5 kg/m^2 PCOS diagnosed using the NIH criteria. Lean defines as a BMI <23 kg/m^2, the Asia-Pacific perspective.	
N	Lean PCOS n = 73 Lean Control n = 34	
Setting	Division of Endocrinology, Department of Internal Medicine, Ewha Woman's University College of Medicine, Seoul, Korea	
Intervention/exposure	Euglycaemic hyperinsulinaemic clamp	
Comparison/control	Normal control women with regular menstrual cycles. Age 25.9 ± 3.3 y; BMI 20.9 ± 3.2 kg/m^2	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Women with PCOS meeting the NIH criteria for diagnosis of PCOS. BMI <23 kg/m^2	
Exclusion Criteria	Diabetes. Controls: Family history of diabetes or PCOS.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	Statements detailing any funding sources or conflicts of interests have not been reported.
Does the study have a clearly focused question?	Yes	The research questions is focused and justified. The appropriate populations and outcomes have been investigated.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	Specific inclusion/exclusion criteria have been detailed (p. S234). However, it is unclear whether they were established prior to recruitment.

If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	The eligibility criteria used were appropriate for the population. However, information regarding the recruitment age of participants and medication taken is not provided.
Were the cases and controls taken from comparable populations?	Not reported	It is unclear of the recruitment method for women with PCOS and controls. Recruitment of participants from hospitals instead of the general population may bias results.
Was case and control status established in a standard, valid and reliable way?	Yes	Women with PCOS were diagnosed using the NIH criteria. Furthermore, women in the control group did not meet the NIH criteria nor did they have a family history of PCOS or diabetes.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Not reported	It is not reported if participants ceased medication affecting important outcomes before beginning the study. A sufficient period of time would be required to 'washout' the effects of medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	There is a large difference between the numbers of women in each group. It is not known if this is due to difficulty in recruiting or participants refusal to participate.
Were the groups comparable with regards to key prognostic variables?	Yes	Groups were comparable in regards to key characteristics including age and BMI.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size and power calculations were not reported.
Were all individuals included in the analysis?	Not reported	
If statistical analysis was undertaken, was this appropriate?	Not reported	Results are presented with p-values, however the methods do not detail what statistical analysis was performed. It is also not reported if data is reported as means ± SD or means ± SEM
Is the paper free of selective outcome reporting?	Yes	All planned outcomes were measured and reported.

Other		
What is the overall risk of bias?	Not enough information	

Results.
The fasting plasma glucose ($p < 0.01$) and post-glucose load plasma insulin ($p < 0.01$) of women with PCOS were higher than those of controls. Glucose disposal rate (*M*-value) was lower in women with PCOS compared to controls ($p < 0.05$). Insulin resistant (IR) and insulin sensitive (IS) PCOS were divided by the *M*-value of 25-percentile (5.5 mg/kg min) in controls. Between IR and IS groups, DHEAS ($p < 0.01$), post-glucose load plasma insulin ($p < 0.05$) showed differences after the adjustment for BMI.

Author's Conclusions.
Our non-obese women with PCOS showed significant insulin resistance compared to their age and BMI comparable control subjects and their insulin resistance may contribute to hyperandrogenism especially via adrenal androgen overproduction.

Our Comments/Summary.
It is difficult to determine risk of bias as not enough information has been provided on methodological quality to be able to determine risk of bias. The population sampled and recruitment strategy was not reported and therefore it is difficult to make generalisations. Statistical analysis was performed but not reported and authors do not report funding sources or conflicts of interest.

Study: Patel K, Coffler MS, Dahan MH, Yoo RY, Lawson MA, Malcom PJ, RJ Chang,. Increased Luteinizing Hormone Secretion in Women with Polycystic Ovary Syndrome Is Unaltered by Prolonged Insulin Infusion, Journal of Clinical Endocrinology and Metabolism. 2003; 88(11): 5456-5461.

Description of study:	
Patient/population	Women with PCOS; (mean ± SEM) age 28.6 ± 0.6 y, BMI 35.3 ± 0.8 kg/m^2. PCOS diagnosed using the Rotterdam criteria.
N	PCOS n = 11 Control n = 9
Setting	General Clinical Research Center at the University of California, San Diego.
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp
Comparison/control	Women with regular menstrual cycles were recruited into the control group; Age 26.3 ± 0.8 y, BMI 27.4 ± 0.7 kg/m^2.
Outcomes	Anthropometric: BMI Nonfertility: Testosterone, LH, FSH. Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp
Inclusion Criteria	Control women had regular menstrual cycles. Women were diagnosed with PCOS based on criteria equivalent to the Rotterdam criteria.
Exclusion Criteria	Receiving hormonal medication for 3 months before the study.

Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Not reported	The study was funded by the National Institute of Child Health and Human Development/NIH through cooperative agreement (U54 HD 12303-20) as part of the Specialized Cooperative Centers Program in Reproduction Research and in part by NIH Grant MO1 RR00827. Any conflicts of interests are not reported.
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcomes have been included.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	The study details specific criteria for the inclusion of women with PCOS (Rotterdam criteria).
If there were specified inclusion/exclusion criteria, were these appropriate?	Partial	Criteria were appropriate but more detail is required, for example age or BMI restrictions.
Were the cases and controls taken from comparable populations?	Not reported	The method of recruitment has not been reported. It is unclear where women with PCOS were recruited from clinics or hospitals seeking treatment for endocrinological complaints, referred to the study by their doctor or the general population.
Was case and control status established in a standard, valid and reliable way?	Yes	Women in the PCOS group were diagnosed with the syndrome using the Rotterdam Criteria. The control women had regular menstrual cycles and androgen levels within the normal range.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Hormonal medications that may affect important outcomes were ceased three months prior to the study. This period of time is likely to be sufficient to ‘washout’ the affects of the medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	The number of participants who refused to participate is not reported. Groups were nearly equal in sample size.

Were the groups comparable with regards to key prognostic variables?	Partial	In PCOS women, the mean (±se) age was 28.6 ± 0.6 y and not significantly greater than that of the normal control group, 26.3 ± 0.8 y. The mean BMI was significantly greater in PCOS subjects compared with that of normal women (35.3 ± 0.8 *vs.* 27.4 ± 0.7, *P* <0.02), whereas the difference in mean waist-to-hip ratio (0.88 ± 0.03 *vs.* 0.80 ± 0.06) failed to achieve statistical significance. Body mass may affect insulin sensitivity.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.
Were all individuals included in the analysis?	Not reported	All participants that participated in the study had their results analysed, however it is unclear if any participants did not complete the study or if data points were missed.
If statistical analysis was undertaken, was this appropriate?	Partial	Statistical analysis is reported on page 5457. A log transformation was applied when appropriate. To determine interaction between group and dose as well as main effects, two-group repeated measures ANOVA and analysis of covariance were used. *Post hoc* testing was done with a Bonferroni correction. Comparisons of mean baseline values between PCOS and normal women were performed using independent Student's *t* tests (SPSS 11.0 software, SPSS Inc., Chicago, IL). Correlations among variables were analysed using the Pearson correlation coefficient method. It may have been more appropriate to conduct a statistical test to determine if insulin sensitivity was still significantly different between groups when BMI was taken into account. BMI may have a confounding affect on insulin sensitivity and therefore women with PCOS may have had lower insulin sensitivity at baseline as a result of obesity.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.

Other		
Risk of Bias	Moderate	

Results.
In the PCOS group, mean steady-state plasma insulin levels, 235 ± 25.5 μU/ml, resulting from the hyperinsulinemic clamp were significantly (P = 0.02) higher than those achieved in normal women, 173 ± 19.3 μU/ml, despite equivalent infusion rates and similar serum glucose concentrations (Fig. 1). Steady-state serum glucose levels were maintained between 85 and 90 mg/dl in both groups. The mean glucose disposal rate in PCOS subjects was significantly less (P < 0.02) than that found in normal women and indicative of insulin resistance.

Author's Conclusions.
These findings demonstrated that in PCOS women, LH secretion and gonadotropin responses to GnRH were not influenced by insulin administration. Insulin infusion had little effect on steroid hormone production with the possible exception of androstenedione. These results suggest that inappropriate LH secretion in PCOS is not a direct consequence of insulin resistance and compensatory hyperinsulinemia.

Our Comments/Summary.
There is a moderate risk of bias. Some of the criteria have been fulfilled and the criteria that have not been reported (recruitment of participants, dropout rates, sample size or power calculations) may affect the conclusions of the study.

Study: Stepto NK, Cassar S, Joham AE, Hutchison SK, Harrison CL, Goldstein RF, Teede HJ,. Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulinaemic clamp, Human Reproduction. 2013; 28(3): 777-784.

Description of study: Cross-sectional

Patient/population	Lean women with PCOS; (mean ± SD) age 27 ± 4 y, BMI 23 ± 2 kg/m^2. Overweight women with PCOS; age 30 ± 6 y, BMI 36 ± 7 kg/m^2 PCOS diagnosed using the Rotterdam criteria.
N	Lean PCOS n = 20 Overweight PCOS n = 20 Lean control n = 19 Overweight control n = 14
Setting	Not reported.
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp
Comparison/control	Authors refer to previous publication
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp
Inclusion Criteria	Authors refer to previous publication
Exclusion Criteria	Authors refer to previous publication

Study Validity.

Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	The authors report that there are no conflicts of interests (p.778) and funding sources (p. 783).
Does the study have a clearly focused question?	Yes	
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	Authors refer to previous publication
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	Authors refer to previous publication for inclusion/exclusion criteria however, criteria are appropriate.
Were the cases and controls taken from comparable populations?	Yes	Cases and controls were both recruited through community advertisements.
Was case and control status established in a standard, valid and reliable way?	Yes	The Rotterdam criteria was applied to discriminate between cases and controls.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Medications affecting end-point measures were ceased three months prior to participation.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Partial	The overweight PCOS group was older than the three other groups. BMI was not different between the lean PCOS and control groups or between the overweight PCOS and control groups.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.

Were all individuals included in the analysis?	Yes	
If statistical analysis was undertaken, was this appropriate?	Yes	The confounding effect of age and BMI on insulin sensitivity was accounted for.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
Risk of Bias	Moderate	

Results.
PCOS women were more insulin resistant than BMI-matched controls (main effect for BMI and PCOS; P <0.001). Insulin resistance was present in 75% of lean PCOS, 62% of overweight controls and 95% of overweight PCOS. Lean controls (mean ± SD; GIR 339 ± 76 mg min^{-1} m^{-2}) were less insulin resistant than lean PCOS (270 ± 66 mg min^{-1} m^{-2}), overweight controls (264 ± 66 mg min^{-1} m^{-2}) and overweight PCOS (175 ± 96 mg min^{-1} m^{-2}). The negative relationship between BMI and insulin resistance reflected by GIR was more marked in PCOS (P < 0.0001) than controls.

Author's Conclusions.
Insulin resistance is exacerbated by increased BMI, supporting intrinsic insulin resistance in PCOS. BMI impact on insulin resistance is greater in PCOS, than in controls, irrespective of visceral fat, prioritizing lifestyle intervention and the need for effective therapeutic interventions to address intrinsic insulin resistance and prevent diabetes in this high-risk population.

Our Comments/Summary.
This study has a moderate risk of bias. Detail regarding inclusion and exclusion is missing and authors refer to previous publications.

Study: Svendsen PF, Nilas L, Norgaard K, Jensen JB, Madsbad S., Obesity, body composition and metabolic disturbances in polycystic ovary syndrome. 2008; 23(9): 2113-2121.		
Description of study:		
Patient/population	Lean women with PCOS; (mean ± SD) age 28 ± 4.7 y, BMI 23 ± 1.5 kg/m^2 Overweight women with PCOS; age 29 ± 3.9 y, BMI 33 ± 4.0 kg/m^2 PCOS diagnosed using the Rotterdam criteria.	
N	Lean PCOS n = 17 Overweight PCOS n = 18 Lean control n = 9 Overweight control n = 16	
Setting	Not reported	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	Lean women with normal menstrual cycles and androgen levels; age 30 ± 4.1 y, BMI 22 ± 1.4 kg/m^2. Overweight women with normal menstrual cycles and androgen levels; age 31 ± 5.4 y, BMI 33 ± 4.0 kg/m^2.	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Control women had regular menstrual cycles and normal androgen levels. Women were diagnosed with PCOS based on the Rotterdam criteria.	
Exclusion Criteria	Women with known chronic diseases. Women who had used oral contraceptives or other drugs known to alter glucose and insulin metabolism within the last 3 months.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Partial	Funding sources are detailed (p.2120), however any conflicts of interests are not reported.
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcomes have been included.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Partial	The study details specific criteria for the inclusion of women with PCOS (Rotterdam criteria).
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	

Were the cases and controls taken from comparable populations?	Yes	Cases and controls were both recruited through advertisements in the local newspaper.
Was case and control status established in a standard, valid and reliable way?	Yes	Women in the PCOS group were diagnosed with the syndrome using the Rotterdam Criteria. The control women had regular menstrual cycles and androgen levels within the normal range.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Hormonal medications that may affect important outcomes were ceased 3 months prior to the study. This period of time is likely to be sufficient to 'washout' the effects of the medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Yes	Groups were age and weight-matched.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.
Were all individuals included in the analysis?	Not reported	All participants that participated in the study had their results analysed, however it is unclear if any participants did not complete the study or if data points were missed.

If statistical analysis was undertaken, was this appropriate?	Partial	Results are presented as mean ± SD or SEM. A two-way ANOVA was performed to determine the effects of PCOS and obesity and a possible interaction between PCOS and obesity on insulin sensitivity index (ISI) and other variables. For comparison of subgroups, the Bonferroni method was used for adjusting P-values. A simple linear regression analysis was performed to investigate the role of androgens on body fat distribution (trunk/peripheral fat) and on insulin sensitivity (GDR, ISI and HOMA) and action (AIRg). Data on VLDL, HOMA-IR and DI did not follow a Gaussian distribution and were therefore log-transformed, and they were thereby approximated by the normal distribution. Levels of significance were set at 0.05%.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
Risk of Bias	Low	

Results.
When adjusted for obesity, PCOS was associated with higher 2-h glucose levels ($P < 0.05$), higher trunk/periphery fat ratio ($P < 0.001$), lower ISI ($P < 0.001$), lower insulin-stimulated glucose oxidation (GOX 2) ($P < 0.05$) and lower non-oxidative glucose metabolism ($P < 0.05$), but a normal acute insulin response to glucose (AIRg) compared with control women. Lean women with PCOS had lower ISI ($P < 0.001$), GOX-2 ($P < 0.05$) and higher trunk/periphery fat ratio ($P < 0.05$) than lean control women. In obese women with PCOS, ISI was reduced with 25% compared with obese control women, whereas trunk/peripheral fat ratio did not differ. AIRg was increased in obese groups compared with lean groups ($P < 0.05$), but was, in all groups, appropriate for the ambient action of insulin.

Author's Conclusions.
PCOS is associated with a low ISI, which in lean women with PCOS may partly be explained by higher trunk/peripheral fat ratio. AIRg was amplified by obesity, but was, in all groups, appropriate for prevailing insulin sensitivity, suggesting a normal β-cell adaptation.

Our Comments/Summary.
There is a low risk of bias. The study presents with sound methodology.

Study: Tosi F1, Dorizzi R, Castello R, Maffeis C, Spiazzi G, Zoppini G, Muggeo M, Moghetti P., Body fat and insulin resistance independently predict increased serum C-reactive protein in hyperandrogenic women with polycystic ovary syndrome. 2009; 161: 737-745		
Description of study:		
Patient/population	Women with PCOS; (mean ± SD) age 22.4 ± 4.1 y, BMI 24 ± 4 kg/m^2 PCOS diagnosed by both the Rotterdam and NIH criteria.	
N	PCOS n = 50 Control n = 35	
Setting	Division of Endocrinology and Metabolism of Verona Hospital, Italy – a tertiary care academic medical center.	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	BMI-matched healthy women; age 25.4 ± 4.6 y, BMI 23.4 ± 5.2 kg/m^2.	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Healthy nonhirsute women, without polycystic ovaries and with regular ovulatory cycles, served as controls. Women diagnosed with PCOS based on the Rotterdam and NIH criteria.	
Exclusion Criteria	Androgen-secreting tumors, congenital adrenal hyperplasia, thyroid dysfunction, hyperprolactinemia, Cushing's syndrome, and diabetes mellitus were ruled out in all subjects. Women taking metformin, oral contraceptives, or any other medications (including statins or acetylsalicylic acid) potentially interfering with the study in the previous 6 months.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Authors report no conflict of interests and funding sources are detailed (p.744).
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcomes have been included.
Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	

Were the cases and controls taken from comparable populations?	No	Women with PCOS were recruited by referrals to the Division of Endocrinology and Metabolism of Verona Hospital for hirsutism and/or hyperandrogenic oligoamenorrhea were included in the study. The control group included women referred to the outpatient clinic of the same medical center for simple obesity. It is unclear how lean control volunteers were recruited.
Was case and control status established in a standard, valid and reliable way?	Yes	Women in the PCOS group were diagnosed with the syndrome using the Rotterdam and NIH Criteria. The control women had regular menstrual cycles and androgen levels within the normal range.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Hormonal medications that may affect important outcomes were ceased 6 months prior to the study. This period of time is likely to be sufficient to 'washout' the effects of the medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Not reported	
Were the groups comparable with regards to key prognostic variables?	Partial	Groups were weight-matched, however the control group was slightly older than the PCOS group.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.
Were all individuals included in the analysis?	Not reported	All participants that participated in the study had their results analysed, however it is unclear if any participants did not complete the study or if data points were missed.

If statistical analysis was undertaken, was this appropriate?	Yes	ANOVA, followed by the Scheffé test for multiple comparisons, was used to compare groups of hyperandrogenic women and controls. Analysis of covariance was carried out to compare between groups CRP values and insulin sensitivity adjusted for age and BMI. Univariate and multiple regression analyses were used to correlate CRP levels with relevant hormonal and metabolic variables. In multiple regression analyses, age, fat mass, insulin sensitivity, and serum androgens (either free or total testosterone) or the PCOS status were included as independent variables. Additional independent variables were also included in some analyses, as indicated. Serum hs-CRP and other skewed variables were log-transformed before analysis to ensure normality of data and then back-transformed to their natural units for presentation in tables and figures.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
Risk of Bias	Moderate	

Results.
Hs-CRP concentrations were higher in PCOS women (3.43±2.01 mg/l) than in HA subjects and healthy women (2.43±1.04, P<0.005; and 2.75±0.86 mg/l, P<0.05 respectively versus PCOS). In multiple regression analyses, increased serum hs-CRP was independently predicted by higher body fat and lower insulin sensitivity. However, in lean women, serum-free testosterone was an additional, negative, predictive variable. Insulin-mediated glucose uptake in the glucose clamp was significantly lower in both groups of hyperandrogenic women (PCOS 10.3±2.8, HA 11.8±3.0 mg/kg fat-free mass×min) than in controls (13.9±2.8 mg/kg fat-free mass×min, P<0.01 versus PCOS, P<0.05 versus HA).

Author's Conclusions.
PCOS is accompanied by a low-grade chronic inflammation. Body fat appears the main determining factor of this finding, which is only partly explained by insulin resistance. At least in lean women, androgen excess per se seems to play an additional, possibly protective, role in this association.

Our Comments/Summary.
There is a moderate risk of bias. The recruitment process could have biased results by recruiting women seeing treatments and therefore more severe symptoms of PCOS.

Study: VrbÍková J, Cibula D, Dvořáková K, Stanická S, Šindelka G , Hill M, Fanta M, Vondra K, and Škrha J., Insulin sensitivity in women with polycystic ovary syndrome. 2004; 89(6): 2942-2945.		
Description of study:		
Patient/population	Lean women with PCOS; (mean ± SD) age 24.2 ± 4.6 y, BMI 21.5 ± 1.8kg/m^2 Obese women with PCOS; age 26.2 ± 4.8 y, BMI 29.6 ± 3.7 kg/m^2 PCOS diagnosed by NIH criteria.	
N	Lean PCOS n = 53 Overweight PCOS n = 30 Control n = 15	
Setting	Institute of Endocrinology and the Faculty Hospital of Charles University in Prague, Prague, Czech Republic.	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	BMI-matched healthy women; age 28.1 ± 6.6 y, BMI 21.5 ± 2.0 kg/m^2.	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Women with PCOS were in good health condition, without any other serious disorders. In one patient, cytologically benign nodular goiter was present; she was on thyroid-suppressive medication with TSH, 0.04 mIU/liter; free T_4, 19 pmol/liter (normal range, 12–22 pmol/liter), and T_3, 1.83 nmol/liter (normal range, 1.30–3.10 nmol/liter). None of the patients had taken oral contraceptives or any other steroid or glucose-metabolism-affecting medication during the preceding 3 months.	
Exclusion Criteria	Women with epilepsy or migraine. In all patients, 17-OH-progesterone was determined in the early follicular phase of their cycle, and if levels were between 5 and 10 nmol/liter, an ACTH test was undergone to exclude late-onset congenital adrenal hyperplasia. Hyperprolactinemia (prolactin levels), hypercortisolism (plasma cortisol, and, if necessary, urinary cortisol excretion per 24 h or short dexamethasone suppression test with 1 mg of dexamethasone at 2200–2300 h), and thyroid dysfunction (TSH, free T_4, antithyroglobulin, and anti-thyroid-peroxidase antibodies) were excluded.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Partial	Funding sources are detailed (p.2944), however authors have not provided a conflict of interest statement.
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcomes have been included.

Is a case control study the appropriate method to answer this question?	Yes	
Does the study have specified inclusion/exclusion criteria?	Yes	
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	
Were the cases and controls taken from comparable populations?	No	Women in the control group were recruited from the healthcare personnel of the hospital and their acquaintances.
Was case and control status established in a standard, valid and reliable way?	Yes	Women in the PCOS group were diagnosed with the syndrome using the NIH Criteria. The control women had regular menstrual cycles and androgen levels within the normal range.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Hormonal medications that may affect important outcomes were ceased 3 months prior to the study. This period of time is likely to be sufficient to 'washout' the effects of the medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	No	
Were the groups comparable with regards to key prognostic variables?	Partial	Groups were weight-matched, however the control group was slightly older than the PCOS group.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.

Were all individuals included in the analysis?	No	Authors detail the number of participants that did not complete a study variable. 5 control women did not have LH data and 1-3 obese PCOS women did not have some hormonal measures.
If statistical analysis was undertaken, was this appropriate?	Yes	Kruskal-Wallis robust ANOVA was used for evaluation of the differences between controls, lean PCOS patients, and obese PCOS patients. The individual differences between the subgroups were evaluated by Kruskal-Wallis robust multiple-comparison z-value test. NCSS 2001 (Number Cruncher Statistical Systems, Kaysville, UT) was used for the calculations.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported. However, there exists a discrepancy in the reporting of age and BMI of the control group. These variables are reported in two different palaces (subjects and methods section and the results section) and are not consistant.
Other		
Risk of Bias	Moderate	

Results.
Basal blood glucose was significantly higher in lean and obese PCOS than in controls (P <0.02). Basal insulin was significantly higher in obese and lean PCOS women than in controls (P <0.000001). M was significantly lower in obese than in lean PCOS and controls (P <0.02), and the same was observed for ISI (P <0.0008). On the other hand, lean PCOS did not differ in M or ISI from controls. MCRI was significantly lower in obese than in lean PCOS (P <0.03) and showed no difference between lean PCOS and controls.

Author's Conclusions.
Lean PCOS women are not more insulin resistant than healthy controls. Insulin hypersecretion, on the other hand, is present even in lean PCOS women.

Our Comments/Summary.
There is a moderate risk of bias. The recruitment process could have biased results. There is also discrepancies in the reporting of data.

Study: Yang S, Li Q, M.D, Song Y, Tian B, Cheng Q, Qing H, Zhong L, Xia W., Serum complement C3 has a stronger association with insulin resistance than high-sensitivity C-reactive protein in women with polycystic ovary syndrome, Fertility and Sterility. 2011; 95(5): 1749-1753.		
Description of study:		
Patient/population	Lean women with PCOS; (mean ± SD) age 25.39 ± 4.27 y, BMI 20.54 ± 1.80 kg/m^2. Overweight/Obese women with PCOS; age 25.89 ± 4.50 y, BMI 27.80 ± 3.39 kg/m^2. PCOS diagnosed using the Rotterdam criteria. BMI defined as lean <24 kg/m^2 and overweight/obese ≥24 kg/m^2	
N	Lean PCOS n = 37 Overweight PCOS n = 64 Lean Control n = 108 Overweight Control n = 8	
Setting	Clinical Research Center, China.	
Intervention/exposure	Euglycaemic-hyperinsulinaemic clamp	
Comparison/control	Healthy age-matched women were recruited as controls. Lean controls; age 25.94 ± 2.73 y, BMI 20.57 ± 1.63 kg/m^2. Overweight/obese controls; age 25.75 ± 1.67 y, BMI 26.20 ± 1.57 kg/m^2.	
Outcomes	Anthropometric: BMI Metabolic: Insulin sensitivity by euglycaemic-hyperinsulinaemic clamp	
Inclusion Criteria	Control women had regular menstrual cycles and no clinical/biochemical hyperandrogenism. Women were diagnosed with PCOS based on criteria equivalent to the Rotterdam criteria.	
Exclusion Criteria	Use of hormonal medication within a month or medication that affect insulin sensitivity within 3 months of study commencement.	
Study Validity.		
Is it clear that there are no conflicts of interest in the writing or funding of this study?	Yes	Both conflicts of interests and funding sources have been reported (p. 1749).
Does the study have a clearly focused question?	Yes	The research question is clearly stated and appropriate participants and outcomes have been included.
Is a case control study the appropriate method to answer this question?	Yes	

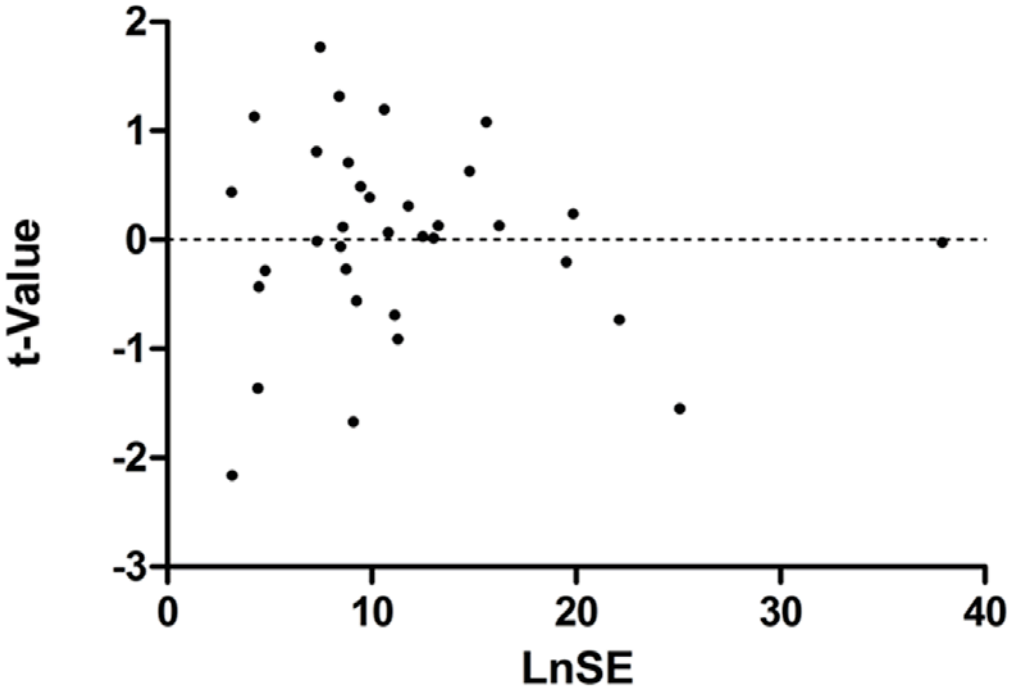
Does the study have specified inclusion/exclusion criteria?	Yes	
If there were specified inclusion/exclusion criteria, were these appropriate?	Yes	
Were the cases and controls taken from comparable populations?	Not reported	Women with PCOS were recruited from patients attending the Department of Endocrinology and the Department of Obstetrics and Gynaecology of the First Affiliated Hospital of Chongqing Medical University. It is unclear how the control group were recruited.
Was case and control status established in a standard, valid and reliable way?	Yes	Women in the PCOS group were diagnosed with the syndrome using the Rotterdam Criteria. The control women had regular menstrual cycles and androgen levels within the normal range.
Was case and control status established by assessors blind to the exposure?	Not reported	
Were the outcomes measured appropriate?	Yes	
Was there sufficient duration of follow-up?	Yes	Hormonal medications that may affect important outcomes were ceased 3 months prior to the study. This period of time is likely to be sufficient to 'washout' the effects of the medication.
Was the percentage of each group (cases and controls) who refused to participate in the study reported?	Partial	It is noted that researchers failed to recruit and overweight or obese participants with PCOS to undergo an insulin clamp.
Were the groups comparable with regards to key prognostic variables?	Yes	Groups had similar age and BMI.
Was there ≤20% drop-out?	Not reported	
Was the study sufficiently powered to detect any differences between the groups?	Not reported	Sample size or power calculations have not been reported.

Were all individuals included in the analysis?	Not reported	All participants that participated in the study had their results analysed, however it is unclear if any participants did not complete the study or if data points were missed.
If statistical analysis was undertaken, was this appropriate?	Partial	Statistical analysis is reported on page 1750. A log transformation was applied when data was not normally distributed. An ANOVA was used when analysis more than two groups; for post hoc analysis, Games-Howell tests were conducted in cases of heterogeneity of variance and SKN tests were used in cases of homogeneity of variance. Independent sample t-tests were used for comparisons between two groups. Correlations among variables were analysed using the Pearson correlation coefficient method. Multiple-linear regression.
Is the paper free of selective outcome reporting?	Yes	Planned outcomes were measured and reported.
Other		
Risk of Bias	Moderate	

Results.
Compared with controls, women with PCOS had a lower M value and higher C3 (1.37 ± 0. 34 vs. 1.10 ± 0.22 g/L) and hs-CRP levels (1.46 ± 2.29 vs. 0.49 ± 0.88 mg/L). In women with PCOS, C3 and hs-CRP negatively correlated with M value ($r = -0.61$ and $r = -0.47$, respectively). By regression analysis, C3 was found to have a greater impact on the M value (standardized coefficient $\beta = -0.24$) than did hs-CRP (standardized coefficient $\beta = -0.13$). After adjusting for body mass index (BMI), women with PCOS in the upper quartile were 4.30 times more likely to exhibit IR compared with those in the lower quartiles, whereas hs-CRP was not a statistically significant predictor of IR in women with PCOS.

Author's Conclusions.
Compared with hs-CRP, serum C3 might be a stronger inflammatory marker of IR in women with PCOS.

Our Comments/Summary.
There is a moderate risk of bias. It is unclear how the controls groups were recruited making generalization about results limited. No women in the overweight PCOS group underwent an insulin clamp.



Supplementary Figure 1 Scatter plot of the t-statistic associated with each study-estimate value contributing to the study-estimate random effect versus the log of the standard error of the effect.

Chapter 3 Women with Polycystic Ovary Syndrome have Intrinsic Insulin Resistance on Euglycaemic-Hyperinsulaemic Clamp.

Declaration of Co-Authorship and Co-Contribution: Paper Incorporated in Thesis by Publication

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

Declaration by: **Signature:** **Date**

Samantha Cassar  27/11/2014

Paper Title:

Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulaemic clamp.

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

Name	Contribution %	Nature of Contribution
Samantha Cassar	70	Collected, analysed and interpreted data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Nigel K. Stepto	10	Involved with conception and design, analysis and interpretation of data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Anju E. Joham	2.5	Collected the data, undertook the critical revision for important intellectual content and approved the final version for publication
Samantha K. Hutchison	2.5	Collected the data, undertook the critical revision for important intellectual content and approved the final version for publication
Cheryce L. Harrison	2.5	Collected the data, undertook the critical revision for important intellectual content and approved the final version for publication.

Rebecca F. Goldstein	2.5	Collected the data, undertook the critical revision for important intellectual content and approved the final version for publication.
Helena J. Teede	10	Involved with conception, design, analysis, interpretation of data undertook the critical revision for important intellectual content and approved the final version for publication.

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University

and will be held for at least five years from the date indicated below.

		Date
Samantha Cassar	[redacted]	27/11/2014
Nigel Stepto		27/11/2014
Anju E. Joham		27/11/2014
Samantha K. Hutchison		27/11/2014
Cheryce L. Harrison		27/11/2014
Rebecca F. Goldstein		27/11/2014
Helena J. Teede		27/11/2014

3.0 Introduction

IR is implicated in the aetiology of PCOS and plays a central role in a range of conditions and clinical sequale including, diabetes, cardiovascular disease, obesity, dislipidemia and ovarian dysfunction, which all relate to PCOS and are areas of public health concern. The aetiology of IR in PCOS is poorly understood contributing to the controversy over diagnostic criteria and optimal treatment strategies. This research informed the systematic review and meta-analysis conducted in Chapter 2, as no other studies provided prevalence rates of IR across different PCOS phenotypes.

3.1 Insulin Resistance in PCOS

The definition of IR is ambiguous and is often described as an impaired biological response to exogenous or endogenous insulin that results in disturbed metabolic and mitogenic (gene transcription, DNA synthesis) processes that cause tissues to become less sensitive to insulin resulting in hyperinsulinaemia to maintain euglycaemic levels (Anonymous, 1998, Muniyappa et al., 2008). Since the late 1980's many studies have reported some degree of IR in women with PCOS using various assessment techniques (Ciampelli et al., 1997, Ciaraldi et al., 2009, Diamanti-Kandarakis et al., 1998, Dunaif et al., 1989, Dunaif et al., 1992, Holte et al., 1994, Morin-Papunen et al., 2000b). In early ground breaking studies, insulin sensitivity measured by an euglycaemic hyperinsulinaemic clamp was reported to be ~35% lower in women with PCOS compared to age and body composition comparable healthy controls (Dunaif et al., 1989, Dunaif et al., 1992). IR is documented to be present in 44% to 70% of women with PCOS when less sensitive surrogate markers are used (Ciampelli et al., 2005, de Paula Martins et al., 2007, Diamanti-Kandarakis and Dunaif, 2012, Fulghesu et al., 2006). There is general consensus that overweight and especially obese women

with PCOS are highly likely to be insulin resistant and therefore should undergo OGTT screening regularly to detect an impaired response to insulin (Teede et al., 2011). When combined with PCOS, obesity has a synergistic and enhanced negative effect on the clinical features and IR in women with PCOS (Dunaif et al., 1989). Highlighting that obesity is an extrinsic factor that contributes to IR in PCOS.

The concept of intrinsic IR remains controversial, with some studies failing to demonstrate IR in lean women with PCOS (Carmina and Lobo, 1999, Mortensen et al., 2009, Azziz, 2004) using the highly sensitive insulin clamp technique. Others contest IR is mainly extrinsic and related to obesity. Intrinsic IR has been supported by recent mechanistic PCOS studies reporting evidence of insulin signalling abnormalities that are both unique to PCOS and BMI separately (Corbould et al., 2005, Diamanti-Kandarakis and Papavassiliou, 2006). Heterogeneity in results may be related to differences in the diagnostic criteria for PCOS, with most studies to date using the NIH diagnostic criteria so the prevalence of IR in new phenotypes defined by the non-NIH criteria (Rotterdam criteria) is less clear (Moran et al., 2009).

Therefore, the aim of this chapter was to comprehensively examine the intrinsic and extrinsic IR in PCOS. To do this we investigated both the prevalence of IR and the impact of obesity in healthy women and women with PCOS and divided them into four groups: lean healthy controls, lean PCOS (intrinsic IR), obese controls (extrinsic IR) and obese PCOS (intrinsic + extrinsic IR) and used gold standard euglycaemic hyperinsulinaemic clamps to assess IR and furthermore assess the prevalence of IR based on diagnostic criteria.

3.2 My Role

Chapter 3 consists of a large cross sectional study investigating IR in women with and without PCOS. I collected the data, independently biochemically analysed insulin concentrations using an enzyme-linked immunosorbent (ELISA) assay, statistically analysed and interpreted the data and wrote the manuscript. As a result of my contribution I am co-first author on this manuscript, which was published in Human Reproduction (Impact Factor 4.59, Q1 Journal).

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human reproduction

ORIGINAL ARTICLE *Reproductive endocrinology*

Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic–hyperinsulaemic clamp

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STUDY QUESTION: What is the prevalence of insulin resistance (IR) and the contributions of intrinsic and extrinsic IR in women diagnosed with polycystic ovary syndrome (PCOS) according to the Rotterdam criteria?

SUMMARY ANSWER: We report novel clamp data in Rotterdam diagnosed PCOS women, using World Health Organization criteria for IR showing that women with PCOS have a high prevalence of IR, strengthening the evidence for an aetiological role of IR in both National Institutes of Health (NIH) and Rotterdam diagnosed PCOS in lean and overweight women.

WHAT IS KNOWN ALREADY: PCOS is a complex endocrine condition with a significant increased risk of gestational diabetes and type 2 diabetes.

STUDY DESIGN, SIZE, DURATION: Using a cross-sectional study design, 20 overweight and 20 lean PCOS (Rotterdam criteria), 14 overweight and 19 lean body mass index (BMI)-matched control non-PCOS women underwent clinical measures of IR after a 3-month withdrawal of insulin sensitizers and the oral contraceptive pill.

MATERIALS, SETTING, METHODS: In an academic clinic setting, glucose infusion rate (GIR) on euglycaemic–hyperinsulinaemic clamp was investigated as a marker of insulin sensitivity.

MAIN RESULTS AND THE ROLE OF CHANCE: PCOS women were more IR than BMI-matched controls (main effect for BMI and PCOS; $P < 0.001$). IR was present in 75% of lean PCOS, 62% of overweight controls and 95% of overweight PCOS. Lean controls (mean $\pm$ SD; GIR 339 $\pm$ 76 mg min^{-1} m^{-2}) were less IR than lean PCOS (270 $\pm$ 66 mg min^{-1} m^{-2}), overweight controls (264 $\pm$ 66 mg min^{-1} m^{-2}) and overweight PCOS (175 $\pm$ 96 mg min^{-1} m^{-2}). The negative relationship between BMI and IR reflected by GIR was more marked in PCOS ($y = 445.1 - 7.7x$, $R^2 = 0.42$ ($P < 0.0001$) than controls ($y = 435.5 - 4.6x$, $R^2 = 0.04$ ($P < 0.01$)).

LIMITATIONS, REASONS FOR CAUTION: The study did not use glucose tracer techniques to completely characterize the IR, as well as the lack of matching for body composition and age.

WIDER IMPLICATIONS OF THE FINDINGS: IR is exacerbated by increased BMI, supporting intrinsic IR in PCOS. BMI impact on IR is greater in PCOS, than in controls, irrespective of visceral fat, prioritizing lifestyle intervention and the need for effective therapeutic interventions to address intrinsic IR and prevent diabetes in this high-risk population.

STUDY FUNDING/COMPETING INTEREST(S): This investigator-initiated trial was supported by grants from the National Health & Medical Research Council (NHMRC) Grant number 606553 (H.J.T., N.K.S. and S.K.H.) as well as Monash University and The Jean Hailes

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CLINICAL TRIAL REGISTRATION: ISRCTN84763265.

Key words: prevalence of insulin resistance / BMI / visceral fat / hyperandrogenism

Introduction

Polycystic ovary syndrome (PCOS) affects 12–21% of reproductive-aged women (March *et al.*, 2010; Boyle *et al.*, 2012) and has major reproductive (leading cause of anovulatory infertility) (Teede *et al.*, 2011), psychological (anxiety and depression) (Deeks *et al.*, 2010) and metabolic (increased type 2 diabetes mellitus and cardiovascular risk factors) (Moran *et al.*, 2010) impacts, representing a substantial health burden (Fig. 1). On meta-analysis the risk of type 2 diabetes in PCOS is increased to 4.43-fold (OR, 95% CI 4.06–4.82; Moran *et al.*, 2010, 2011) even after correcting for body mass index (BMI). Despite PCOS prevalence and health implications, the aetiology and ideal therapies for PCOS remain unclear. Insulin resistance (IR) is a central characteristic in the majority of affected women (Teede *et al.*, 2007), driving both hyperandrogenism and clinical features. Underlying mechanisms of IR remain ill-defined (Teede *et al.*, 2011), contributing to controversy over diagnostic criteria, and a lack of optimal therapies. Therapeutic strategies in PCOS include medical therapy (metformin) (Meyer *et al.*, 2005), exercise (Hutchison *et al.*, 2011; Harrison *et al.*, 2012) and diet-induced weight loss, which all reduce, yet do not reverse IR and fail to optimally treat PCOS. In this context, greater insight into the aetiology of IR in PCOS is needed.

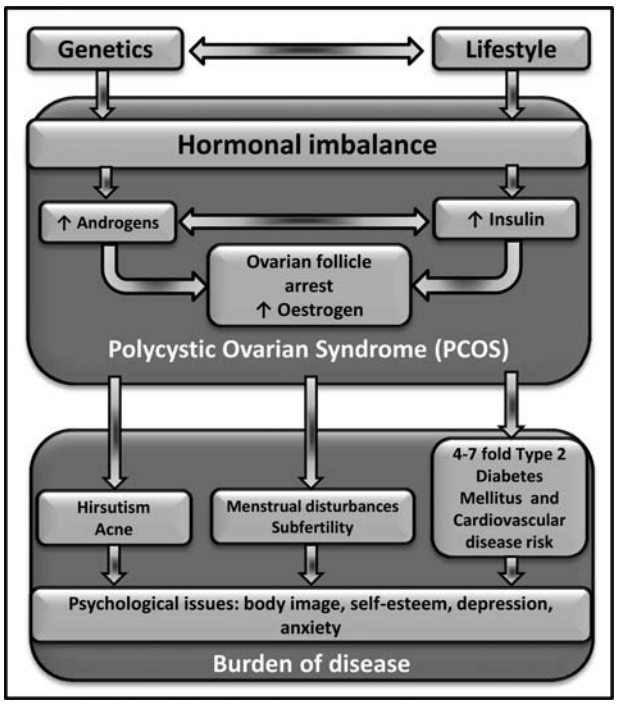


Figure 1. Schema of the aetiology, clinical features and health burden of PCOS (reproduced from Teede *et al.*, 2011 with permission).

Since the sentinel publication by Dunaif *et al.* (1989) noting increased IR in PCOS, reported prevalence of IR in PCOS has varied widely, attributable to the arbitrary and inconsistent definition of IR, the variable and often inaccurate methodologies, the heterogeneity of PCOS and the evolving diagnostic criteria. The Rotterdam criteria include women with milder reproductive and metabolic features of PCOS and while theoretically IR may be less prevalent in women diagnosed via Rotterdam criteria, the prevalence of IR on clamps studies has not been reported (Moran and Teede, 2009).

While not useful in the clinical setting, euglycaemic–hyperinsulinaemic clamps remain the gold standard for research-based assessment of IR. Based on non-clamp data, prevalence of IR has been reported to range from 50 to 70% in women with PCOS (Carmina *et al.*, 1992; Legro *et al.*, 1998). Traditionally, this IR was attributed to obesity in PCOS (Rachon and Teede, 2010), yet it has been hypothesized that intrinsic or unique PCOS-related IR is present and is compounded by separate extrinsic or BMI-related IR (Dunaif *et al.*, 1989; Diamanti-Kandarakis and Papavassiliou 2006; Teede *et al.*, 2007). The concept of intrinsic IR remains controversial in the setting of conflicting literature, with inadequate sample size and application of inaccurate methods to test IR (Dunaif *et al.*, 1989; Mancini *et al.*, 2009; Rabøl *et al.*, 2011). Intrinsic IR has been supported by recent mechanistic PCOS studies including evidence of insulin signalling abnormalities with both unique PCOS- and common BMI-related abnormalities (Corbould *et al.*, 2005, 2006; Diamanti-Kandarakis and Papavassiliou, 2006). Prior work by our group suggests that intrinsic IR in PCOS may in part be related to selectively increased visceral fat deposition in overweight women with National Institutes of Health (NIH)-diagnosed PCOS. To progress understanding on aetiology of PCOS, IR in PCOS needs to be examined in larger studies, using gold standard clamp methods, comprehensive analysis of visceral fat and needs to include women diagnosed by Rotterdam criteria and women across the BMI range.

In this context, we hypothesize that the majority of women with PCOS diagnosed via Rotterdam criteria will be IR and that PCOS involves both intrinsic PCOS-specific IR seen in lean women, compounded by extrinsic BMI-related IR in overweight women. We aimed to comprehensively examine both IR prevalence and impact of BMI across four groups: lean non-PCOS controls, lean PCOS (intrinsic IR), obese non-PCOS controls (extrinsic IR) and obese PCOS women (intrinsic + extrinsic IR), using gold standard insulin clamps.

Materials and Methods

Participants

Seventy-three premenopausal women with and without PCOS were recruited through community advertisements. The women were categorized according to PCOS status and matched for BMI. Categorization into BMI groups was based on the threshold BMI of 27 kg m^{-2}, as an *a priori* decision, as this is the inflexion point in the relationship between BMI and IR (Garca-Estevez *et al.*, 2004) and as previously published by our group (Hutchison *et al.*, 2011, 2012; Harrison *et al.*, 2012). Diagnosis of PCOS was undertaken by expert endocrinologists (S.K.H., A.E.J. and H.J.T.) based on Rotterdam criteria with two of (i) irregular menstrual cycles (<21 or >35 days), (ii) clinical (hirsutism, acne) or biochemical (elevation of at least one circulating ovarian androgen) hyperandrogenism and (iii) PCO on ultrasound (Group, 2004). As this work expands on a previous smaller overweight PCOS study, the exclusion criteria and screening for other causes of hyperandrogenism have been previously described (Hutchison *et al.*, 2011). The Southern Health Research Advisory and Ethics Committee approved the study and participants gave written informed consent. The clinical trial registration number is ISRCTN84763265.

Study design

At screening (3 months prior to testing), standard diet and lifestyle advice were delivered (Heart Foundation recommendations (www.heartfoundation.org.au)) and medications affecting end-points including insulin sensitizers, anti-androgens and hormonal contraceptives were ceased. Data were collected in the follicular phase of the menstrual cycle where feasible.

Clinical and biochemical measurements

Participants anthropometric assessments including body weight, height, waist and hip circumference and computed axial tomography (CT) scans for visceral fat assessments were conducted as previously reported (Hutchison *et al.*, 2011).

Insulin sensitivity was assessed by the euglycaemic–hyperinsulinaemic clamp technique as previously reported (Hutchison *et al.*, 2011). Briefly, the clamp was performed 72 h after a standardized high-carbohydrate diet prior to an overnight fast. Venous fasting blood samples were collected, analysed and stored as appropriate after arterialization. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 mU m^{-2} min^{-1} for 120 min generating an elevated, stable insulin concentration from 10 to 120 min, with plasma glucose maintained at ~5 mmol/l, using variable infusion. Glucose was assessed every 5 min and the glucose infusion rate (GIR) was calculated during last 30 min of the insulin-stimulated period and expressed as glucose (mg) per body surface area (m^2) per min.

Stored blood samples were batch analysed for serum fasting glucose, total cholesterol, high-density lipoprotein(HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, insulin and testosterone and glycosylated haemoglobin (HbA1c) as previously reported (Meyer *et al.*, 2005). LDL and the homeostatic model IR assessment (HOMA) were calculated as previously described (Meyer *et al.*, 2005).

Statistics

All data are presented as mean ± SD. Results are presented for 73 participants. Two-tailed statistical analysis was performed using SPSS for Windows 20.0 software (SPSS, Inc., Chicago, IL, USA) with statistical significance was accepted when $P \leq 0.05$. Data were assessed for normality and log transformed where appropriate and analysed using univariate analysis of variance (ANOVA) (PCOS status × body weight status) using age as a covariate. Correlations of BMI and GIR with the lipid profile parameters, and GIR with free androgen index (FAI) were determined using the Pearson's product moment correlation coefficient (r). Hierarchical linear regression was used to investigate the influence of visceral fat on GIR and to account for the significant age contributions to the accumulated visceral fat in all women. Split linear regressions were used to demonstrate the *a priori* distinction of lean and obese groups based on a BMI threshold of 27 kg m^{-2} for the exacerbation of IR in the whole group.

Results

We confirmed the *a priori* BMI categorization into lean and overweight/obese women, based on a BMI cut-off of 27 kg m^{-2}, demonstrating a stronger impact of BMI on GIR equal to or above a BMI of 27 kg m^{-2} across all groups (Fig. 2A). Specifically, all women with a BMI <27 kg m^{-2} demonstrated that for every 1 BMI unit increase, GIR was 2.6 units lower ($R^2 = 0.005$ ($P = 0.7$)) compared with the 7.0 units lower for every BMI unit increase in women with a BMI ≥27 kg m^{-2} ($R^2 = 0.212$ ($P = 0.007$); Fig. 2A).

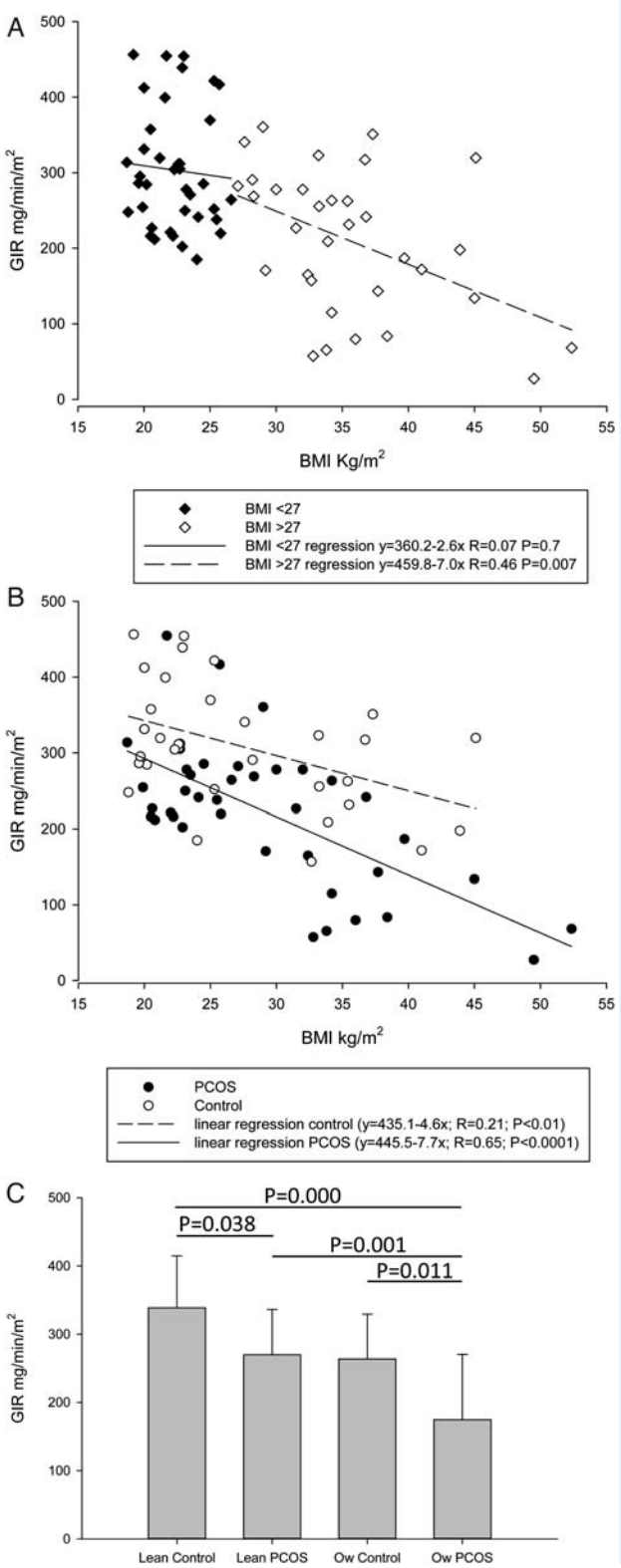
We analysed 34 overweight women ($n = 20$ PCOS and $n = 14$ controls with a BMI ≥27 kg m^{-2}) and 39 lean women ($n = 20$ PCOS and $n = 19$ controls with a BMI <27 kg m^{-2}) with characteristics reported in Table I. The lean women with and without PCOS, and overweight women with PCOS were well matched for age (~28 years). Overweight control women were older than other groups ($P < 0.001$). Using age as a covariate, we noted that age did not influence outcome variables measured ($P > 0.05$) except visceral fat ($P < 0.001$).

Women were primarily Caucasian (68%), but the cohort also included women with a European (14%), Asian/Indian (12%) and a mixed race (6%) background. BMI, body weight, waist and hip circumference, fasting glucose, HOMA, HbA1c, triglycerides, HDL, LDL, LDL:HDL ratio, abdominal subcutaneous and visceral fat were significantly different between the combined groups of lean and obese women (main effect of BMI, $P < 0.05$; Table I) and were not clearly related to PCOS status. Overall, BMI and GIR correlated with triglycerides ($r = 0.39$ ($P = 0.001$) and $r = -0.39$ ($P = 0.001$)), HDL ($r = -0.61$ ($P < 0.001$) and $r = 0.56$ ($P < 0.001$)) and the LDL/HDL ratio ($r = 0.53$($P < 0.001$) and $r = -0.55$ ($P < 0.001$)), respectively.

Testosterone was different between lean and overweight women with PCOS (main effect of PCOS, $P = 0.001$ and $P = 0.04$ respectively; Table I), and fasting insulin was different for lean and overweight women with and without PCOS (main effect PCOS, $P = 0.04$; main effect BMI, $P < 0.001$; Table I). Both BMI and PCOS were related to FAI (Table I, PCOS and BMI, $P < 0.001$, PCOS × BMI $P < 0.05$). IR was correlated to androgen status (FAI) where $r = -0.44$ ($P < 0.001$) and $r = -0.52$ ($P < 0.001$) for all women and women with PCOS, respectively.

IR is a continuous measure and is defined arbitrarily. We defined IR on clamp-derived GIR levels as less than the 25th centile of lean matched controls (non-PCOS specific World Health Organization (WHO) criteria) (Grundy *et al.*, 2004). IR as determined by GIR normalized to body surface area showed that overall PCOS women were more IR than BMI-matched controls, even after correction for age (main effect for PCOS and BMI $P < 0.001$; Fig. 2B).

Specifically, lean controls (339 ± 76 mg min^{-1} m^{-2}) were less IR than lean PCOS (269 ± 66 mg min^{-1} m^{-2}), overweight controls (264 ± 66 mg min^{-1} m^{-2}) and overweight PCOS (175 ± 96 mg min^{-1} m^{-2}), respectively (Fig. 2C). There was no significant difference in IR between lean PCOS women and overweight controls.



Also, overweight women with PCOS were significantly more IR than all groups including overweight controls (Fig. 2C). IR was present in 75% of lean PCOS, 62% of overweight controls and 95% of overweight PCOS (Fig. 3A). The increased IR in PCOS is highlighted by the frequency distribution curve for GIR which is shifted to the left in PCOS (Fig. 3B).

Lean PCOS phenotypes in this community-recruited study included 5/19 with NIH PCOS and 14/19 with Rotterdam PCOS only who did not meet NIH criteria. In the overweight women, 17/20 had NIH PCOS and 3/20 had Rotterdam criteria alone. All participants diagnosed with PCOS according to the Rotterdam criteria in both the lean and overweight groups had irregular menstrual cycles and PCO on ultrasound, with none having hyperandrogenism clinically or biochemically. Overall 53% of PCOS women met NIH criteria. IR was present in 70% of lean Rotterdam, non-NIH PCOS and 80% of lean NIH PCOS with both of these lean subgroups demonstrating lower GIR's of 279 ± 74 and 248 ± 41 mg min^{-1} m^{-2} compared with lean controls (339 ± 76 mg m^{-2} min^{-1}), respectively ($P < 0.05$). Once corrected for BMI, we noted insulin sensitivity for all women was different between controls (301 ± 89 mg min^{-1} m^{-2}) and both NIH (195 ± 91 mg min^{-1} m^{-2}, $P < 0.005$) and Rotterdam only (PCOS + irregular cycles) PCOS phenotypes (260 ± 89 mg min^{-1} m^{-2}, $P < 0.04$).

There was a negative relationship between BMI and IR (GIR; Fig. 2B), which is more marked in women with PCOS (PCOS $R^2 = 0.42$ ($P < 0.0001$) versus controls $R^2 = 0.04$ ($P < 0.01$)), with every 1 unit increase in BMI associated with 7.7 unit lower GIR versus the 4.6 units in control women (Fig. 2B). Visceral fat, a known major contributor to IR and assessed here via visceral fat area on CT, was negatively related to GIR, whereby after accounting for the unequal variance and age, visceral fat accounted for 39, 31 and 39% of the GIR variance overall (adjusted $r^2 = 0.390$; $P < 0.001$), in controls (adjusted $r^2 = 0.312$; $P = 0.002$) and in PCOS (adjusted $r^2 = 0.392$; $P < 0.001$) women, respectively.

Discussion

Here using gold standard clamp techniques, we confirm that PCOS women, irrespective of BMI, are more IR (Dunaif *et al.*, 1989, Ovalle and Azziz, 2002) and report novel data that the prevalence of IR in PCOS based on the WHO definition (<25th centile of GIR in healthy lean controls) is 75% in lean PCOS, 62% in overweight controls and 95% in overweight PCOS in a largely Caucasian population. Overall, we show significantly higher IR in lean PCOS women versus lean controls, supporting the hypothesis that a unique 'intrinsic-related

Figure 2. The relationship between BMI and IR as determined by the GIR in the last 30 min of the 120 min hyperinsulinaemic–euglycaemic clamp. (**A**) Scatterplot of GIR versus BMI where women are separated by BMI at the threshold of 27 kg m^{-2} and associated regressions lines. (**B**) Scatterplot of GIR versus BMI where women are separated by PCOS status, with associated regression lines. (**C**) Mean GIR ± SD data for lean control ($n = 19$), lean PCOS ($n = 20$), overweight/obese (ow) control ($n = 14$) and ow PCOS ($n = 20$) women. Groups defined by the ends of the horizontal bars were significantly different from each other (univariate ANOVA).

Table I. Clinical characteristics of lean (BMI <27 kg m^{-2}) and overweight (BMI >27 kg m^{-2}) women with and without PCOS.

Clinical feature	Lean controls (n = 19)	Lean PCOS (n = 20)	Overweight controls (n = 14)	Overweight PCOS (n = 20)	P value main effect of PCOS	P value main effect of BMI
General characteristics						
Age (years)	28 ± 6	27 ± 4	35 ± 4	30 ± 6	0.028	<0.001
Height (cm)[a]	165 ± 7	166 ± 7	164 ± 4	164 ± 5	0.627	0.221
Body weight (kg)[a]	59 ± 7	63 ± 8	94 ± 16	95 ± 18	0.316	<0.001
BMI (kg m^{-2})[a]	22 ± 2	23 ± 2	35 ± 6	36 ± 7	0.349	<0.001
Waist (cm)[a]	71 ± 5	74 ± 7	102 ± 14	101 ± 11	0.157	<0.001
Hip (cm)[a]	85 ± 7	88 ± 9	119 ± 15	120 ± 14	0.329	<0.001
WHR[a]	0.83 ± 0.04	0.85 ± 0.04	0.85 ± 0.10	0.85 ± 0.06	0.591	0.538
Insulin sensitivity						
Fasting glucose (mmol l^{-1})[a]	4.6 ± 0.3	4.5 ± 0.3	4.9 ± 0.4	4.8 ± 0.6	0.788	0.015
Fasting insulin (pmol l^{-1})[a,b]	24 ± 9	26 ± 10	120 ± 60	172 ± 83	0.043	<0.001
HOMA[a,b]	0.8 ± 0.3	0.8 ± 0.3	4.4 ± 2.6	6.3 ± 3.2	0.143	0.044
HbA1c (%)[a]	4.7 ± 1.2	5.0 ± 0.1	5.4 ± 0.3	5.4 ± 0.4	0.439	0.002
Body composition						
CT abdominal visceral fat (cm^2)[d]	32 ± 20	35 ± 10	122 ± 35	118 ± 59		
Log CT abdominal visceral fat[a]	1.45 ± 0.20	1.53 ± 0.15	2.07 ± 0.13	2.01 ± 0.25	0.257	<0.001
CT abdominal subcutaneous fat (cm^2)[a]	183 ± 69	234 ± 71	550 ± 169	535 ± 175	0.635	<0.001
Hormonal status						
Testosterone (nmol l^{-1})[a]	1.7 ± 0.5	2.1 ± 0.8	1.5 ± 0.8	2.6 ± 0.8	0.001	0.060
SHBG (nmol l^{-1})[a]	79 ± 19	69 ± 34	46 ± 29	32 ± 11	0.070	<0.001
FAI[a,c]	2.3 ± 1.0	3.5 ± 1.8	4.4 ± 3.5	9.2 ± 4.5	<0.001	<0.001
Lipid profile						
Cholesterol (mmol l^{-1})[a]	4.7 ± 0.6	4.9 ± 0.7	4.8 ± 0.9	4.9 ± 1.1	0.382	0.915
Triglycerides (mmol l^{-1})[a]	0.8 ± 0.6	0.8 ± 0.7	1.1 ± 0.3	1.4 ± 0.9	0.350	0.015
HDL (mmol l^{-1})[a]	1.7 ± 0.4	1.7 ± 0.4	1.3 ± 0.3	1.1 ± 0.3	0.596	0.001
LDL (mmol l^{-1})[a]	2.6 ± 0.5	2.9 ± 0.6	3.1 ± 0.7	3.2 ± 0.9	0.299	0.075
LDL:HDL ratio[a]	1.7 ± 0.6	1.7 ± 0.5	2.5 ± 0.7	3.1 ± 1.4	0.086	<0.001

Data are mean ± SD.
BMI, body mass index; CT, computed axial tomography; FAI, free androgen index (([testosterone]/[SHBG]) × 100); HbA1c, glycosylated haemoglobin; HDL, high-density lipoprotein; HOMA, homeostatic model assessment of IR; LDL, low-density lipoprotein; SHBG, steroid hormone binding globulin; WHR, waist-to-hip ratio.
[a]Data analysis used age as covariate due to the significant difference between groups.
[b]Statistical analysis reported for the log-transformed data due to unequal variance.
[c]PCOS × BMI interaction $P < 0.05$.
[d]Unequal variance of data was log transformed for statistical analysis.

IR exists in women with PCOS'. We also confirm that extrinsic BMI-related IR occurs in both control and PCOS women and demonstrate that BMI has a more potent extrinsic IR impact than is seen in controls. On phenotypic subgroup analysis, we also demonstrated that 14/19 lean Rotterdam diagnosed PCOS women who had the PCO and irregular cycle phenotype without hyperandrogenism, and did not meet NIH diagnostic criteria, still greater IR on insulin clamps than did lean controls. Finally, we report that unlike IR, lipid abnormalities appear to be primarily related to BMI and are not significantly related to PCOS status *per se*.

IR is defined as an impaired biological response to exogenous or endogenous insulin, reflecting disturbed metabolic and mitogenic

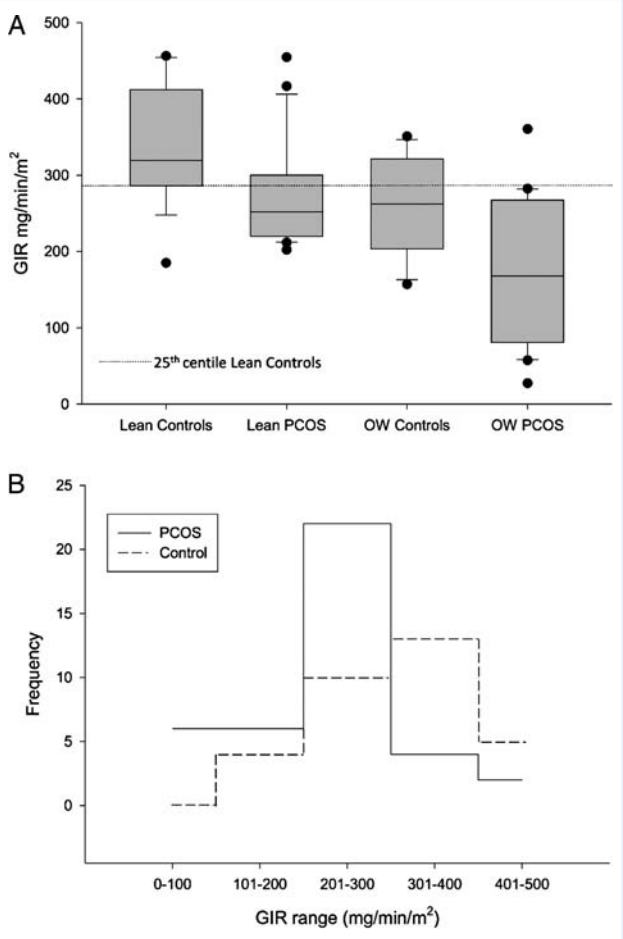


Figure 3. IR prevalence demonstrated by (**A**) box and whisker plots showing the median (central line), range (whiskers), 25th to 75th centiles (box) and individual outliers (dots) of the GIRs for lean control ($n = 19$), lean PCOS ($n = 20$), overweight/obese (ow) control ($n = 14$) and ow PCOS ($n = 20$) women with thresholds for IR in lean and ow PCOS women (WHO defined as below the 25th centiles of the lean control group) and (**B**) the shift in frequency to lower GIR in women with PCOS independent of BMI.

processes (Consensus Development Conference on Insulin Resistance (1998)). IR is a continuous variable measured with a range of different methodologies and defined based on controversial cut-off values. Studies on IR in PCOS rarely use gold standard clamp techniques and do not conventionally include a control group to define IR based on cut-offs in healthy controls, in a given population (Grundy *et al.*, 2004). Given the important role that IR plays in PCOS and the high risk of type 2 diabetes, we have studied the prevalence of IR in lean and overweight PCOS women recruited from the community, using gold standard clamp methods and defined IR using WHO criteria as a GIR below the lowest quartile for the appropriate control population (Grundy *et al.*, 2004). We also used an age-appropriate lean healthy group of women as the control group. In this context we present novel data demonstrating that overall 85% of women with PCOS were IR, with 75% of lean and 95% of obese women having WHO-defined IR. Overall our data show a higher prevalence of IR in PCOS compared with other studies using clamps (Dunaif *et al.*, 1989; Ovalle and Azziz, 2002; Rabøl *et al.*, 2011), the insulin tolerance test (68–76%; Carmina *et al.*, 1992) or frequently sample intravenous glucose tolerance test (53%; Legro *et al.*, 1998) or indeed the ethnicity independent consensus of 50–70% prevalence (Ovalle and Azziz, 2002). These discrepancies in reported IR prevalence in PCOS across the BMI range cannot only be attributed methodological differences but also the lack of a consistent definition of IR and the variable use of control populations. Given the current data, in the context of previous literature, we conclude that IR is present in the large majority of women with PCOS independent of BMI. Understanding of the high prevalence of IR in this condition arguably reduces the heterogeneity of hormonal abnormalities that contribute to metabolic and reproductive consequences of PCOS and highlights the need for greater research into the mechanistic underpinnings of IR to progress the understanding of PCOS aetiology.

Conflicting results on the prevalence of IR in PCOS also stem from the evolution of the diagnosis of PCOS, from NIH to the Rotterdam criteria. Clamp data on IR in Rotterdam-diagnosed PCOS women compared with controls across the BMI range have not been published to date. Rotterdam criteria remain controversial, with the additional diagnostic criteria of PCO on ultrasound resulting in more women diagnosed with PCOS and in the inclusion of women with milder reproductive and metabolic PCOS features compared with those diagnosed by NIH criteria (Moran *et al.*, 2011). However, we have previously demonstrated that Rotterdam, non-NIH PCOS cases still have metabolic abnormalities compared with controls (Moran and Teede, 2009). Here we advance knowledge in this area further by demonstrating for the first time that 70% of lean women diagnosed with PCOS on Rotterdam criteria, most of whom do not meet NIH criteria and who represent a milder reproductive PCOS phenotype, are still IR compared with BMI-matched controls and have a more severe metabolic phenotype than controls. Indeed subgroup analysis of the PCO and irregular cycle phenotype without hyperandrogenism (non-NIH PCOS), corrected for BMI, still had higher IR lean controls in the current study. Consistent with this finding, prior studies using less accurate measures of IR have shown that metabolic and endocrine differences including increased IR are present in women with irregular cycles and PCO (Welt *et al.*, 2006), regardless of the androgen status, although these features may be milder compared with women with hyperandrogenic phenotypes (Dewailly *et al.*, 2006). Another study using HOMA scores did not demonstrate a difference in IR between control and PCOS based on irregular cycles and PCO on ultrasound (Barber *et al.*, 2007); however, insulin clamps used in the current study are a more accurate reflection of IR than HOMA scores. It appears that the more controversial Rotterdam phenotype of PCO and irregular cycles does have elevated IR when measured using accurate methods. As controversy over PCOS diagnostic criteria persists, this finding in lean women is important and suggests that even reproductively milder subgroups with PCOS do have IR and metabolic abnormalities independent of obesity. Clinical implications of this include the need to screen for metabolic complications in both NIH and Rotterdam-diagnosed women, across the BMI range (Teede *et al.*, 2011); however,when to start and how often to screen using which tests still require further research including a better understanding of the natural history of PCOS including the different phenotypes of the condition.

PCOS-associated (intrinsic) IR has been proposed as a contributor to PCOS aetiology for over two decades, where significant IR was noted to occur independent of BMI (Dunaif *et al.*, 1989). Others have suggested that there is a significant IR in lean PCOS women compared with lean controls (Li and Li, 2012). However, intrinsic IR in PCOS has been contentious with a lack of consistent results, potentially related to limited quality of the data including variable use of inaccurate methods to assess and define IR in PCOS (Mancini *et al.*, 2009). The current study, using gold standard methodology and an internationally accepted definition of IR, demonstrates significantly higher IR in lean PCOS women versus lean controls, supporting the hypothesis that a unique 'intrinsic IR' exists in women with PCOS. In this setting, greater understanding of the underlying mechanisms and genetic basis for intrinsic PCOS-related IR is needed. Limited mechanistic IR research in PCOS suggests aberrant peripheral insulin signalling through insulin receptor substrate 1 in PCOS, compared with controls (Corbould *et al.*, 2005, 2006; Diamanti-Kandarakis and Papavassiliou, 2006). Other proposed mechanisms of intrinsic IR may include reduced mitochondrial biogenesis (Skov *et al.*, 2007) and/or function (Rabøl *et al.*, 2011), but the results thus to date are not supportive of this hypothesis (Hutchison *et al.*, 2012). Further investigation into potential mechanisms is warranted to progress understanding of PCOS aetiology and to identify potential future therapeutic targets in this common condition. Indeed, current literature suggests that metformin, an insulin sensitizer, may be more effective in non-obese women with PCOS (Misso *et al.*, 2013), suggesting that therapies may selectively target intrinsic and extrinsic IR differentially. Likewise, the impact of lifestyle intervention may primarily target extrinsic BMI-related IR in PCOS, with further research needed to clarify mechanisms of therapeutic action in PCOS.

Obesity is well known to increase extrinsic IR in the general population, with the impact of BMI on IR being more marked once BMI increases beyond 27 $kg\,m^{-2}$ (Garca-Estevez *et al.*, 2004). As we confirm here, obesity exacerbates IR in PCOS (Teede *et al.*, 2007) with overweight women with PCOS having higher IR (Dunaif *et al.*, 1989; Mancini *et al.*, 2009; Hutchison *et al.*, 2011). Our current data also highlight the novel finding that there is an increased impact of BMI on IR, in women with PCOS, compared with in BMI-matched controls. As visceral fat has been implicated in the aetiology of IR in in PCOS (Lord *et al.*, 2006; Hutchison *et al.*, 2011), we investigated if visceral fat accounted for the differences in IR between PCOS and controls. Our data demonstrated that visceral fat makes similar contributions to IR in PCOS as it does in control women, indicating that visceral fat is more likely a contributor to extrinsic IR and also showing that visceral fat is not the only driver of differences in IR between PCOS and controls. The impact of BMI and visceral fat on the interaction between extrinsic and intrinsic IR in PCOS is not yet well understood and warrants further research. Overall, increased BMI and increased visceral fat in PCOS reflect a significant health concern and the current data strengthen the argument for aggressive lifestyle intervention to prevent weight gain and induce weight loss to minimize associated extrinsic IR (Teede *et al.*, 2011). Notably, the similar degree of IR in lean PCOS and overweight control women is consistent with the high risk of diabetes in PCOS, independent of BMI and reinforces the need for screening for glucose intolerance even in lean PCOS women (Meyer *et al.*, 2005; Moran *et al.*, 2010; Teede *et al.*, 2011). In contrast, we did not observe a significant relationship between lipids and PCOS status, with lipids primarily related to BMI status, again highlighting the need for aggressive weight management.

The strengths of the current study include a community-recruited cohort of PCOS women, the extension of PCOS diagnostic criteria to include those with Rotterdam-diagnosed PCOS, the use of the hyperinsulinaemic–euglycaemic clamp methodology with pre-clamp dietary control and the inclusion of healthy controls who were matched for BMI and were not taking any medication. Limitations include not using glucose tracer techniques to completely characterize the IR, and the lack of matching for body composition and age. Also there were proportionately more women diagnosed by Rotterdam, but not NIH criteria, in the lean compared with in the overweight PCOS group.

We report for the first time the prevalence of IR on clamp studies in women with Rotterdam-diagnosed PCOS, where 75% of lean and 95% of overweight women with PCOS are IR, based on WHO criteria, using age-appropriate lean healthy control women. We show that the overwhelming majority of women with PCOS are IR including those who are lean and those who meet Rotterdam criteria but not NIH diagnostic criteria for PCOS, specifically those with the PCO and irregular cycle, non-hyperandrogenic PCOS phenotype. Additionally, we confirm that IR is higher in women with PCOS in the presence of an inherent, intrinsic IR that is further worsened with increasing BMI and demonstrate a more potent extrinsic IR impact of BMI in PCOS compared with controls. Given the clinical implications of IR including a high risk of type 2 diabetes, future research is needed into mechanisms of intrinsic and extrinsic IR in PCOS and into novel targeted therapies. Potentially, lifestyle change may best manage extrinsic IR (Hutchison *et al.*, 2011; Harrison *et al.*, 2012) and pharmacological interventions, such as metformin, may best target intrinsic PCOS-related IR; however, more research is needed.

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Authors' roles

N.K.S. and H.J.T. were involved with conception and design, analysis and interpretation of data. N.K.S. and S.C. analysed and interpreted data and wrote the manuscript. S.C., A.E.J., S.K.H., C.L.H. and R.F.G. researched the data. All authors undertook the critical revision for important intellectual content and approved the final version for publication.

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Conflict of interest

None declared.

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Chapter 4a Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity.

Declaration of Co-Authorship and Co-Contribution: Paper Incorporated in Thesis by Publication

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

Declaration by: **Signature:** **Date**

Samantha Cassar  27/11/2014

Paper Title:

Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity.

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

Name	Contribution %	Nature of Contribution
Samantha Cassar	70	Involved in the conception, collected data, performed laboratory analysis, statistically analysed and interpreted data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Helena J. Teede	10	Involved with conception and design, analysis and interpretation of data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Cheryce L. Harrison	3	Collected data, undertook critical revision for important intellectual content and approved the final version for publication
Anju E. Joham	3	Collected the data, undertook critical revision for important intellectual content and approved the final version for publication.

Lisa J. Moran	4	Collected the data, assisted with statistical analysis, undertook critical revision for important intellectual content and approved the final version for publication.
Nigel K. Stepto	10	Involved with conception and design, analysis and interpretation of data, undertook the critical revision for important intellectual content and approved the final version for publication.

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University

and will be held for at least five years from the date indicated below.

			Date
Samantha Cassar	[redacted]		27/11/2014
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4.0 Introduction

In Chapters 2 and 3 of this thesis, it was demonstrated that when assessed with the gold standard euglycemic-hyperinsulinemic technique, IR is an intrinsic feature of PCOS. IR is known to play a key role in the pathogenesis of metabolic disorders such as CVD and T2DM (DeFronzo and Abdul-Ghani, 2011, Knowler et al., 2002). Early detection and treatment of IR through lifestyle or pharmacological interventions can delay the onset and reduce the severity of the associated cardio-metabolic conditions. As IR is a prominent feature of PCOS (Group, 2012) and hyperinsulinaemia is directly linked to a number of the clinical features present in PCOS including hyperandrogenism and reproductive dysfunction (Fauser et al., 2012), treatment of IR is important in reducing the severity of the metabolic and reproductive complications in PCOS. Therefore effective early detection of IR in women with PCOS is clinically important.

The AES and the ESHRE/ASRM position statements recommend patients with PCOS be screened for IGT and diabetes by OGTT. However ESHRE/ASRM conceded that there is no utility for measuring insulin in most cases of women with PCOS (Fauser et al., 2012). This highlights the complexity of measuring IR.

The assessment of IR can be performed by numerous direct and indirect methods, each having their own advantages and limitations. Although the euglycemic-hyperinsulinemic clamp is considered to be the gold standard technique to quantitatively assess IR its complexity makes it impractical to be performed in a clinical setting. Simple surrogate measures to determine IR based on fasting plasma glucose and insulin concentrations have been developed. HOMA correlates well with

the euglycaemic-hyperinsulinaemic clamp in a general population but tends to become inaccurate in insulin resistant populations especially when basal insulin levels are within the normal, high-normal, or slightly elevated ranges (Ciampelli et al., 2005, Diamanti-Kandarakis et al., 2004). Furthermore, these surrogate measures are reported to be not sensitive enough to detected IR in women with PCOS (Ciampelli et al., 2005, Diamanti-Kandarakis et al., 2004). Therefore, clinicians would benefit from a simple, feasible, reliable and accurate way to detect and monitor IR including in PCOS.

4.1 Potential Markers of Insulin Resistance

It has been observed that various metabolic hormones, cytokines and adipokines, such as ghrelin, resistin, visfatin, glucagon-like peptide- 1 (GLP-1), leptin, PAI-1, glucose-dependant insulinotropic polypeptide (GIP) and C-Peptide, are associated with IR (Gnacinska et al., 2009). These hormones play pivotal roles in appetite and body weight regulation, glucose homeostasis, insulin sensitivity, lipid metabolism and CVD risk. As such, they are potentially relevant to the elevated cardio-metabolic risk present in PCOS (Chaput and Tremblay, 2006, de Luca and Olefsky, 2008, Shoelson et al., 2007, Wang et al., 2008). Importantly, they may also provide novel biomarkers of IR that can be measured from a single blood sample. Therefore, measurement of these markers may provide a quick and simple way to identify women who have IR and follow up with further diagnosis and early treatment. They may also differentiate women with PCOS who are at risk of metabolic disease.

The association of these potential biomarkers with IR and metabolic features in both lean and overweight women are not fully understood. Traditionally, these biomarkers have been measured in isolation using single assays. IR has a complex

pathophysiology which is not fully understood in PCOS. Therefore an integrated approach (multiplex based assays) to investigate adipokines, gut hormones and other metabolic biomarkers relating to IR may provide a better option to predict the presence of IR than isolated measurements. In 2008, the Bio-Plex human diabetes multiplex immunoassay was developed to allow simultaneous quantification of 10 proteins involved in glucose metabolism with relevance for diabetes including C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, PAI-1, resistin and visfatin.

4.2 Bio-Plex Immunoassay

The Bio-Plex human diabetes multiplex immunoassay procedure is similar to a sandwich enzyme-linked immunoabsorbent assay (ELISA). Magnetic beads on a 96 well plate are utilised to analyse multiple biomarkers in 3 to 4 hours using approximately 12.5 µl of serum or plasma samples per well. The magnetic beads are conjugated with specific antibodies targeting the biomarker of interest in the sample. Following incubation of a biotynilated detection antibody, the final detection reaction is completed with the addition of a streptavidin-phycoerythrin conjugate. Detection of phycoerythrin fluorescence is then performed using the Bio-Plex microplate reader system (Biorad Laboratories, Hercules, CA, USA). The beads and phycoerythrin are excited with separate specific wavelengths of light and detected with a photomultiplier tube. The intensity of the fluorescence detected is proportional to the concentration of the specific biomarkers in each sample (Bio-Plex Assay Reader). The analyte concentration is calculated against an internal standard curve, using software provided by the manufacturer (Bio-Plex Manager Software). Reproducibility (intra and inter coefficient of variation 3-6% and 2-6%), reliability and high

sensitivity are the best features of this assay (Amato et al., 2014, Costantini et al., 2012).

Although IR is present in up to 85% women with PCOS (Stepto et al., 2013), it is not currently part of the diagnostic criteria and it is not routinely measured in clinical practice. There is current agreement that IR should not yet be measured in the clinical setting given methodological limitations. Therefore clinicians will benefit from other measures that could reliably predict IR in PCOS and metabolic biomarkers offers a potential solution.

4.3 Summary

The aim of this chapter was to determine whether metabolic biomarkers involved in the pathophysiology of IR can be used to predict insulin sensitivity measured with the euglycemic-hyperinsulinemic clamp. We also aimed to compare levels of metabolic biomarkers between lean and overweight women with and without PCOS to explore associations between these biomarkers and PCOS phenotype, IR and adiposity.

4.4 My Role

My role in this chapter was to collect and statistically analyse and interpret the data. I set up the Bio-Plex human diabetes multiplex immunoassay in our laboratory, optimised the technique over a two month period and performed the biochemical analysis. I also primarily wrote and submitted the manuscript for publication, which was accepted by Clinical Endocrinology (Impact Factor 3.35, Q1 Journal).

ORIGINAL ARTICLE

Biomarkers and insulin sensitivity in women with Polycystic Ovary Syndrome: Characteristics and predictive capacity

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Summary

Objective Polycystic ovary syndrome (PCOS) is a common endocrine disorder associated with metabolic complications. Metabolic biomarkers with roles in obesity, glycaemic control and lipid metabolism are potentially relevant in PCOS. The aim was to investigate metabolic biomarkers in lean and overweight women with and without PCOS and to determine whether any biomarker was able to predict insulin resistance in PCOS.

Design Cross-sectional study.

Patients Eighty-four women (22 overweight and 22 lean women with PCOS, 18 overweight and 22 lean women without PCOS) were recruited from the community and categorized based on PCOS and BMI status.

Measurements Primary outcomes were metabolic biomarkers [ghrelin, resistin, visfatin, glucagon-like peptide-1 (GLP-1), leptin, plasminogen activator inhibitor -1 (PAI-1), glucose-dependent insulinotropic polypeptide (GIP) and C-Peptide] measured using the Bio-Plex Pro Diabetes assay and insulin sensitivity as assessed by glucose infusion rate on euglycaemic–hyperinsulinaemic clamp.

Results The biomarkers C-peptide, leptin, ghrelin and visfatin were different between overweight and lean women, irrespective of PCOS status. The concentration of circulating biomarkers did not differ between women with PCOS diagnosed by the Rotterdam criteria or National Institute of Health criteria. PAI-1 was the only biomarker that significantly predicted insulin resistance in both control women ($P = 0{\cdot}04$) and women with PCOS ($P = 0{\cdot}01$).

Conclusions Biomarkers associated with metabolic diseases appear more strongly associated with obesity rather than PCOS status. PAI-1 may also be a novel independent biomarker and predictor of insulin resistance in women with and without PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting up to 18% of reproductive aged women, depending on the diagnostic criteria used and population studied.[1] Typical clinical features of the syndrome include hyperandrogenism, oligomenorrhoea, anovulation and polycystic ovaries. These features have major reproductive, psychological and metabolic consequences, representing a considerable health and economic burden across the lifespan.[2,3]

Women with PCOS have intrinsic insulin resistance (IR) and compensatory hyperinsulinaemia that is independent of, but exacerbated by obesity.[4] Insulin resistance can predispose women with PCOS to diabetes, metabolic syndrome and cardiovascular disease (CVD) risk factors including dyslipidaemia, hypertension and impaired glucose tolerance[2,5–8] and is thought to drive hyperandrogenism in PCOS, underpinning the metabolic and reproductive features of the condition.[9] Altered insulin sensitivity in PCOS can be attributed to insulin receptor and/or post-binding defect in the insulin signalling pathways.[10,11] IR, reflected by hyperinsulinaemia, can lead to elevated circulating levels of free fatty acids (FFA), triglycerides and LDL cholesterol and decreased levels of HDL cholesterol. The release of FFA activates release of various inflammatory cytokines, which may play a role in IR and cause a disruption in glucose homeostasis by mediating the insulin signaling pathways in muscle, adipocytes and liver.[12] Cytokines have the ability to disrupt insulin signaling pathways either directly by inhibiting serine phosphorylation of insulin receptor substrate (IRS) or indirectly through inflammatory pathways including the c-Jun N-terminal kinase (JNK) and I-kappa B kinase β (IKKβ)/NFκB pathways.[13,14] Obesity, diabetes and metabolic syndrome are associated with chronic inflammation;[15] however, the presence of inflammation and its association with IR in PCOS is still contentious.

Given that PCOS is a condition with a range of metabolic complications related to IR, it is important to measure various circulating markers that may be associated with IR[16] and inflammation and that play pivotal roles in appetite and body weight regulation, glucose homeostasis, lipid metabolism and CVD risk.[17] These biomarkers include leptin, due to its ability to promote insulin sensitivity and resistin, which can impair insulin sensitivity.[18–20] These measures may also be novel biomarkers reflecting IR, which is otherwise difficult to accurately measure in clinical practice, and may help to identify women with PCOS who are at risk of metabolic disease. However, these biomarkers have mainly been measured in isolation, which limits the understanding of the relative interaction and detailed pathways contributing to metabolic dysfunction in this condition.

The aim of this study was to identify differences in plasma biomarker levels between lean and overweight women with and without PCOS and evaluate the usefulness of these biomarkers to predict IR in this cohort of women. We also compare the predictive capacity of these biomarkers with that of clinical indices of IR, waist circumference and SHBG. We hypothesized that women with PCOS will display an adverse biomarker profile independent of obesity and that novel biomarkers will be independent predictors of IR.

Methods

Participants

Lean (BMI <25 kg/m^2) and overweight/obese (BMI >27 kg/m^2), premenopausal women aged 20–40 years with and without PCOS were recruited through community advertisements. This study is an extension of detailed mechanistic studies examining insulin resistance in women with PCOS compared to healthy controls.[4,21] with additional overweight participants with and without PCOS (n = 23) from a separate study to expand the sample size.[22] To achieve this sample size, eligible participants according to the above inclusion criteria were selected at random. Only the first 23 samples that met the eligibility criteria and that were appropriately collected and stored were used in this analysis. This work expands on a previous smaller study[22] in overweight women only, adding lean women with and without PCOS and end-points measured. In total, 84 premenopausal women with and without PCOS were investigated.

Participants were categorized into four groups: lean control, lean PCOS, overweight control and overweight PCOS. Women were diagnosed as having PCOS by expert endocrinologists (AEJ, RFG, SKH and HJT) based on the Rotterdam criteria, two of the three features of clinical or biochemical hyperandrogenism, [Ferriman-Gallwey score >8, total T > 2·7 nmol/l, or free androgen index (FAI) >4·5], polycystic ovaries (PCO) on ultrasound and irregular menstrual cycles (cycle length outside 21–35 days or <8 cycles per year) with the exclusion of other aetiologies (congenital adrenal hyperplasia, androgen-secreting tumours, Cushing's syndrome, hyperprolactinemia, thyroid dysfunction and adrenal disorders).[6] Women with PCOS were also divided into groups based on *a priori* phenotypes and BMI. Control women had no history of diagnosed PCOS and did not present with any features of PCOS.

Exclusion criteria included pregnancy, smoking, diabetes, uncontrolled hypertension, lipid-lowering, hormonal (e.g. oral contraceptive pill) or insulin-sensitizing medication unless willing to cease medications for 3 months before study commencement. This study received Institutional Review Board approval (Standing Committee on Ethics in Research Involving Humans for Monash University and Human Research Ethics Committee of Southern Health), and all participants gave written informed consent prior to participation.

Study design

At screening, participants were assessed to determine whether they met diagnostic and inclusion criteria and medications affecting end-points were ceased.[12] After a 3-month washout, data were collected in the mid-follicular phase of the menstrual cycle where feasible.

Clinical and biochemical measurements

Participant's anthropometric assessments of BMI and percentage body fat were measured by dual-energy X-ray absorptiometry (DEXA) [GE Lunar Prodigy (GE Lunar Corp., Madison, WI, USA) using operating system version 9] as previously reported.[4,21,22]

Insulin sensitivity was assessed in a subgroup of 61 participants by the euglycemic-hyperinsulinemic clamp technique as previously reported.[4] Briefly, the clamp was performed 72 h after a standardized high-carbohydrate diet following an overnight fast. Venous fasting blood samples were collected, analysed and stored as appropriate. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 mU/m^2 per minute for 120 min generating an elevated, stable insulin concentration from 10–120 min, with plasma glucose maintained at approximately 5 mmol/l, using variable infusion. Glucose was assessed every 5 min (YSI 2300 STAT PLUS; YSI Life Sciences, Yellow Springs, OH, USA), and the glucose infusion rate (GIR) was calculated during last 30 min of the insulin-stimulated period and expressed as glucose (mg), per body surface area (m^2) per minute.

Blood was collected in a fasting state in various anticoagulant and serum clot activator blood tubes. Serum tubes were allowed to clot at room temperature for 30 min prior to being centrifuged. Tubes were centrifuged at 3000 revolutions per minute for 15 min at 4 °C. The plasma or serum was then transferred to a clean polypropylene tube and frozen at −80 °C for future analysis. Stored plasma and serum samples were batch analysed as appropriate for fasting glucose, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides, insulin and testosterone as previously reported.[6]

For the quantification of biomarkers, serum samples were thawed and again centrifuged. Each sample was analysed in

duplicate for ghrelin, resistin, visfatin, glucagon-like peptide 1 (GLP-1), leptin, plasminogen activator inhibitor-1 (PAI-1), glucose-dependent insulinotropic polypeptide (GIP) and C-Peptide and was performed using the Bio-Plex Pro Diabetes assay (CAT#171-A7001M; Biorad Laboratories, Hercules, CA, USA) according to manufacturer's instructions.

Statistics

All data are presented as mean ± SD or means and 95% confidence intervals (CI). Results are presented for 84 participants for all variables except DEXA (81 participants) and insulin clamp (61 participants; 28 control and 33 women with PCOS) results. The Shapiro–Wilk statistic was chosen to assess normality and some metabolic biomarkers were log-transformed to overcome issues of heteroscedascity. Statistical analysis was performed on the log-transformed data; and back transformed data has been reported for ease of interpretation. Comparisons between groups were performed using a one-way analysis of variance (ANOVA) and a one-way analysis of covariance (ANCOVA; age or age and BMI), with *post hoc* multiple comparisons performed using LSD correction. A receiver operating characteristic (ROC) curve was constructed to examine whether a biomarker could be used as a diagnostic test to predict IR in women with PCOS and suggest cut-off values. Sensitivity (*y*-axis) against 1-specificity (*x*-axis) was plotted and the area under the curve (AUC) was computed. The AUC quantifies the diagnostic value of the test; the greater the AUC, the better the performance of the test. An AUC value of 0·5 or less means the test result is no better than chance, a value of 1·0 means the predictive value of the biomarker is perfect. The accuracy of each metabolic biomarker is assessed in terms of the probability that the test correctly classifies a noninsulin resistant subject as negative (specificity) and the probability that the metabolic biomarker correctly classifies an insulin resistant subject as positive (sensitivity)[23]. For ROC curve analysis, IR was defined based on insulin clamp derived GIR levels as less than the 25th percentile of lean matched controls (non-PCOS-specific World Health Organization criteria[24]), as previously published by our group.[11] Statistical analysis was conducted using SPSS for Windows 20.0 software (SPSS Inc., Chicago, IL, USA) and significance was accepted at $P < 0{\cdot}05$.

Calculation of prospective sample size for C-peptide and leptin were conducted *a priori* using data reported by Morin Papunen *et al.*[25] Based on these results a total sample size of $n = 68$ was required to observe differences in C-peptide at 95% power ($P < 0{\cdot}05$) and $n = 44$ was required to observe differences in leptin at 95% power ($P < 0{\cdot}05$). At the time of participant recruitment, it was difficult to calculate the exact sample size needed for this study as limited previous literature has assessed the end-point measures of the other biomarkers in lean and overweight women with and without PCOS. Previous work[26] has investigated lean and overweight controls in comparison to lean and overweight PCOS women divided based on phenotype for PAI-1. Based on these results using overweight and lean controls and lean Rotterdam and overweight NIH groups, a total sample size of $n = 56$ was required to observe differences in PAI-1 at 80% power ($P < 0{\cdot}05$). On *post hoc* power analysis, we were powered ($\alpha = 0{\cdot}05$) to detect observed differences in C-peptide (95%), leptin (72%), visfatin (50%), resistin (40%), GIP (35%), ghrelin (34%), PAI-1 (24%) and GLP-1 (5%) between four groups.

Results

Baseline characteristics

The characteristics and biomarkers of lean and overweight PCOS and control participants are shown in Table 1. From the women who underwent an insulin clamp, 64% of lean PCOS women, 56% of overweight control women and 91% of overweight PCOS women were classified as insulin resistant. The overweight control group were older ($P < 0{\cdot}001$) than the lean control, lean PCOS and overweight control groups. By definition the overweight control and PCOS groups had a higher BMI ($P < 0{\cdot}001$). The overweight groups had higher levels of C-peptide and leptin and lower levels of ghrelin compared to lean groups, after correcting for age (Table 2). Visfatin was elevated in the overweight control group compared to the lean control and lean PCOS group ($P < 0{\cdot}05$).

Diagnostic criteria

Participants were divided into subgroups based on PCOS diagnostic criteria with the NIH PCOS group being heavier (BMI; 33 ± 9 kg/m^2) compared to the Rotterdam PCOS group (25 ± 4 kg/m^2, $P < 0{\cdot}001$) and controls (26 ± 5 kg/m^2, $P < 0{\cdot}001$). On assessment of reproductive and metabolic differences between the groups and with adjustment for age and BMI; testosterone, FAI, and LDL:HDL ratio were higher and SHBG levels were lower in the NIH group compared to the Rotterdam and control groups ($P < 0{\cdot}05$). Furthermore, insulin sensitivity was lower in the NIH (195 ± 80 mg/min/m^2) and Rotterdam groups (269 ± 86 mg/min/m^2) compared to controls (312 ± 89 mg/min/m^2; $P < 0{\cdot}05$). There were no differences in the concentration of biomarkers between the three groups (Table 3).

Receiver operating characteristic curves

A ROC curve analysis was performed to assess the ability of each biomarker to distinguish between women with and without IR based on euglycaemic–hyperinsulinemic clamp in all women ($n = 61$) and in PCOS and control women separately (Table 4). In all participants, the ROC curve indicated that leptin, ghrelin and PAI1 were able to predict IR with cut-off points at 1·9 ng/ml (sensitivity 69% and specificity 71%), 0·21 ng/ml (sensitivity 66% and specificity 65%) and 10·03 ng/ml (sensitivity 77% and specificity 54%) respectively (Fig. 1a). ROC curve analysis indicated that leptin, ghrelin and PAI1 were able to predict IR in the control group (Fig. 1b) with cut-off levels of 2·67 ng/ml (sensitivity 83% and specificity 89%), 0·21 ng/ml (sensitivity 83% and specificity 68%), and 13·6 ng/ml (sensitivity 83% and

Table 1. Anthropometric, metabolic and hormonal characteristics of the PCOS affected women according BMI matching or diagnostic criteria (NIH or Rotterdam) grouping

Clinical features	Lean control $n = 22$	Lean PCOS $n = 22$	Overweight control $n = 18$	Overweight PCOS $n = 22$	*P*-value unadjusted	*P*-Value Age adjusted	Control $n = 40$	Rotterdam criteria $n = 23$	NIH criteria $n = 21$	*P*-value	*P*-value Age/BMI adjusted
General characteristics											
Age (years)	28 ± 6[c]	27 ± 4[c]	35 ± 5[a,b,d]	29 ± 4[c]	<0·001*	–	31 ± 6	28 ± 5	28 ± 4	0·08	–
BMI (kg/m^2)	22 ± 2[c,d]	23 ± 2[c,d]	31 ± 3[a,b,d]	35 ± 7[a,b,c]	<0·001*	<0·001*	26 ± 5[g]	25 ± 4[g]	33 ± 9[e,f]	<0·001*	–
Waist Circumference (cm)	72 ± 7[c,d]	74 ± 7[c,d]	95 ± 9[a,b,d]	100 ± 16[a,b,d]	<0·001*	<0·001*	83 ± 14	78 ± 8	97 ± 20	<0·001*	0·746
Total fat mass (%)	27 ± 6[c,d]	31 ± 7[c,d]	45 ± 6[a,b]	46 ± 6[a,b]	<0·001*	<0·001*	36 ± 11	34 ± 9	44 ± 10	0·01*	0·86
Insulin Sensitivity											
Fasting Glucose (mmol/l)	4·6 ± 0·3	4·6 ± 0·4	4·9 ± 0·4	4·8 ± 0·6	0·09	0·31	4·7 ± 0·4	4·6 ± 0·4	4·7 ± 0·6	0·56	0·63
Fasting Insulin (pmol/l^1)	5·2 ± 3·6[c,d]	4·0 ± 1·8[c,d]	17·8 ± 9·3[a,b,d]	25·3 ± 13·5[a,b,c]	<0·001*	<0·001*	10·9 ± 9·2	9·9 ± 14·5	19·9 ± 12·4	0·01*	0·93
GIR (mg/min/m^2)	339 ± 76[b,c,d]	276 ± 67[a,d]	258 ± 93[a,d]	167 ± 88[a,b,c]	<0·001*	<0·001*	312 ± 89[f,g]	269 ± 86[e,g]	195 ± 80[e,f]	0·001*	0·05*
Androgen Profile											
Testosterone (nmol/l)	1·7 ± 0·5[d]	2·1 ± 0·8[d]	1·4 ± 0·7[d]	2·5 ± 1·0[a,b,c]	<0·001*	0·001	1·6 ± 0·6[g]	1·9 ± 0·8[g]	2·7 ± 0·9[e,f]	<0·001*	0·001*
SHBG (nmol/l)	78 ± 19[c,d]	71 ± 34[c,d]	41 ± 16[a,b]	35 ± 11[a,b]	<0·001*	<0·001*	61 ± 26	67 ± 35	38 ± 16	0·001*	0·40
FAI	2·3 ± 1·0[c,d]	3·4 ± 1·8[d]	4·0 ± 2·9[a,d]	8·0 ± 4·1[a,b,c]	<0·001*	<0·001*	3·1 ± 2·2[g]	3·5 ± 2·4[g]	8·2 ± 3·9[e,f]	<0·001*	0·002*
Lipid Profile											
Cholesterol	4·7 ± 0·6	4·9 ± 0·7	4·7 ± 0·8	4·9 ± 0·9	0·74	0·75	4·7 ± 0·7	4·8 ± 0·7	5·0 ± 0·9	0·47	0·26
Triglycerides	0·8 ± 0·6	0·8 ± 0·3	1·2 ± 0·6	1·1 ± 0·6	0·04*	0·26	1·0 ± 0·6	0·8 ± 0·3	1·1 ± 0·6	0·20	0·62
LDL:HDL Ratio	1·1 ± 0·01[b,c,d]	1·7 ± 0·5[a,c,d]	2·4 ± 0·6[a,b]	2·5 ± 0·8[a,b]	<0·001*	<0·001*	1·7 ± 0·8[g]	1·8 ± 0·5	2·5 ± 0·8[e]	<0·001*	0·03*

BMI, body mass index; FAI, fasting androgen index; GIR, glucose infusion rate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SHBG, sex hormone binding globulin.
*Indicates a significant difference ($P < 0·05$) across all groups as assessed by the ANCOVA.
[a]Indicates a significant difference from lean control ($P < 0·05$).
[b]Indicates a significant difference from lean PCOS ($P < 0·05$).
[c]Indicates a significant difference from overweight control ($P < 0·05$).
[d]Indicates a significant difference from overweight PCOS ($P < 0·05$).
[e]Indicates a significant difference from the control group ($P < 0·05$).
[f]Indicates a significant difference from the Rotterdam criteria group ($P < 0·05$).
[g]Indicates a significant difference from the NIH group ($P < 0·05$).

Table 2. Biomarker concentrations in lean and overweight PCOS and non-PCOS participants

Biomarkers	Lean control $n = 22$	Lean PCOS $n = 22$	Overweight control $n = 18$	Overweight PCOS $n = 22$	*P*-value unadjusted	*P*-value Age adjusted
PAI-1# (ng/ml)	10·5 (7·7–14·2)	8·98 (6·4–12·5)	10·5 (7·4–14·9)	7·62 (5·7–10·3)	0·41	0·44
Resistin# (ng/ml)	1·0 ± (0·78–1·28)	0·97 (0·75–1·25)	1·19 (0·84–1·69)	1·43 (0·99–1·52)	0·32	0·26
C-Peptide (ng/ml)	0·16 ± 0·07[c,d]	0·15 ± 0·05[c,d]	0·25 ± 0·15[a,b]	0·32 ± 0·22[a,b]	<0·001*	<0·001*
Ghrelin (ng/ml)	0·25 ± 0·05[c,d]	0·25 ± 0·07[c,d]	0·16 ± 0·07[a,b]	0·17 ± 0·08[a,b]	<0·001*	<0·001*
GIP (ng/ml)	0·03 ± 0·01	0·03 ± 0·02	0·03 ± 0·02	0·04 ± 0·02	0·53	0·22
GLP1 (ng/ml)	0·06 ± 0·01	0·06 ± 0·01	0·06 ± 0·02	0·06 ± 0·01	0·79	0·475
Leptin#(ng/ml)	1·2 ± (0·88–1·6)[c,d]	1·3 ± (0·93–1·8)[c,d]	6·1 (4·4–8·6)[a,b]	7·1 (5·4–9·5)[a,b]	<0·001*	<0·001*
Visfatin (ng/ml)	0·75 ± 0·15[c]	0·70 ± 0·16[c]	0·83 ± 0·33[a,b]	0·81 ± 0·32	0·07	0·33

GIP, glucose dependent insulinotrophic polypeptide; GLP-1 glucagon-like peptide-1; PAI-1, plasminogen activator inhibitor-1.
Data reported as mean ± SD, except for log-transformed variables which are reported as back transformed mean with 95% CI.
#Indicates the variable has been log-transformed.
*Indicates a significant difference ($P < 0·05$) across all groups.
[a]Indicates a significant difference from lean control ($P < 0·05$).
[b]Indicates a significant difference from lean PCOS ($P < 0·05$).
[c]Indicates a significant difference from overweight control ($P < 0·05$).
[d]Indicates a significant difference from overweight PCOS ($P < 0·05$).

specificity 63%) respectively, however this was only true for PAI-1 in PCOS with a cut-off level of 10·03 ng/ml (sensitivity 70% and specificity 89%; Fig. 1c). Waist circumference was able to predict IR in all groups and SHBG was able to predict IR in PCOS (Table 4).

Discussion

The present study expands on prior research by examining metabolic biomarkers in lean and overweight women with and without PCOS and exploring their ability to predict IR. With regards to novel metabolic biomarkers, C-peptide, ghrelin and leptin were different between overweight and lean groups suggesting the role of adiposity in metabolic dysfunction. Biomarker concentrations were not different between women diagnosed with PCOS using the Rotterdam or NIH criteria. While a number of biomarkers were related to IR in controls (leptin, ghrelin, PAI-1), we report that PAI-1 was the only biomarker that was related to IR in women with PCOS.

Consistent with prior literature, women with PCOS in the current study were more insulin resistant compared to the control group.[27] Other conditions associated with IR, including obesity and diabetes, have adverse biomarker profiles with impaired GIP and GLP activity and elevated PAI-1, visfatin, leptin and resistin.[28] As PCOS is a unique insulin resistant condition with intrinsic IR independent of obesity, markers for CVD and diabetes may be more prevalent. However, contrary to our hypothesis, we report here that C-peptide, leptin, ghrelin and visfatin differed between the lean and overweight groups irrespective of PCOS status, but did not differ by PCOS status. It is difficult to explain the lack of difference in these biomarkers between the control and PCOS groups. The underlying cause of intrinsic IR in PCOS is unknown. In the current study, many of the biomarkers predict IR in all subjects and are abnormal in

Table 3. Biomarker concentration based on PCOS diagnostic criteria

Biomarkers	Control $n = 40$	Rotterdam criteria $n = 23$	NIH criteria $n = 21$	*P*-value unadjusted	*P*-value age/ BMI adjusted
PAI-1# (ng/ml)	10·5 (6·7–12·7)	7·6 (5·6–10·4)	9·2 (6·7–12·7)	0·22	0·53
Resistin# (ng/ml)	1·08 (0·89–1·3)	0·97 (0·76–1·24)	1·25 (1·01–1·55)	0·35	0·18
C-Peptide(ng/ml)	0·20 ± 0·13	0·16 ± 0·07	0·33 ± 0·23	0·001*	0·17
Ghrelin (ng/ml)	0·21 ± 0·07	0·22 ± 0·07	0·20 ± 0·10	0·66	0·43
GIP (ng/ml)	0·03 ± 0·02	0·03 ± 0·02	0·04 ± 0·02	0·23	0·85
GLP1 (ng/ml)	0·06 ± 0·01	0·06 ± 0·01	0·06 ± 0·01	0·97	0·86
Leptin#(ng/ml)	2·55 (1·82–3·58)	1·57 (1·09–2·24)	6·33 (4·21–9·50)	<0·001*	0·18
Visfatin (ng/ml)	0·78 ± 0·3	0·72 ± 0·2	0·86 ± 0·3	0·23	0·49

GIP, glucose-dependent insulinotrophic polypeptide; GLP-1 glucagon-like peptide-1; PAI-1, plasminogen activator inhibitor-1.
Data reported as mean ± SD, except for log-transformed variables which are reported as back transformed mean with 95% CI.
#Indicates the variable has been log-transformed.
*Indicates a significant difference ($P < 0·05$) across all groups.

Table 4. Receiver operator characteristic curve analysis

	All Participants		Control		PCOS	
Predicting variable	Area under ROC curve Mean (95% CI)	*P*-value	Area under ROC curve Mean (95% CI)	*P*-value	Area under ROC curve Mean (95% CI)	*P*-value
Waist Circumference	0·740 (0·612–0·869)	0·001*	0·773 (0·515–1·000)	0·041*	0·734 (0·554–0·913)	0·044*
SHBG	0·744 (0·614–0·873)	0·001*	0·652 (0·448–0·855)	0·263	0·877 (0·759–0·996)	0·001*
C-peptide	0·615 (0·463–0·767)	0·146	0·447 (0·101–0·794)	0·703	0·617 (0·382–0·851)	0·322
Ghrelin	0·728 (0·593–0·863)	0·004*	0·868 (0·714–1·000)	0·008*	0·667(0·446–0·887)	0·157
GIP	0·524 (0·368–0·680)	0·762	0·373 (0·093–0·653)	0·356	0·511 (0·279–0·743)	0·925
GLP1	0·396 (0·242–0·551)	0·191	0·781 (0·583–0·979)	0·042*	0·458 (0·246–0·671)	0·724
Leptin	0·735 (0·596–0·874)	0·003*	0·816 (0·551–1·000)	0·022*	0·717 (0·522–0·911)	0·066
PAI-1	0·665 (0·519–0·811)	0·038*	0·781 (0·586–0·976)	0·042*	0·806 (0·647–0·964)	0·010*
Resistin	0·500 (0·344–0·656)	1·000	0·430 (0·185–0·675)	0·611	0·600 (0·372–0·828)	0·396
Visfatin	0·416 (0·259–0·572)	0·287	0·320 (0·097–0·543)	0·192	0·511 (0·294–0·728)	0·925

GIP, glucose dependant insulinotrophic polypeptide; GLP-1 glucagon like peptide-1; PAI-1, plasminogen activator inhibitor-1.
*Indicates significance ($P < 0·05$).

overweight groups. As many of the biomarkers do not predict IR in PCOS, it is possible that inflammatory cytokines play a more significant role in IR associated with high BMI rather than PCOS. However, we acknowledge that a type 2 statistical error may be an alternative explanation as to why metabolic markers were not abnormal in PCOS.

Insulin resistance can be defined as an impaired physiological response to exogenous and endogenous insulin, reflecting disturbed metabolic and mitogenic processes.[29] The euglycaemic–hyperinsulinaemic clamp is the gold standard technique for assessing insulin sensitivity yet is invasive, time consuming and not practical in a clinical setting. Thus, given the high prevalence of IR in women with PCOS, the use of surrogate markers to determine IR has become important. The majority of,[26,30,31] but not all[22,32] literature report elevated levels of PAI-1 in lean and overweight women with PCOS. Elevated PAI-1 concentration is also a risk factor for diabetes and CVD and is present prior to the development of clinical features of these conditions.[16] It was expected that PAI-1 levels would be elevated in PCOS with strong associations with obesity, IR and menstrual disturbances, all common features of PCOS.[26,30,31]

Although we did not find any statistical differences in PAI-1 levels between groups, we identified PAI-1 as the only biomarker related to IR in both PCOS and controls. Furthermore, PAI-1 was able to predict IR with relatively high accuracy (≥70%). Waist circumference and SHBG were similar in their ability to predict IR, except in the control groups where PAI-1 outperformed SHBG. It has been suggested that markers of dyslipidemia (triglycerides and HDL cholesterol) may be prerequisites to high plasma PAI-1 concentrations and maybe therefore impact PAI-1's ability to predict IR.[33] PAI-1 regulates fibrinolysis and is produced in adipose tissue and endothelial cells.[32] PAI-1 may play a role in endothelial dysfunction and indirectly in IR through reduction of insulin-mediated skeletal muscle glucose uptake by decreasing the blood flow, and as a consequence glucose extraction and glucose/insulin delivery, in insulin sensitive tissues like skeletal muscles.[34] It therefore may become important in reflecting IR in women with PCOS.

Ghrelin concentrations were reduced whereas leptin concentrations were markedly elevated in the overweight group *vs* the lean group. Furthermore, ghrelin and leptin were able to predict IR in the control group only. Leptin and ghrelin have been associated with obesity and insulin resistant conditions and play important roles in appetite and body weight regulation.[35,36] Elevated levels of ghrelin stimulate appetite whereas elevated levels of leptin suppress appetite, increase energy expenditure, and promote fatty acid oxidation.[37] Lower levels of ghrelin in obesity may be a compensatory adaptation to reduce food intake and aid in weight regulation[38] or leptin may be elevated in obesity due to leptin resistance.[39] Ghrelin and leptin also have a role in glucose and fat metabolism, inflammation, vasodilation and ovarian function. Therefore it is feasible that chronically altered ghrelin and leptin in overweight women may contribute to metabolic and reproductive dysfunction in women with and without PCOS.[40,41] Studies have reported administration of high doses of insulin reduces ghrelin secretion and vice-versa, highlighting an interplay between the two hormones.[42] Leptin levels have been reported to strongly correlate with IR independently of obesity.[42] Importantly, although leptin and ghrelin appear to be good markers of IR in the general population, they do not predict the presence of intrinsic IR in PCOS and are substandard to SHBG and waist circumference in predicting IR in this population.

We highlight the importance of further research into IR and clinically applicable potential markers that reflect IR. However, the study does have some limitations. Firstly it has a small sample size, did not divide participants based on phenotypes rather diagnostic criteria and had incomplete euglycemic-hyperinsulinemic clamp data. Secondly, this study was a cross-sectional study rather than a longitudinal study, therefore caution needs to be taken when interpreting the results. Long-term prospective studies are required to confirm if these biomarkers are able to predict the future development of IR. The strengths of this study

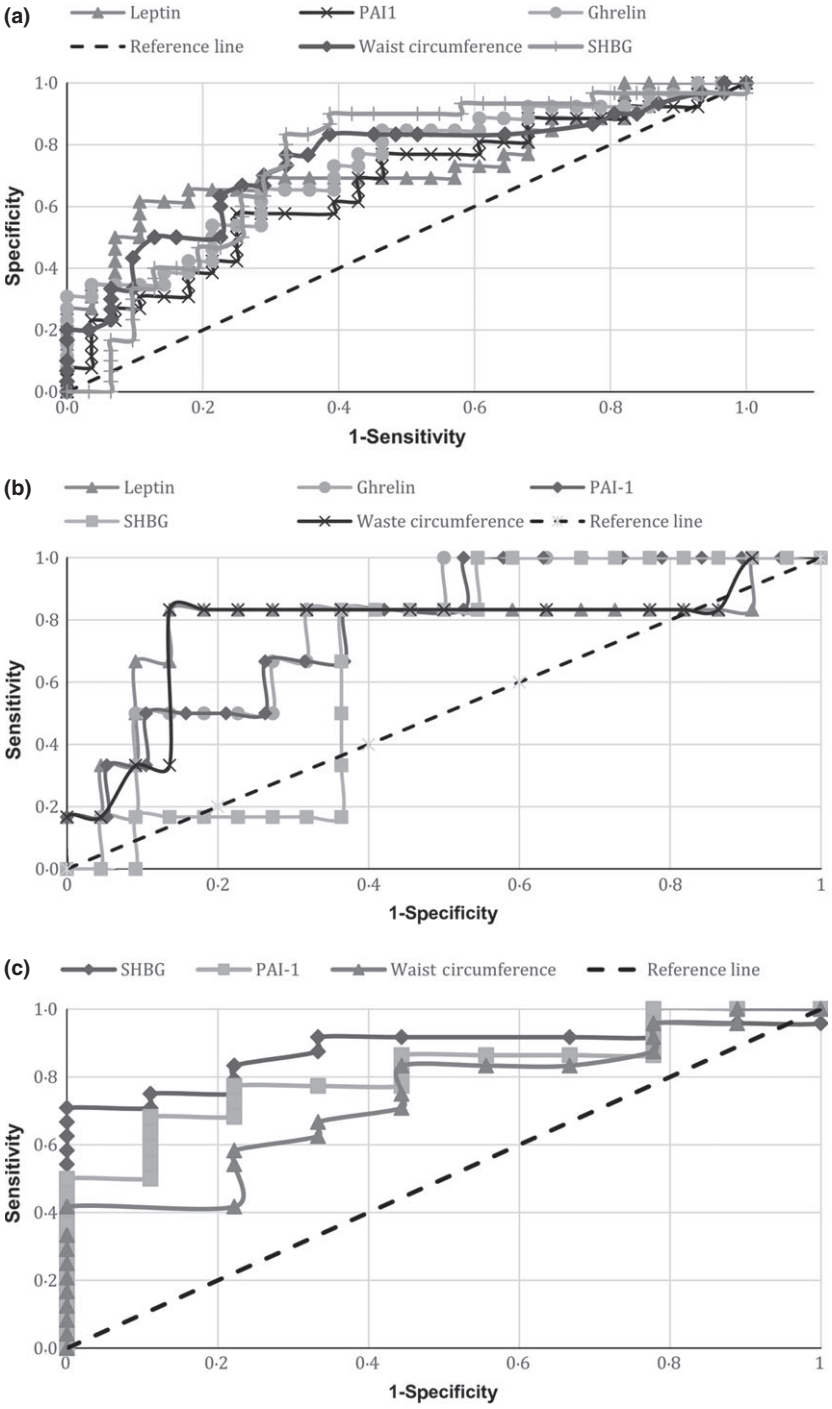

Fig. 1 ROCcurve representing metabolic biomarker ability to identify IR in all (a), control (b) and PCOS (c) participants.

include a cohort of women that were recruited through the community, the inclusion of both lean and overweight women and the use of the two different diagnostic criteria. Here, insulin clamps were performed on lean and overweight women with and without PCOS to provide an evaluation of IR, which is uncommon in the PCOS biomarker literature. Furthermore, this is one of the first studies to take a universal approach investigating biomarkers that play a role in glucose, insulin and lipid metabolism and CVD risk factors, together with IR on clamp studies. Overall this is a tightly defined study population, which provides the opportunity to assess the effects of both obesity and of PCOS status on the relevant biomarkers of diabetes and CVD.

Conclusion

In the current study, we found that biomarkers associated with metabolic diseases appear more strongly associated with adiposity rather than PCOS status, across the four well defined groups of lean and overweight women with and with-

out PCOS. PCOS is a common condition underpinned by IR and hyperandrogenism, strongly associated with obesity and has a high prevalence of metabolic abnormalities. We note here that PAI-1, leptin and ghrelin levels may emerge as relatively accurate predictors of IR in the general population, but PAI-1 is potentially a biomarker specific to PCOS. However, waist circumference and SHBG were equally able to identify IR, especially in PCOS. This study has important implications not only in identifying novel biomarkers that predict IR in women with and without PCOS, but also in improving the understanding of the metabolic dysfunction and pathways affected by PCOS. Clinicians would benefit from simple and reliable markers to identify IR and metabolic risk in PCOS and more research to identify and evaluate such biomarkers is needed. Future research could assess the longitudinal association of these biomarkers with PCOS phenotypes and in other population groups, including healthy men and women and those with diabetes, to determine if these biomarkers have clinical utility in detecting and predicting IR.

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Chapter 4b Response to ‘Insulin Sensitivity and Leptin in Women with PCOS.’

Declaration of Co-Authorship and Co-Contribution: Paper Incorporated in Thesis by Publication

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

Declaration by: **Signature:** **Date**

Samantha Cassar  27/11/2014

Paper Title:

Response to 'insulin sensitivity and leptin in women with PCOS.'

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

Name	Contribution %	Nature of Contribution
Samantha Cassar	70	Wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Helena J. Teede	11	Assisted with writing the letter to the editor, undertook critical revision for important intellectual content and approved the final version for publication.
Cheryce L. Harrison	3.3	Provided critical revision for important intellectual content and approved the final version for publication
Anju E. Joham	3.3	Provided critical revision for important intellectual content and approved the final version for publication
Lisa J. Moran	3.3	Provided critical revision for important intellectual content and approved the final version for publication.
Nigel K. Stepto	9	Provided critical revision for important intellectual content and approved the final version for publication.

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University

and will be held for at least five years from the date indicated below.

		Date
Samantha Cassar	[redacted]	27/11/2014
Helena J. Teede		27/11/2014
Cheryce L. Harrison		27/11/2014
Anju E. Joham		27/11/2014
Lisa J. Moran		27/11/2014
Nigel K. Stepto		27/11/2014

4.5 Introduction

The submission of the manuscript titled ‘Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity’ in Chapter 4a generated some discussion amongst the research community and as a result a letter to the editor was written by Agilli et al., titled ‘Insulin sensitivity and leptin in women with PCOS’ (Agilli et al., 2014). This letter to the editor expressed concern regarding the reporting of confounding factors that may have impacted on my results and therefore interpretation of data. This chapter is the response letter written to the editor that addresses the concerns expressed by the authors (Agilli et al., 2014).

4.6 My Role

I primarily wrote this manuscript, which was edited by my supervisors Professor Helena Teede and Associate Professor Nigel Stepto.

Article Type: 11 Letter

Title: Response to 'insulin sensitivity and leptin in women with PCOS'

Key words: polycystic ovary syndrome, insulin resistance

Short title: Leptin and PCOS

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Conflict of interest: The authors declare no conflicts of interest.

Dear Editor,

We thank Agilli et al.[1] for their comments and the opportunity to clarify a number of points from our work entitled 'Biomarkers and insulin sensitivity in women with PCOS: Characteristics and predictive capacity.[2]

Agilli et al., raised concerns regarding potential confounding factors for leptin.[1] We noted in our study by Cassar et al., and made reference to other papers outlining this cohort, that participants underwent a screening process to assess eligibility.[2, 3] The medical screening process was conducted by experienced endocrinologists and involved the assessment of the overall health and wellbeing of each participant, discussing previous and current medical conditions, medications and vitamins taken and family history. We confirm that none of the participants had liver disease, inflammatory diseases, rheumatoid arthritis, systemic lupus erythematosus, or a history of Helicobacter pylori infection. None were taking glucocorticoids, antipsychotics, antihypertensive, hormonal or insulin sensitising medication, linoleic acid, zinc, vitamin E or vitamin A. On detailed review of the medical files, three participants were taking antidepressant medication, two were taking vitamin D supplements and five were taking omega-3 fatty acid supplements.

We agree with Agilli et al., that potential confounding factors should ideally be excluded, or be reported. In our cohort of otherwise healthy women, except for their

PCOS, the large majority were not on any medications or supplements with limited projected impact on results. We repeated our statistical evaluation as outlined in the manuscript, omitting the three and five participants on antidepressants and omega-3 fatty acid supplements respectively, and leptin relationships remained similar.

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Chapter 5 Mitochondrial Dysfunction as a Potential Mechanism Underlying Intrinsic Insulin Resistance in Polycystic Ovary Syndrome

5.0 Introduction

The mechanisms underlying intrinsic IR in PCOS are largely unknown. Skeletal muscle play a major role in glucose uptake and whole-body energy homeostasis (DeFronzo et al., 1985). A reduction in insulin-stimulated glucose uptake into muscle is often observed in metabolic conditions such as IR, obesity, and T2DM and this reduction has been attributed to a decrease in skeletal muscle mitochondrial content, size and function (Joseph et al., 2012)

5.1 Role of mitochondria in insulin resistance

The predominant function of mitochondria is ATP generation by OXPHOS (Brand and Nicholls, 2011). Other functions include fatty acid oxidation, mediating oxidative stress and generating cellular signalling molecules (Goodpaster, 2013). Metabolic homeostasis is tightly controlled by the mitochondria through the oxidisation of both carbohydrates and lipids and by transitioning between these substrates in response to insulin, substrate concentrations and the contractile status (rest versus contraction) of the muscle (Kelley et al., 1993, Jeukendrup, 2002). Abnormality in any of these processes can be termed mitochondrial dysfunction (Brand and Nicholls, 2011). IR is associated with mitochondrial dysfunction including, decreased mitochondrial number, abnormal morphology, lower levels of mitochondrial oxidative enzymes, accumulation of intracellular lipid content, and reduced adenosine triphosphate (ATP) synthesis (Kim et al., 2000, Petersen et al., 2004, Ritov et al., 2005). Intracellular lipid accumulation causes reduced mitochondrial oxidative capacity in skeletal muscle of T2DM patients and their offspring (Befroy et al., 2007, Mogensen et al., 2007) and acute lipid infusion or chronic elevation of plasma free fatty acids (FFA) causes hepatic insulin resistance (Brehm et al., 2006). Patients with nonalcoholic fatty liver

disease (NAFLD) also exhibit mitochondrial abnormalities including lesions, depletion of mitochondrial DNA (mtDNA), decreased activity of respiratory complexes (Perez-Carreras et al., 2003, Pessayre and Fromenty, 2005) and impaired mitochondrial β-oxidation, which contribute to liver injury (Befroy et al., 2007, Fromenty and Pessayre, 1995). Furthermore, excessive adipose tissue as seen in obesity increases lipolysis, and the release of FFA further contribute to mitochondrial dysfunction. Thus, mitochondrial dysfunction is associated with IR not only in skeletal muscle, but also in the liver and adipose tissue (Kim et al., 2008, Samuel et al., 2004, Steinberg et al., 1996). Whether the development of metabolic conditions or their progression is a result of mitochondrial dysfunction or whether mitochondrial dysfunction is a consequence of the disease is currently under debate.

5.2 Mitochondrial dysfunction and intrinsic insulin resistance in PCOS

Conflicting data exists on whether IR is caused by mitochondrial dysfunction in PCOS. A reduction is skeletal muscle OXPHOS gene expression has been reported in obese insulin resistant women with PCOS compared to age and BMI-matched healthy controls (Skov et al., 2007). This decrease was also accompanied by reduced levels of peroxisome proliferator-activated receptor γ coactivator α (PGC-1α) leading authors to conclude that PGC-1α mediated the reduction in OXPHOS gene expression, which may predispose women with PCOS to T2DM (Skov et al., 2007). Moreover, thiazolidinedione (pioglitazone) treatment has the ability to improve insulin sensitivity, in part, by upregulating the genes involved in mitochondrial OXPHOS

(Skov et al., 2007). Mitochondrial complex I respiration is also reduced in leukocytes obtained from lean women with PCOS compared with age and BMI-matched healthy control women (Victor et al., 2009). However, caution needs to be taken when interpreting these results as leukocyte mitochondrial function may be different to mitochondrial function in skeletal muscle (Brand and Nicholls, 2011).

In contrast, others have not reported mitochondrial dysfunction in PCOS. Obese women with PCOS had no difference in gene expression or protein abundance of OXPHOS enzymes compared to BMI matched controls (Hutchison et al., 2011). There were also similarities in mitochondrial function, content and mass in isolated mitochondria obtained from myotubes of obese women with PCOS (Eriksen et al., 2011). The authors concluded that any dysfunction was secondary to IR rather than a primary cause. In a recent study investigating mitochondrial function in lean and obese women with and without PCOS (Rabol et al., 2011), mitochondrial function, investigated using high resolution respirometry, and content were similar in both groups. A limitation of the majority of studies investigating mitochondrial function in PCOS is the lack of account for fitness (Rabol et al., 2011). Fitness levels have been shown to effect mitochondrial function (Frisard and Ravussin, 2006, Ritz and Berrut, 2005).

5.3 Techniques to measure Mitochondrial Function

Various techniques have been employed to assess mitochondrial function and for the purposes of this chapter, mitochondrial function will be defined as the ability of the mitochondria to consume oxygen (Brand and Nicholls, 2011). High resolution respiration is one technique that has the ability to measure mitochondrial oxidative capacity in small amounts of isolated mitochondria or permeabilized muscle fibers

(Lanza and Nair, 2009) in a twin chamber instrument manufactured by Oroboros Instruments (Innsbruk, Austria). This method allows detailed analysis of different states of the Kreb cycle and ETC by the addition of various substrates and inhibitors. An advantage of this approach is the ability to assess multiple levels of the ETC in the same tissue sample (Brand and Nicholls, 2011).

5.4 Summary

In summary, the role of mitochondrial function in IR is still under debate and few studies have investigated the contribution of mitochondrial dysfunction to intrinsic IR in PCOS. Therefore Chapter 5 will aim to improve our knowledge in this area.

5.5 My Role

My role in this chapter was to conduct fitness testing and prepare the resting muscle biopsy for analysis. Following the euglycameic-hyperinulinaemic clamp, I transported the muscle biopsy sample from Monash University to Victoria University and immediately performed mitochondrial respiration. I was also responsible for ordering all the consumables and optimizing the respiration technique in our laboratory. I independently optimised and completed western blot analysis and cytochrome C analysis in the laboratory. I wrote the manuscript and constructed all the tables and figures. This manuscript is unpublished work.

Mitochondrial dysfunction as a potential mechanism underlying intrinsic insulin resistance in Polycystic Ovary Syndrome

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic disorder of premenopausal reproductive aged women and is associated with numerous clinical sequelae including metabolic (obesity, T2DM, impaired glucose tolerance), reproductive (infertility, irregular menstrual cycles), psychological (depression and anxiety) and social health implications across the lifespan (Azziz et al., 2006, Teede et al., 2010). The aetiology of PCOS is unknown, complex and multifactorial. A combination of intrinsic (genetic) and environmental factors play a role in the development of the syndrome (Dunaif et al., 1989, Norman et al., 2007, Stepto et al., 2013). Lean and obese women with PCOS are less insulin sensitive than body weight matched controls, indicating that insulin resistance is intrinsic to the condition (Dunaif et al., 1989, Stepto et al., 2013). Furthermore, obesity exacerbates the degree of insulin resistance in both healthy and PCOS women, worsening the clinical characteristics of hyperandrogenism in the condition (Teede et al., 2010). However, the molecular mechanisms underlying intrinsic IR in PCOS is unknown.

Mitochondrial dysfunction has been linked to the development of skeletal muscle IR in certain conditions (Peterson 2006). The mitochondria play an important role in energy homeostasis by metabolising nutrients in order to produce energy, in the form of ATP. An imbalance between energy intake and expenditure can lead to mitochondrial dysfunction and chronic diseases (Kim 2008). Mitochondrial dysfunction describes a reduction in mitochondrial content and/or reduced mitochondrial respiratory capacity. It may inhibit insulin sensitivity through lower

fatty acid oxidation, accumulation of intracellular lipid species, incomplete β-oxidation, and elevated generation of reactive oxygen species (Kim et al., 2000, Petersen et al., 2004, Ritov et al., 2005). Therefore underlying mitochondrial dysfunction may contribute to the pathophysiology of many chronic diseases with underlying IR including diabetes (Mogensen et al., 2007).

At present there are few studies that have investigated the contribution of skeletal muscle mitochondria to insulin resistance in PCOS. A reduction in OXPHOS gene expression has been reported in obese insulin resistant women with PCOS compared to age and BMI-matched healthy controls (Skov et al., 2007). Furthermore, in a recent study investigating mitochondrial function in lean and obese women with and without PCOS, mitochondrial function (investigated by high resolution respiration [HRR]) and content were similar between all groups (Rabol et al., 2011).

A limitation of the majority of studies investigating mitochondrial function in skeletal muscle of women with PCOS is the lack of attention given to lean women with the syndrome and a disregard for potential confounding factors including level of fitness. Fitness levels have been shown to affect mitochondrial function and should be answered and reported (Ritz and Berrut, 2005).

Since IR is intrinsic to PCOS we wanted to investigate whether intrinsic IR was accompanied by skeletal muscle mitochondrial dysfunction in lean women with PCOS. We did this by using HRR, citrate synthase assay and immunoblotting of skeletal muscle from lean women with and without PCOS in conjunction with assessment of insulin sensitivity using the gold standard euglycemic-hyperinsulinemic

clamp. The advantage of studying this cohort of women is that they have less confounding factors that may contribute to IR (e.g. obesity). Furthermore, using HHR allows us to measure each of the protein complexes of the electron transport chain in isolation to determine specific dysfunctions in the mitochondria.

Methods

Participants

Twenty-eight healthy weight (BMI > 25) premenopausal women with (n=13) and without (n=15) PCOS were recruited. PCOS was diagnosed by endocrinologists based on Rotterdam criteria with two of (i) irregular menstrual cycles, (ii) clinical (hirsutism, acne) or biochemical (elevation of at least one circulating ovarian androgen) hyperandrogenism and (iii) PCO on ultrasound (Group, 2004). As this work expands on previous studies, the exclusion criteria and screening for other causes of hyperandrogenism have been previously described (Stepto et al., 2013, Hutchison et al., 2011). The Southern Health Research Advisory and Ethics Committee approved the study and participants gave written informed consent. The clinical trial registration number is ISRCTN84763265.

Study design

At screening (3 months prior to testing), standard diet and lifestyle advice were delivered (Heart Foundation recommendations (www.heartfoundation.org.au)) and medications affecting end-points including insulin sensitizers, anti-androgens and hormonal contraceptives were ceased. Data were collected in the follicular phase of the menstrual cycle where feasible.

Clinical and biochemical measurements

Fasting venous blood samples were collected and analysed for glucose, insulin, testosterone and sex hormone binding globulan (SHBG). Plasma insulin was measured using a commercial human insulin-specific RIA kit (Linco Research, St. Charles, MO). Testosterone (reference range 0-2.7 nmol/L) was measured on Beckman Coulter Unicel DXI 800 analyser (Beckman Coulter Diagnostics Australia, Gladesville, Australia) using an automated competitive binding immunoenzymatic assay. Serum SHBG (reference range 18-136 nmol/L) was measured by automated enzyme immunoassay on a Diagnostic Products Corporation Immulite analyser (Diagnostic Products Corp., Los Angeles, CA).

Euglycaemic–Hyperinsulinaemic Clamp

Insulin sensitivity was assessed by the euglycaemic–hyperinsulinaemic clamp technique as previously reported (Hutchison et al., 2011). Briefly, the clamp was performed 72 h after a standardized high-carbohydrate diet prior to an overnight fast. Venous fasting blood samples were collected, analysed and stored as appropriate after arterialization. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 mU/min/m^2 for 120 minutes generating an elevated, stable insulin concentration from 10 to 120 minutes, with plasma glucose maintained at 5 mmol/L, using variable infusion. Glucose was assessed every 5 minutes and the glucose infusion rate (GIR) was calculated during the last 30 minutes of the insulin-stimulated period and expressed as glucose (mg) per body surface area (m^2) per min. HOMA was calculated as previously described (Meyer et al., 2005b).

High-Resolution Respirometry

Mitochondrial function was investigated by using mitochondrial respiration. Muscle biopsies were taken under local anaesthesia from the *vastas lateralis* muscle using the Bergstrom needle technique. Muscle biopsies were taken at baseline before the insulin clamp commenced. A small portion of the biopsy was placed in ice cold relaxing BIOPS (Pesta and Gnaiger, 2012); The solution contains 10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5,77 mM ATP, 15 mM phosphocreatine and has a pH 7.1 medium, where the muscle is able to survive for up to 12 hours before integrity is compromised (unpublished data). All muscle was analysed within 3 hours of being taken. The muscle was mechanically separated under a microscope while kept in ice-cold BIOPS media using fine forceps. Following separation, the muscle was permeabilised with saponin (50μg/ml in BIOPS) for 30 minutes. Following permeabilisation, the muscle was rinsed for 20 minutes in MiRO5 (g 0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH2P04, 20 mM HEPES, 110 mM Sucrose, 1 g/l BSA, at pH 7.1) to ensure saponin is removed (Rabol, 2011). Each muscle was weighed following the removal of liquid by blotting on paper.

Mitochondrial Respiration Protocol

Duplicate muscle samples (2-3.5 mg) were placed in Oxygraph HHR (Oroboros, Innsbruck, Austria) chambers containing MIRO5 and catalase (Pesta and Gnaiger, 2012). All analysis was conducted following hyper-oxygenation (approximately 500 nmol O_2/mL) in respiration chambers and the oxygen level in the chambers was maintained between 200 and 500 mM O_2. Substrates and inhibitors were added consecutively. Complex I leak state (CI leak) was assessed following the addition of

pyruvate (5mmol/L), malate (2 mmol/L), glutamate (10 mmol/l). Oxidative phosphoralation (OXPHOS) with electron flux through CI was assessed by titrating ADP (0.25, 0.75, 2.5,and 5 mM). The integrity of the mitochondrial membrane was then assessed by the addition of cytochrome C (10 mmol/L). If respiration increased more than 20%, it was deemed that the mitochondrial membrane had been permealised or damaged and therefore the results were not included in analysis. Complex I and Complex II coupled respiration was assessed by adding succinate (10 mmol/L). We examined uncoupled respiration by addition of protonophore carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (1 mmol/L) in a stepwise titration. Tissue wet weight was used as the denominator for oxygen flux rates. Finally, sodium azide was added to the chambers and allowed to stabilise before residual oxygen consumption (ROX) was measured. All respiration measurements were completed in duplicate and an average of the two were taken.

Mitochondrial Content

Citrate synthase activity has been established as a reliable marker for mitochondrial density (Wang et al., 1999). Skeletal muscle frozen at -80ºC was used for analysis. Samples were thawed and mechanically homogenized on ice for 30 s and freeze-thawed a further 3 times. To each well of a 96-well microtitier plate, 10 µL of sample was loaded along with 190 µL of working solution (final concentrations in mM: 72.5 tris buffer, 0.1 DTNB, 0.45 acetyl co-A, 0.25% Triton X-100) followed by 10 µL of oxaloacetic acid (0.5 mM) before the reaction was read at 412 nm in a spectrophotometer (x-Mark, Bio-Rad laboratories, Inc., Hercules, California, USA). CS activity values are reported normalised to protein concentration determined via Bradford assay.

Immunoblotting

Approximately 60 mg of frozen muscle samples was homogenized in ice-cold buffer containing 20 mM of Tris, pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM of NaCl, 2.7 mM of KCl (Merck, Darmstadt, Germany), 1 mM of $MgCl_2$, 5 mM of $Na_4O_7P_2$, 10 mM of NaF, 1% Triton X-100, 10% glycerol (Ajax Finechem, Taren Point, Australia), 0.5 mM of Na_4VO_3, 1 μgmL^{-1} of leupeptin, 1 μgmL^{-1} of aprotinin, 200 mM of Phenylmethanesulfonyl fluoride, 1 mM of DL-Dithiothreitol, and 1 mM of benzamidine. Samples were homogenized (1:37.5 dilution (w/v)) for 2 x 20 s, using a tissue homogenizer (TH220; Omni International, Kennesaw, GA). Homogenates were then rotated for 60 minutes at 4°C and centrifuged at 15,000g for 10 minutes at 4°C, and protein concentration of the resulting supernatant was determined using a DC Protein Assay kit (Bio-Rad). Aliquots of the muscle lysate were mixed with Laemmli sample buffer and optimal protein loading was determined (Figure 1). From the optimisation results, 10 µg of total protein per sample was separated by 7.5% Criterion TGX Precast Midi Gels (Bio-Rad Laboratories Inc., USA) for 1 hour at constant rate of 200 volts in a standard vertical electrophoresis unit (SE 600 Chroma; Hoefer, Inc., Holliston, MA). After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (PVDF; Bio-Rad Laboratories Inc.) for 7 min using the Trans-Blot Turbo Transfer system (Bio-Rad Laboratories Inc.) and a low molecular weight protocol was selected. Membranes were blocked in TBST buffer (10 mM of Tris, 100 mM of NaCl, 0.02% Tween 20) containing 7.5% non-fat milk, for 1 hour at room temperature. After being washed 3 x 10 min in TBST, membranes were incubated overnight at 4°C with primary antibodies for complexes I-V of the mitochondrial electron transport system (MitoProfile® total

OXPHOS human antibody cocktail, Abcam, Sapphire Biosciences, Waterloo, NSW, Australia, ab110411). The primary antibody was diluted 1:1,000 in TBS containing 0.1 % NaN_3 and 0.1 % albumin bovine serum. Following incubation with the primary antibody, membranes were incubated with an anti-mouse (PerkinElmer, NEF822001EA) horseradish peroxidase (HRP)-conjugated secondary antibody, for 1 hour at room temperature (1:10,000 in TBST with 5% non-fat milk). Immunoreactivity was detected using a chemiluminescence reagent (Clarity Western Star C ECL substrate, Biorad) and quantified by densitometric scanning (VersaDoc^{TM} MP4000, Bio-Rad). Only linear adjustments to the whole images were performed (Quantity One, Bio-Rad), with no modifications to gamma settings. Loading control was performed using a modified version of the Coomassie stain protocol (Welinder and Ekblad, 2011). Briefly, after removing the HRP substrate, membranes were incubated with a Coomassie stain solution (0.1 % Brilliant Blue R-250 in 1:1 methanol/distilled water) for 2 minutes, followed by 5 minutes of de-staining (1:5:4 acetic acid/ethanol/distilled water). After washing, the membranes were analysed as described above.

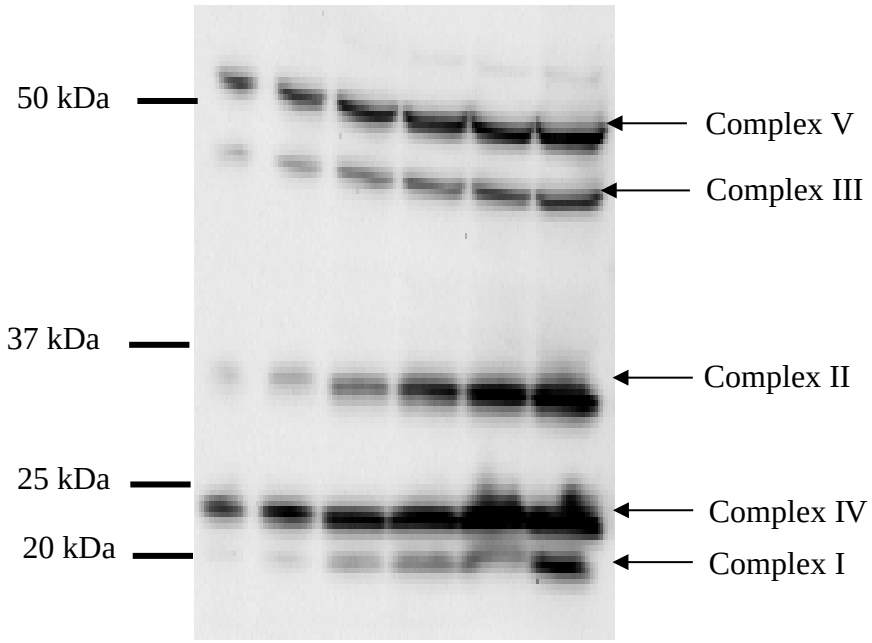

Figure 1 OXPHOS antibody linearity gel to determine optimal amount of protein to load. The amount of protein loaded in each well from left to right are; 4µg, 8µg, 20µg, 40µg, 60µg and 80µg.

Maximal Aerobic Capacity (VO_{2max})

Prior to testing, the participant's height and weight were recorded. Maximal aerobic capacity was measured telemetrically using a portable gas analyser (Cosmed K4B2, Italy), which was warmed up for at least 20 minutes before use. Following the warm up period, the pneumotach was calibrated with ten samples from a 3L calibration syringe and the gas analysers were calibrated using medically certified reference gases of known concentrations for oxygen (16%) and carbon dioxide (4%). Each participant was fitted with an appropriately sized anatomical silicone face mask to sample expired air, a sensor unit to test ventilation, oxygen, and carbon dioxide concentrations in the expired air, and a battery pack before exercise. A modified Bruce Protocol graded exercise test (Bruce et al., 1973) was performed on a treadmill (Biodex RTM 500, model no. 945-295, New York, USA). Termination of the test

occurred if the participant; a) reached volitional exhaustion and asked to stop, b) reached a plateau in VO_2 with increases in work load, c) was unable to maintain the required running speed on the treadmill, d) reached age-predicted maximum heart rate (220–age) determine through the use of a Polar heart rate monitor. VO_{2max} was defined as the highest oxygen uptake during a 1-min sampling period.

Statistical Analysis

All values are expressed as means and SD. An independent sample t-test was used to compare groups for all analysis. All statistical analysis was performed using SPSS software (version 22). Significance is reported where $P < 0.05$

Results

Baseline Characteristics

The baseline characteristics of participants are presented in Table 1. The control and PCOS groups were similar in age (P=0.6) and BMI (P=0.2). Fasting plasma glucose (P=0.5), fasting plasma insulin (P=0.2) and SHGB (p=0.5) were not different between control and PCOS groups. The glucose infusion rate (GIR), as measured by the insulin clamp, was significantly lower in the PCOS group compared to the control group (279 ± 76 vs 337 ± 65 mg/min/m^2; P=0.02), indicating some degree of insulin resistance. Testosterone was elevated in the PCOS compared to control group (2.3 ± 0.8 vs 1.7 ± 0.5 nmol/l^{-1}, P=0.04). Fitness levels tended to be higher in the control group, but this did not reach significance (P=0.07).

Table 1 – Participant characteristics

Characteristics	Control	PCOS
Age (years)	26.4 ± 5.3	27.3 ± 5.0
BMI (kg/m^2)	21.9 ± 2.2	23.0 ± 2.2
Fasting Glucose (mmol/L)	4.5 ± 0.3	4.4 ± 0.3
Fasting Insulin (pmol/L)	3.7 ±1.1	4.4 ±1.7
GIR* ($mg/min/m^2$)	337 ± 65	270 ± 76
Testosterone* (nmol/L)	1.7 ± 0.5	2.3 ± 0.8
SHBG (nmol/L)	78.8 ± 22.0	71.4 ± 33.8
FAI*	2.5 ± 1.1	3.6 ± 1.8
VO_{2max} (ml/kg/min)	39.4 ± 5.6	34.5 ± 7.0

Results are reported as mean ± SD.

* indicates significant difference (P <0.05).

GIR, glucose infusion rate; SHBG, sex hormone binding globulin; FAI, free androgen index.

Mitochondrial Efficiency

Mitochondrial respiration for Complex I leak state (P=0.4), Complex 1 OXPHOS (P=0.2), Complex I + II OXPHOS (P=0.3) and ETC maximal uncoupled respiration (P=0.1) did not differ between control and PCOS groups (Figure 2). Furthermore, ADP-stimulated basal respiration between the two groups did not differ (Figure 3). There were also no significant differences in the abundance of OXPHOS proteins in Complex I through to Complex V between groups (Figure 4a and 4b). Citrate synthase, a marker of mitochondrial density, was also not different between groups (P=0.8; Figure 5).

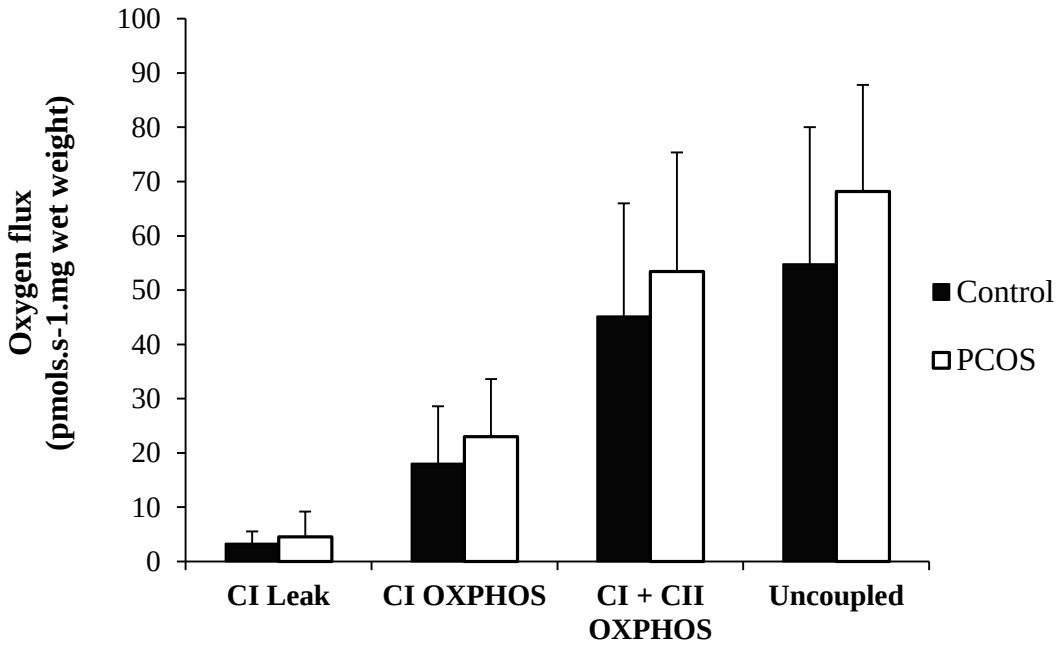

Figure 2 Mitochondrial respiration as measured by high resolution respirometry in lean women with and without PCOS

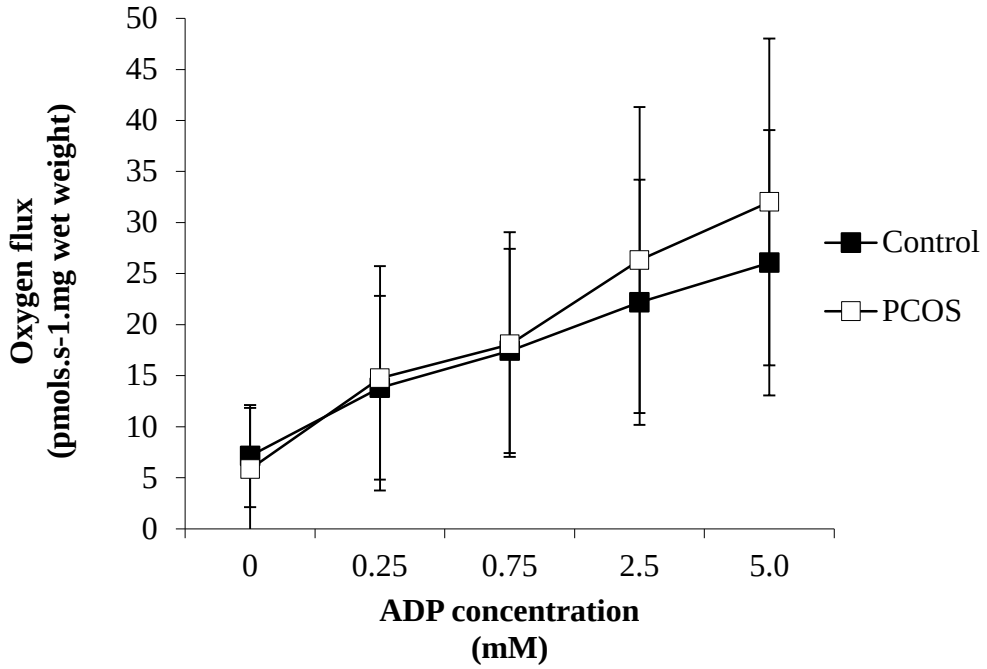

Figure 3 Mitochondrial respiration changes as a result of the addition of ADP.

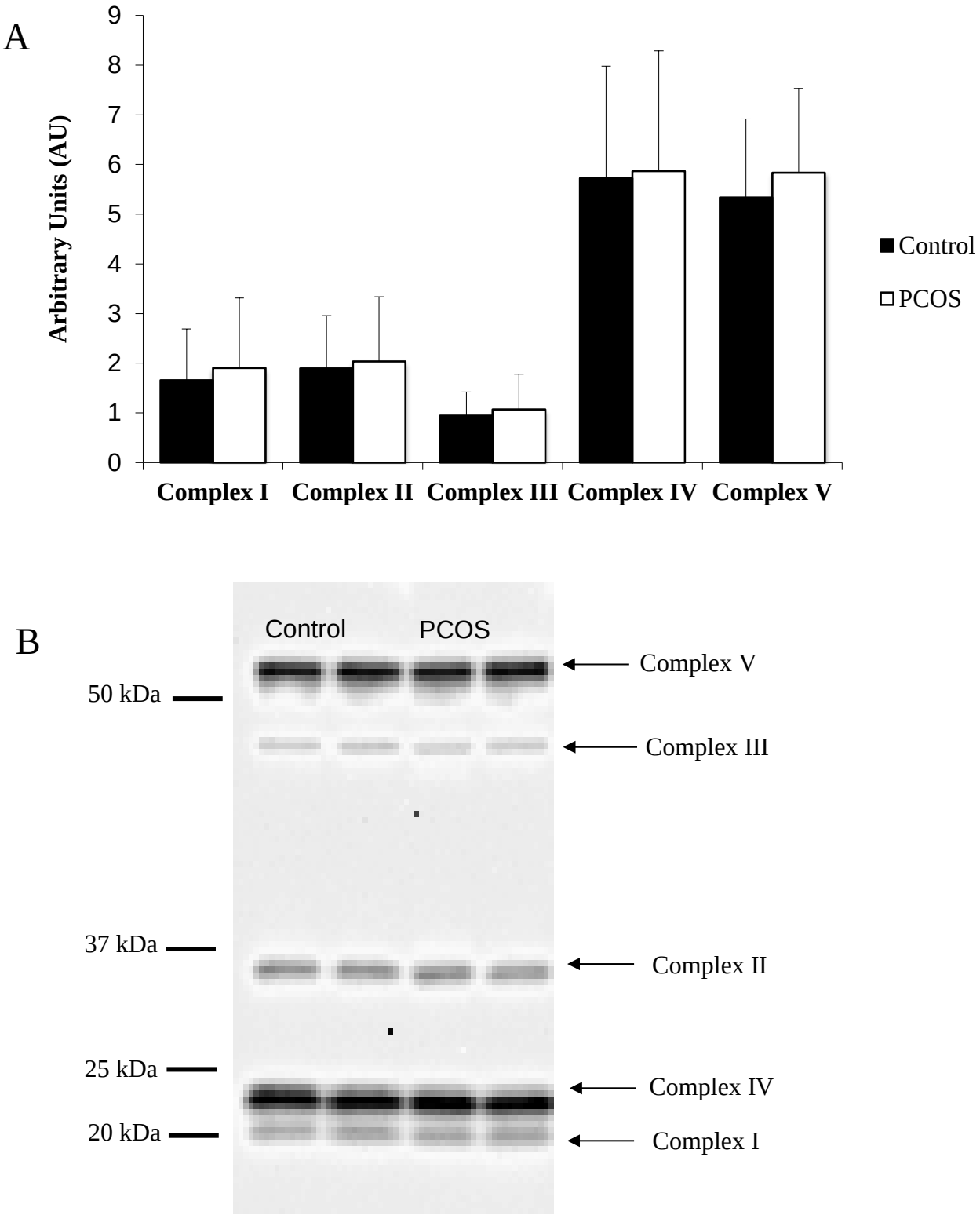

Figure 4 Mitochondrial protein expression in women with and without PCOS (A). Representative blot of mitochondrial protein expression for Complexes I-V (B).

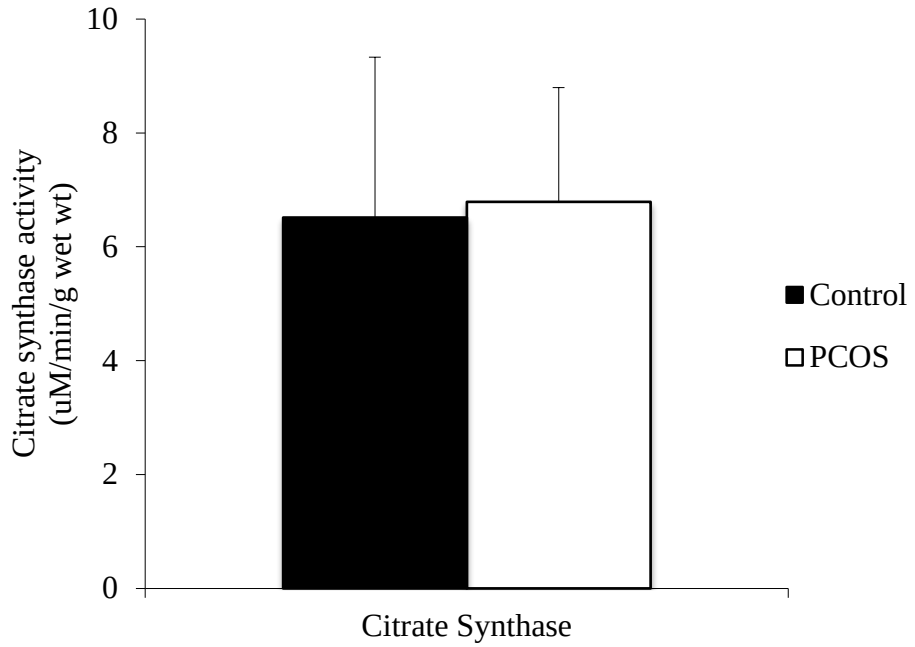

Figure 5 Citrate synthase activity as an indication of mitochondrial abundance in women with and without PCOS

Discussion

Mitochondrial dysfunction has recently been implicated in the aetiology of IR and T2DM. However, it is not yet elucidated whether intrinsic mitochondrial defects cause intrinsic IR in PCOS. Therefore, the present study examined the mechanistic role of mitochondria in intrinsic IR in PCOS. We report no evidence of skeletal muscle mitochondrial dysfunction as measured by HRR, mitochondrial protein abundance and mitochondrial content in lean insulin resistant women with PCOS, compared to a healthy control group with similar age, BMI and cardiorespiratory fitness.

Using the gold standard technique for measuring insulin sensitivity, the euglycemic-hyperinsulinemic clamp, we report that lean women with PCOS show significantly

lower insulin sensitivity supporting the hypothesis that IR is intrinsic to the condition (Dunaif et al., 1989, Stepto et al., 2013). IR can be defined as an impaired biological response to insulin as a result of metabolic and mitogenic dysfunctions (Rotterdam, 2004). Skeletal muscle accounts for the majority of insulin-mediated glucose uptake, therefore defects in skeletal muscle are likely to contribute to insulin resistance in PCOS (DeFronzo and Tripathy, 2009)

Mitochondria have been implicated in the pathophysiology of metabolic diseases (Goodpaster, 2013) and the proposed mechanisms by which mitochondrial dysfunction induces IR include a decrease in mitochondrial density and size, which results in a reduction in oxidative capacity, a reduction in the expression of mitochondrial proteins encoded by both the mitochondrial genome and nucleus, alterations in gene expression related to fatty acid oxidation, accumulation of lipid metabolites and incomplete beta oxidation (Ritz and Berrut, 2005, Skov et al., 2007). We investigated mitochondrial dysfunction using techniques to assess mitochondrial content, enzyme abundance and respiration in women with PCOS to determine if mitochondrial processes are associated with intrinsic IR.

High-resolution respirometry was used to measure mitochondrial oxidative capacity in skeletal muscle of insulin resistant lean women with PCOS. To date, only one study has used this technique in PCOS and reported no evidence of dysfunctional mitochondria respiration in lean women with PCOS (Rabol et al., 2011). In this study, lean women with PCOS did not differ in insulin sensitivity from lean controls. We therefore extend prior knowledge in this area by investigating mitochondrial oxidative capacity in insulin resistant lean women with PCOS. We report Complex I, Complex I

and II and uncoupled respiration to be similar in lean insulin resistant women with PCOS compared to lean healthy controls.

Decreased mitochondrial function as a result of reduced mitochondrial content is present in first degree relatives of type 2 diabetics, indicating its potential role in the pathogenesis of insulin resistance and T2DM (Kelley et al., 2002, Morino et al., 2005). In line with these findings, the abundance of mitochondrial proteins is also reported to be lower in obese insulin resistant and obese type 2 diabetics compared to insulin sensitive controls (Larsen et al., 2011). In the present study we assessed mitochondrial content with the surrogate marker of CS activity (Porter and Wall, 2012). However, we did not detect any differences in citrate synthase activity, or protein abundance of mitochondrial electron transport proteins in lean women with PCOS. These findings are in agreement with a previous study, which found no differences in mitochondrial protein content or mitochondrial content in obese women with PCOS (Hutchison et al., 2012)

Interestingly, Complex II respiration, uncoupled respiration (Rabol et al., 2009) and OXPHOS gene expression (Skov et al., 2007) were reduced in the skeletal muscle of obese women with PCOS. Also, insulin sensitizing treatment improves insulin sensitivity, in part due to upregulating the OXPHOS genes (Skov et al., 2008). Notably, obesity promotes lipid accumulation in skeletal muscle which is linked to reduced insulin-stimulated glucose disposal (Kim et al., 2008). Furthermore, adipocytes release adipokines including leptin, resistin and PAI-1, which have a regulatory role in metabolism (Kershaw and Flier, 2004). Therefore, the functional capacity of the mitochondria may be reduced as a result of the lipid oversupply that is

present in obesity (Muoio and Koves, 2007). It is therefore possible that any mitochondrial dysfunction in PCOS is secondary to the development of obesity. As insulin resistance in PCOS is exacerbated by weight gain, obesity-related mitochondrial dysfunction may promote further deterioration of IR in PCOS.

We report no differences in mitochondrial respiration, protein abundance in complexes I to V or mitochondrial content in lean women with PCOS and lower insulin sensitivity. Therefore, mitochondrial dysfunction does not appear to be responsible for the intrinsic IR in PCOS and may be secondary to insulin resistance and/or obesity. The complexity of IR is unlikely to be explained by a single mechanism, but rather various factors (e.g., obesity, insulin signalling defects, genetics, environmental conditions etc.,) acting synergistically. Furthermore, the mechanisms involved in IR in diabetic and obese individuals are likely to differ from those that contribute to IR in lean individuals with PCOS. Further and more comprehensive assessments of mitochondrial function are needed to elucidate the role mitochondrial play in insulin resistant conditions.

This study had a number of limitations. Firstly, we had a small sample size and data from high resolution respirometry can be variable (Rabol, 2011). Therefore, small but important differences between PCOS and control groups may have been missed. Another limitation of the study is the use of different parts of the muscle samples for respiration (fresh muscle) and the citrate synthase assay (frozen sub-muscle). The muscle from respiration analysis should have been used to quantify citrate synthase.. These latter limitations should be considered as it cannot be assumed that an equal distribution of mitochondria throughout the entire muscle sample. Although, we had a

small sample size and different muscle samples were used to measure mitochondrial respiration and content, mitochondrial function was comprehensively measured by three different techniques and findings were consistent between techniques. Strengths of the study include; comparisons were made between groups of similar age and BMI. Importantly, skeletal muscle mitochondrial function was accompanied by the physical fitness/VO_2max of each participant.

In conclusion, the results of this study show that skeletal muscle mitochondrial respiratory capacity, as measured by high-resolution respirometry, is not decreased in lean insulin resistant women with PCOS. Furthermore, we report no differences in mitochondrial protein abundance in complexes I to V or in mitochondrial density as assessed by citrate synthase activity. Therefore, mitochondrial dysfunction does not appear to be the cause of intrinsic IR but mitochondrial function may be a key target tissue in the prevention of progression of PCOS into more severe phenotypes by actively targeting muscle to promote mitochondrial function. Further work is required to understand the molecular mechanisms underlying the causes of intrinsic IR in women with PCOS and whether muscle mitochondria respond to exercise training in the same way that women without PCOS respond to exercise.

Chapter 6 Polycystic ovary syndrome and Anti-Müllerian hormone: Role of insulin resistance, androgens, obesity and gonadotropins.

Declaration of Co-Authorship and Co-Contribution: Paper Incorporated in Thesis by Publication

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

Declaration by: **Signature:** **Date**

Samantha Cassar  27/11/2014

Paper Title:

Polycystic ovary syndrome and Anti-Müllerian hormone: Role of insulin resistance, androgens, obesity and gonadotropins.

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

Name	Contribution %	Nature of Contribution
Samantha Cassar	70	Involved in the conception, collected data, performed laboratory analysis, statistically analysed and interpreted data, wrote the manuscript, constructed all graphs and tables, undertook critical revision for important intellectual content and approved the final version for publication.
Helena J. Teede	8	Involved with conception and design, interpreted data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.
Lisa J. Moran	5	Collected data, assisted with statistical analysis, interpreted data, wrote the manuscript, undertook critical revision for important intellectual content and approved the final version for publication.

Anju E. Joham	2	Collected data, undertook critical revision for important intellectual content and approved the final version for publication
Cheryce L. Harrison	2	Collected the data, undertook critical revision for important intellectual content and approved the final version for publication
Boyd J. Strauss	5	Performed and analysed body composition measurements, undertook critical revision for important intellectual content and approved the final version for publication.
Nigel K. Stepto	8	Involved with conception and design, analysis and interpretation of data, undertook the critical revision for important intellectual content and approved the final version for publication.

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University

and will be held for at least five years from the date indicated below.

			Date
Samantha Cassar	[redacted]		27/11/2014
Helena J. Teede			27/11/2014
Lisa J. Moran			27/11/2014
Anju E. Joham			27/11/2014
Cheryce L. Harrison			27/11/2014
Boyd J. Strauss			27/11/2014
Nigel K. Stepto			27/11/2014

6.0 Introduction

PCOS is typically characterised by IR, which is independent of but exacerbated by obesity, and hyperandrogenism. Anovulation and PCOM in PCOS can be attributed to an increased rate of early follicular growth and/or follicular arrest (Jonard and Dewailly, 2004). Anti-Mullerian hormone AMH is a glycoprotein that belongs to the transforming growth factor-β (TGF-β) superfamily of glycoproteins that play a role in inhibiting the initial recruitment of follicles and promoting follicular arrest (Cate et al., 1986, Durlinger et al., 2002, Durlinger et al., 2001, La Marca and Volpe, 2006). In women, AMH is produced by the granulosa cells during the development of the primary follicle and continues until the follicles reach approximately 8 mm in diameter (Weenen et al., 2004). Consequently, there is a strong correlation between AMH and antral follicle count (de Vet et al., 2002, Fanchin et al., 2003, Laven et al., 2004, van Disseldorp et al., 2010). Antral follicles are currently a key feature in PCOS diagnosis based on the Rotterdam ultrasound criteria (Jonard et al., 2003). However there are problems with cost, accuracy and accessibility of ultrasound, which reflect ovarian structure only and better functional markers of ovarian dysfunction are needed.

6.1 AMH in Polycystic Ovary Syndrome

Women with PCOS have a 2-6-fold increase in the number of primary growing follicles and higher AMH levels compared to healthy controls (Norman et al., 2007). AMH levels appear reflective of the severity of PCOS; AMH levels being higher in anovulatory and hyperandrogenic women with ‘classical’ PCOS, compared with both ovulatory women with PCO and hyperandrogenemia and anovulatory women with PCO but normal androgen levels (the newer phenotypes diagnosed under the

Rotterdam criteria) (Piouka et al., 2009).

6.2 AMH and Hyperandrogenism

Androgens have been proposed as a mechanism for PCOM and elevated AMH levels in PCOS. Using animal models, the female fetus of Rhesus monkeys display characteristic morphology of a PCO when exposed to high concentrations of testosterone in utero (Abbott et al., 2005). Furthermore, the administration of androgens through injections to rodents produced a polycystic morphology in a normal ovary (Beloosesky et al., 2004). Androgens are reported to accelerate the progression of early follicular development as well as improve granulosa cell sensitivity to FSH, an important step in dominant follicle selection (Hillier and Tetsuka, 1997, Pigny et al., 2003). However, in PCOS the follicles arrest in development and this may be due to the increase in atrial follicle production caused by androgens and as a consequence, increased production of AMH, which inhibits FSH action (Pigny et al., 2006, Pigny et al., 2003). AMH concentrations in women with PCOS are independently correlated with testosterone and the number of small antral follicles (Pigny et al., 2003, Piouka et al., 2009, Laven et al., 2004).

6.3 AMH and Insulin Resistance

In PCOS, despite the systemic IR and compensatory hyperinsulinaemia, insulin sensitivity in the ovaries tends to be preserved or increased (Baillargeon and Nestler, 2006, Poretsky, 2006). However, current literature is contradictory with respect to providing a clear association between AMH and IR. A positive correlation has been reported between the number of ovarian antral follicles and ovarian volume with hyperinsulinemia in PCOS and in women with T2DM (Carmina et al., 2005b, Pache

et al., 1993, Codner et al., 2006). Similarly a correlation has been reported between AMH levels and HOMA in women with PCOS (Chen et al., 2008a, La Marca et al., 2004). However, some studies have not reported any associations between PCOM, AMH concentrations and insulin sensitivity (Legro et al., 2005, Loucks et al., 2000). It is proposed that hyperinsulinemia may contribute to AMH production by enhancing androgen production in theca cells, stimulate the development of antral follicles, increase the sensitivity of granulosa cells to FSH, and thus increase the number of follicles and ovarian volume (Franks et al., 1999, Fulghesu et al., 1997). The role of IR in AMH production and ovarian dysfunction remains an area of contention and is hampered by a lack of research using gold standard measures of IR.

6.4 AMH and Obesity

Serum AMH levels are negatively correlated with BMI in PCOS, however there are limited studies evaluating the impact of obesity on AMH in PCOS, with few studies focusing on lean women (Caglar et al., 2013). AMH levels are traditionally lower in overweight and obese women with PCOS compared to lean women with and without PCOS (Piouka et al., 2009).

6.5 AMH and Gonadotropins

Gonadotropins are responsible for the later stages of follicular growth when a dominant follicle is selected. In PCOS, this process is disrupted and it may be as a result of FSH inhibition. FSH concentrations in PCOS are usually in the low to normal range and the administration of FSH restores follicular growth (Pasquali et al., 2011). Furthermore, FSH has been shown, in rat tissue, to down regulate the expression of AMH in granulosa cells (Baarends et al., 1995).

LH also plays a role in follicular development. A positive association between AMH and LH concentration has been reported in PCOS (Panidis et al., 2005). Cells from normal ovaries have little response to LH, but gonadotropin stimulated AMH production is approximately four times greater in cells obtained from PCO, suggesting that AMH concentrations are positively influenced by LH levels in women with PCOS and LH could also contribute to follicular arrest (Piouka et al., 2009). A strong independent positive relationship between LH and AMH production has been reported. However AMH levels do not seem to be correlated with FSH levels in women with PCOS (Piouka et al., 2009). Furthermore, higher AMH levels are reported in lean women, who have been shown to have higher LH levels compared with obese and overweight women (Piouka et al., 2009).

6.6 Summary

This chapter aims to improve knowledge in the area of PCOS by investigating the potential use of AMH as a diagnostic marker for PCOS. Given the lack of research of AMH in lean women with PCOS, the role of obesity, gonadotropins, insulin resistance and androgens in AHM production is also investigated.

6.7 My Role

I collected data, ordered all consumables and independently conducted laboratory optimisation and analysis in the form of an enzyme-linked immunosorbent assay for the quantification of AMH. I also statistically analysed and interpreted the data and wrote and submitted the manuscript to Clinical Endocrinology (Impact Factor 3.35, Q1 Journal) for publication.

Clinical Endocrinology (2014) **81**, 899–906 doi: 10.1111/cen.12557

ORIGINAL ARTICLE

Polycystic ovary syndrome and anti-Müllerian hormone: role of insulin resistance, androgens, obesity and gonadotrophins

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Summary

Objective Polycystic ovary syndrome (PCOS) is a complex endocrine disorder associated with insulin resistance, hyperandrogenism, obesity, altered gonadotrophin release and anovulatory infertility. Anti-Müllerian hormone (AMH) has been proposed as a marker of ovarian function and fertility. Across a cohort of lean and overweight women with and without PCOS, we investigated the association of AMH with insulin resistance and body composition using gold standard measures. A secondary aim was to examine whether AMH was useful to determine PCOS status.

Design Cross-sectional study.

Patients A total of 22 lean and 21 overweight women with PCOS and 19 lean and 16 overweight non-PCOS healthy controls were recruited. PCOS was diagnosed based on the Rotterdam criteria.

Measurements Euglycaemic-hyperinsulinaemic clamp for assessing insulin resistance, dual energy X-ray absorptiometry and computed tomography for assessing adiposity, and blood sampling for the assessment of androgens, gonadotrophins and AMH.

Results Anti-Müllerian hormone levels were increased in women with PCOS ($P < 0{\cdot}001$) regardless of adiposity, with this increase associated with testosterone ($P < 0{\cdot}001$) rather than insulin resistance ($P = 0{\cdot}79$), adiposity ($P = 0{\cdot}98$) or gonadotrophins. In assessing the ability of AMH to predict PCOS, a value of 30 pmol/l or higher indicated 79% of women with PCOS were correctly identified as having the condition.

Conclusion Anti-Müllerian hormone appears primarily related to androgen status suggesting a direct and predominant role of androgens in the pathophysiology of reproductive dysfunction in PCOS. As AMH reflects PCOS status, it may also be useful in PCOS diagnosis.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting 6–21% of reproductive aged women, depending on population studied and diagnostic criteria applied.[1–3] The syndrome has reproductive, metabolic and psychological features and is the most common cause of anovulatory infertility.[4] Furthermore, women with PCOS are commonly insulin-resistant (up to 75% of lean and 95% of overweight women with PCOS) and are 2–4 times more likely to be obese and develop type 2 diabetes.[5,6] This highlights PCOS as a common condition with significant health challenges.

Anti-Müllerian hormone (AMH) is produced predominantly in the ovarian granulosa cells of pre-antral and antral follicles.[7] It has been proposed as a marker of ovarian dysfunction by disrupting folliculogenesis through diminishing follicular sensitivity to follicle stimulating hormone (FSH) and inhibiting follicle recruitment and growth.[7] A growing body of literature reports increased AMH concentrations in PCOS,[8–11] which may be related to increased number of pre-antral and antral follicles or an increased production of AMH by these follicles.[12]

The mechanisms resulting in increased AMH in PCOS are poorly understood and have been attributed to obesity, insulin resistance (IR), hyperandrogenism, gonadotrophins and their complex interactions.[8,11] IR, both inherent in and exacerbated by obesity in PCOS, augments luteinising hormone (LH) and ovarian androgen production.[11] Hyperandrogenism accelerates pre-antral and antral follicular growth in the ovary, and increased LH results in premature luteinization causing follicular arrest[13] driving increased AMH levels. It is also postulated that

obesity has an additional inhibitory effect on gonadotrophin release due to an increase in aromatization of androgens in adipose tissue resulting in the suppression of LH and the consequent inhibition of the dominant follicle.[14] There is little consensus among researchers as to the primary mechanisms associated with altered AMH in PCOS.[11] There is also limited research examining the contribution of these potential regulatory features, particularly using gold standard techniques such as the euglycaemic-hyperinsulinaemic clamp or computed tomography (CT). Furthermore, there is limited research examining AMH levels in lean women with PCOS, with the current research predominantly focusing on overweight or obese women with the condition.[8,15,16]

AMH levels may be related to the severity of PCOS,[11] with higher concentrations seen in women with features including polycystic ovaries (PCO), anovulation, hyperandrogenism and IR. The diagnosis of PCOS is also challenged by the lack of updated polycystic ovary morphology criteria, with up to 80% of young women having PCO on ultrasound using modern technology.[3,17] AMH has therefore been proposed as a potential alternative diagnostic marker for PCOS, especially in circumstances when an internal transvaginal ultrasound is inappropriate.[10] However, prior research focusing on whether AMH can be used as a diagnostic feature for PCOS has predominantly recruited PCOS and healthy women seeking treatment for infertility, which may bias the results towards a higher AMH cut-off value for PCOS diagnosis.[18] There is limited research assessing the utility of AMH as a diagnostic feature for PCOS in community-recruited cohorts.

Across a community-based cohort of lean and overweight women with and without PCOS, this study aimed to comprehensively assess the relationships between AMH, IR, androgens, obesity and gonadotrophins. We also aimed to assess the ability of AMH to predict PCOS status.

Materials and methods

Participants

This cross-sectional study was conducted from July 2008 to February 2012 and is a substudy of a detailed mechanistic study in women with PCOS compared to controls[6,8,19] with samples available for measurement of AMH and IR. We studied 78 premenopausal women with and without PCOS who were recruited through community advertisements. The women were categorized according to PCOS status and body mass index (BMI), based on the threshold BMI of 27 kg/m^2, as an *a priori* decision, given that this is the inflexion point in the relationship between BMI and IR.[20] We have previously reported increased IR at this BMI cut-off in PCOS.[6] Diagnosis of PCOS was based on Rotterdam criteria with two of (1) irregular menstrual cycles (<21 or >35 days), (2) clinical (hirsutism, acne) or biochemical (increase in at least one circulating ovarian androgen) hyperandrogenism and (3) PCO on ultrasound and exclusion of related disorders.[21] Where participants had both criteria 1 and 2, they did not undergo an ultrasound, as they had met diagnostic criteria. All PCOS participants were assessed to determine whether they also meet the National Institute of Health (NIH) diagnostic criteria, defined as irregular menstrual cycles and clinical or biochemical hyperandrogenism given AMH concentrations are reported to be associated with the severity of the syndrome.[11] The exclusion criteria have been previously described.[19] Control women had regular menses and no hyperandrogenism. The Southern Health Research Advisory and Ethics Committee approved the study, and participants gave written informed consent.

Clinical and biochemical measurements

At screening (3 months prior to testing), medications affecting end-points including insulin sensitisers, anti-androgens and hormonal contraceptives were ceased. As previously published, we measured end-points during the follicular phase in controls and where possible in PCOS. In those with irregular cycles, this was not always possible; however, AMH does not vary considerably across the menstrual cycle.[22] End-point measurements including weight, BMI, total and android fat mass (dual energy X-ray absorptiometry [DXA]), visceral and subcutaneous abdominal adiposity (single slice CT), fasting glucose, insulin, lipids, SHBG, homoeostatic model of insulin resistance (HOMA-IR), insulin sensitivity by glucose infusion rate (GIR) calculated during the last 30 min of the euglycaemic-hyperinsulinaemic clamp and AMH were assessed as previously reported.[8,19] Briefly, visceral abdominal adiposity (VA) and subcutaneous abdominal adiposity (SA) were measured by a single slice CT axial image (General Electric Lightspeed CT; GE Medical Systems, Milwaukee, WI, USA) of the abdomen acquired at L4–L5 intervertebral disk space level without angulation, using a lateral pilot for location.[19] Images were saved in DICOM format and analysed using Slice-O-Matic version 4.3 software (TomoVision, Magog, Canada) where visceral fat was defined as the innermost aspect of the abdominal and oblique muscle walls and the posterior aspect of the vertebral body, as previously published.[19] Total fat mass and android fat mass were measured using a GE Lunar Prodigy DXA scan and analysed with System 11 software. The android region was defined as the area between the ribs and the pelvis (iliac crest). HOMA-IR was calculated as fasting serum insulin (mIU/l) × fasting plasma glucose (mmol/l)/22·5]. Plasma insulin was measured using a commercial human insulin-specific RIA kit (Linco Research, St. Charles, MO, USA). Serum SHBG (reference range 18–136 nmol/l) was measured by automated enzyme immunoassay on a Diagnostic Products Corporation Immulite analyzer (Diagnostic Products Corp., Los Angeles, CA, USA). Testosterone (reference range 0–2·7 nmol/l) was measured on Beckman Coulter Unicel DXI 800 analyzer (Beckman Coulter Diagnostics Australia, Gladesville, Australia) using an automated competitive binding immunoenzymatic assay. Free androgen index (FAI) was calculated as testosterone/SHBG × 100 (reference range 0·65–10·90). AMH was analysed using an enzyme-linked immunosorbent assay (A16507, Immunotech, Beckman and Coulter Company) in a single batch from

frozen serum samples with an intra- and interassay variability 8·9% and 6·9%, respectively.

Statistical analysis

Results are presented as mean ± SD with 95% confidence intervals (CI) where appropriate. Normality was assessed using the Shapiro–Wilk statistic. Statistical analysis was performed on the log-transformed data; however, raw data has been reported for ease of interpretation. Differences between the four groups (lean control, lean PCOS, overweight control and overweight PCOS) were determined using one-way ANCOVA (adjusting for age) with LSD post-hoc corrections. Relationships between variables were assessed using two-tailed Pearson's product moment correlation coefficient (r). Multiple linear regression (enter model with simultaneous entry of preselected predictor variables) was performed to assess relationships between the dependant variable of AMH and independent variables of age, abdominal VA:SA ratio, insulin sensitivity (GIR), testosterone, SHBG, LH and FSH for all participants combined. A receiver operating characteristic (ROC) curve was generated to evaluate sensitivity and specificity of AMH as a diagnostic test for PCOS. An area under the curve (AUC) of ≤0·5 indicates the test result is no better than chance. All statistical analysis was performed using SPSS for Windows 20.0 software (SPSS Inc, version 20.0 software, Chicago, IL, USA) with statistical significance accepted when $P < 0{\cdot}05$.

Results

Clinical and biochemical characteristics

In this community-recruited study, all women with PCOS met the Rotterdam diagnostic criteria, with demographic characteristics presented in Table 1. Phenotypes varied across lean and overweight women with PCOS; 23% (5/22) of lean women and 86% (18/21) of overweight women met the narrower NIH criteria based on hyperandrogenism and irregular menstrual cycles, and 77% (17/22) of lean women and 14% (3/21) of overweight women met only Rotterdam but not NIH criteria (irregular menstrual cycles and PCO alone).

Overall analysis. Investigation of the overall cohort showed no differences in BMI, total fat mass, android fat mass, abdominal VA:SA ratio, fasting insulin and glucose, HOMA-IR, SHBG and gonadotrophins between PCOS and control women. Women with PCOS were slightly younger (28 ± 5 *vs* 31 ± 6 years; $P = 0{\cdot}04$) and had lower GIR (232 ± 78 *vs* 295 ± 78 mg/min/m; $P = 0{\cdot}001$) and higher testosterone (2·3 ± 0·7 *vs* 1·7 ± 0·7 nmol/l; $P = 0{\cdot}001$) and FAI (6·1 ± 3·3 *vs* 3·6 ± 3·2; $P = 0{\cdot}003$).

BMI subgroup analysis. Overweight control women were older (35·5 ± 4·2 years) than the lean control (25·7 ± 6·1 years, $P < 0{\cdot}001$), lean PCOS (25·7 ± 4·3 years, $P < 0{\cdot}001$) and overweight PCOS (29·3 ± 5·9 years, $P = 0{\cdot}004$) groups.

Table 1. Anthropometric, hormonal and metabolic features of lean and overweight women with and without PCOS

Characteristics	Lean Control ($n = 19$)	Lean PCOS ($n = 22$)	Overweight Control ($n = 16$)	Overweight PCOS ($n = 21$)	*P* Value	*P* Value Adjusted
Age (years)	27 ± 6§	27 ± 4§	35 ± 4†,‡,¶	29 ± 5§	<0·001	–
BMI (kg/m^2)	21·9 ± 4·6§,¶	23·0 ± 4·7§,¶	34·3 ± 5·1†,‡	34·2 ± 4·6†,‡	<0·001	<0·001
WHR	0·84 ± 0·06	0·85 ± 0·06	0·85 ± 0·07	0·85 ± 0·06	0·613	0·819
Total fat mass (%)	27·0 ± 6·3§,¶	30·6 ± 6·0§,¶	48·2 ± 7·1†,‡	47·7 ± 6·3†,‡	<0·001	<0·001
Android fat mass: weight ratio*	0·18 ± 0·08§,¶	0·22 ± 0·08§,¶	0·42 ± 0·09†,‡	0·44 ± 0·08†,‡	<0·001	<0·001
Abdominal VA:SA* ratio	0·20 ± 0·1	0·18 ± 0·1	0·23 ± 0·1	0·22 ± 0·1	0·505	0·408
Insulin sensitivity						
Fasting Glucose (mmol/l)	4·6 ± 0·4¶	4·6 ± 0·4¶	4·8 ± 0·5	4·9 ± 0·4†,‡	0·013	0·082
Fasting Insulin (pmol/l)	4·0 ± 8·9§,¶	4·3 ± 8·9§,¶	17·9 ± 9·7†,‡,¶	28·7 ± 8·7†,‡,§	<0·001	<0·001
HOMA-IR	0·9 ± 2·1§,¶	0·9 ± 2·1§,¶	3·8 ± 2·3†,‡,¶	6·3 ± 2·0†,‡,§	<0·001	<0·001
GIR (mg/min/m)	337 ± 82‡,§,¶	275 ± 82†,¶	253 ± 89†,¶	175 ± 81†,‡,§	<0·001	<0·001
Hormonal profile						
Testosterone (nmol/l)	1·6 ± 0·7¶	2·0 ± 0·7¶	1·7 ± 0·8¶	2·7 ± 0·7†,‡,§	<0·001	<0·001
SHBG (nmol/l)	79·2 ± 25·1§,¶	71·5 ± 25·2§,¶	42·3 ± 27·7†,‡	31·7 ± 24·6†,‡	<0·001	<0·001
FAI	2·1 ± 3·1§,¶	3·2 ± 3·3¶	4·8 ± 3·6†,¶	9·7 ± 3·2†,‡,§	<0·001	<0·001
LH (IU/l)	9·0 ± 9·1	8·3 ± 9·1	9·5 ± 10·0	9·3 ± 8·9	0·992	0·982
FSH (IU/l)	7·0 ± 2·6	6·7 ± 2·7	7·8 ± 3·0	6·4 ± 2·6	0·417	0·552
AMH*	29·8 ± 30·5‡,¶	64·7 ± 29·8†,§	17·8 ± 33·7‡,¶	54·4 ± 30·2†,§	<0·001	<0·001

Values are means ± SD and adjusted for age. *Denotes log-transformed variable. Significant difference $P < 0{\cdot}05$ compared with; †Lean control, ‡Lean PCOS, §Overweight Control and ¶Overweight PCOS group.
BMI, body mass index; WHR, waist to hip ratio; VA, visceral adiposity; SA, subcutaneous adiposity; HOMA-IR, homeostasis model of assessment of insulin resistance, GIR, glucose infusion rate; SHBG, sex hormone binding globulin; FAI, free androgen index; AMH, anti-Mullerian hormone.

Regardless of PCOS status, BMI and total and android fat mass were lower and SHBG higher in lean women compared with overweight women (P <0·001 for all variables). As previously reported[6], the overweight PCOS group was the most IR based on GIR, compared with the overweight control (P = 0·05) and lean PCOS (P = 0·001) group. There was no difference in IR between the lean PCOS and overweight control groups (P = 0·65).

The overweight PCOS group had higher testosterone levels compared with the lean PCOS (P <0·01), lean control (P <0·001) and overweight control (P <0·001) groups. FAI was higher in the overweight PCOS group compared with the other three groups (P <0·001). There were no differences in LH (P = 0·992) and FSH (P = 0·417) or LH/FSH ratio (P = 0·232) between groups.

AMH concentrations

The NIH PCOS phenotype tended to have lower levels of AMH compared with the non-NIH PCOS phenotype (NIH PCOS 52·05 ± 27·3 pmol/l *vs* non-NIH PCOS 69·5 ± 41·6 pmol/l, P = 0·06). Significant differences in AMH concentrations were evident between the overall cohort of PCOS (60·1 ± 35·6 pmol/l) *vs* control women (24·1 ± 21·8 pmol/l, P <0·001), accounting for age and BMI. AMH concentrations were higher in both the lean (65·5 ± 37·5 pmol/l) and overweight (54·6 ± 33·1 pmol/l) PCOS group, compared with the lean (30·6 ± 26·0 pmol/l) and overweight (15·4 ± 9·6 pmol/l) control groups (Fig. 1; P <0·001). When these comparisons were made between groups with a BMI of 25 kg/m^2 to define overweight, these differences in AMH concentrations between groups persisted (data not shown).

AMH relationships

PCOS status (r = 0·51, P <0·001), age (r = −0·24, P = 0·04) and testosterone (r = 0·43, P <0·001) had a moderate to strong correlation with AMH. AMH levels did not correlate with BMI (P = 0·3), abdominal VA:SA (P = 0·1), GIR (P = 0·9), SHBG (P = 0·6), FSH (P = 0·6) or LH (P = 0·3) (Table 2).

Linear regression analysis

Multiple linear regression analysis was conducted for women with and without PCOS combined, assessing the contribution of age, abdominal VA:SA, insulin sensitivity (GIR), hyperandrogenism and gonadotrophins to AMH. The model predicted 13% (adjusted r^2 = 0·13) of the variation in AMH (P = 0·03), and testosterone was the only independent correlate of AMH (β = 0·465; P = 0·001) (Table 3).

Table 2. Correlations of AMH concentration with clinical and biochemical variables

	Correlation coefficient	*P* Value
PCOS	0·513	<0·001
Age	−0·241	0·04
BMI	−0·115	0·32
Abdominal VA:SA	−0·180	0·13
GIR	−0·012	0·92
Testosterone	0·426	<0·001
SHBG	0·058	0·62
FAI	0·156	0·18
FSH	0·056	0·63
LH	0·114	0·33

P <0·05 is significant. PCOS, polycystic ovary syndrome; BMI, body mass index; GIR, glucose infusion rate; SHBG, sex hormone binding globulin; FAI, free androgen index; FSH, follicle stimulating hormone; LH, luteinising hormone; VA, visceral adiposity; SA, subcutaneous adiposity.

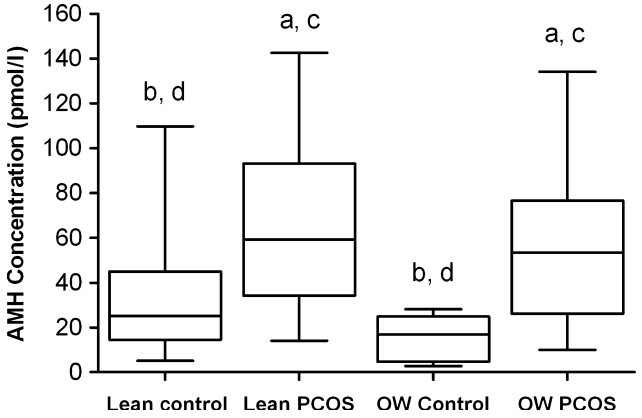

Fig. 1 Concentration of AMH is lean and overweight women with and without PCOS demonstrated by a box and whisker plot illustrating the median (central line), range (whiskers) and 25 and 75th percentiles (box). Abbreviations: OW, overweight. Significant difference P <0·05 compared with the [a]lean control, [b]lean PCOS, [c]overweight control, [d]overweight PCOS group.

Table 3. Regression analysis to establish independent determinants of AMH concentrations

Independent variable	β Standardized coefficients	*P* Value	95% Confidence intervals
Age	−0·038	0·78	−1·80 to 1·33
Abdominal VA:SA	−0·003	0·98	−51·5 to 50·5
GIR	−0·035	0·79	−0·10 to 0·08
FSH	0·199	0·22	−1·69 to 7·21
LH	−0·059	0·71	−1·51 to 1·04
Testosterone	0·465	0·001	8·65 to 31·4
SHBG	0·159	0·23	−0·12 to 0·48

Multiple linear regression with simultaneous entry of preselected predictor variables was performed with AMH as the dependent variable and age, abdominal VA: SA ratio, GIR, FSH, LH, testosterone and SHBG as independent variables. P <0·05 is significant. GIR, glucose infusion rate; SHBG, sex hormone binding globulin; FSH, follicle stimulating hormone; LH, luteinising hormone; VA, visceral adiposity; SA, subcutaneous adiposity.

ROC curve analysis

The ROC curve assessing the ability of AMH to distinguish between women with and without PCOS (Fig. 2) indicated an AUC of 0·829 (95% CI 0·736–0·923, P <0·001), and a threshold value of 30 pmol/l or higher for identifying PCOS. At this cut-off point, 79% (specificity) of women with PCOS and 82% (sensitivity) of women without PCOS will be correctly identified if AMH was used as the sole diagnostic criteria for PCOS.

Discussion

In a community-recruited population not presenting with infertility, including lean and overweight women, we report that women with PCOS have increased circulating AMH compared with non-PCOS women, regardless of IR and adiposity. Exploring the relationships between AMH, IR, androgens, adiposity and gonadotrophins in women with both NIH and non-NIH PCOS and controls, we report that testosterone was the only significant independent predictor of AMH across all groups. We found that AMH was not related to IR measured on clamp studies, gondotrophin levels and body composition on CT and DXA.

AMH may have a role in PCOS diagnosis and in assessing severity. While improvement in AMH assay quality and an international standard is warranted,[23,24] currently, cut-off values for AMH in the diagnosis of PCOS vary widely.[23] In the current study, AMH results were compared across lean and overweight women with and without PCOS. Our results suggest a threshold AMH level of 30 pmol/l in discriminating between women with and without PCOS, which is similar to an AMH cut-off reported in a recent systematic review and meta-analysis of 33·6 pmol/l.[18] We note this meta-analysis predominantly included participants recruited from fertility clinics, with more severe reproductive features. This study extends previous literature to confirm this threshold in a community-based population, not presenting with fertility challenges. Once assay issues are resolved, it is anticipated that AMH levels will be integrated into PCOS community-based assessment to reflect the severity of the condition and to complement PCOS diagnosis.[23]

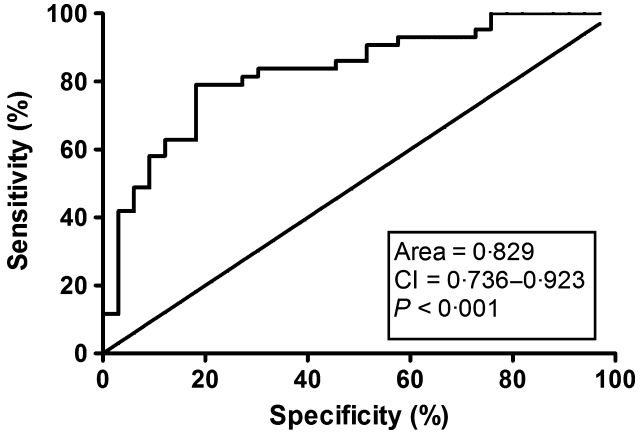

Fig. 2 ROC curve obtained from analysis of the AMH results. The sensitivity (true positive rate, y-axis) is plotted against the false positive rate or specificity (1-sensitivity, x = axis).

The severity of PCO morphology on ultrasound is a key correlate of increased AMH in PCOS.[23] We acknowledge the lack of data on PCO status in our study, as it was only performed if required for diagnosis, and we do not have complete quality ultrasound data from a single operator to compare between AMH and PCO. However, consistent with prior literature, here, AMH was reasonably sensitive and specific for PCOS diagnosis.[18]

We report no association between AMH and IR assessed by the euglycaemic-hyperinsulinaemic clamp. The relationship between AMH concentration and IR remains controversial, with some researchers reporting positive association,[25,26] while others reporting no association[10,11] when using indirect measures of IR. Furthermore, we extend prior research[27] to include milder phenotypes only diagnosed using Rotterdam (but not NIH) criteria. The majority of other studies have focused on overweight women, and to our knowledge, this is one of only two studies to assess the relationship between clamp-based IR and AMH in lean and overweight women with and without PCOS.[27] We report the novel finding that based on the gold standard measurement of IR, there is no direct relationship between AMH and IR. IR drives ovarian androgen production[28], and our findings suggest that associations between AMH and IR in PCOS may reflect indirect regulation through androgens.

We confirm previous studies reporting an association between androgens[10,11,15,16] and report testosterone as the only significant independent predictor of AMH overall in women with PCOS; FAI was not independently related to AMH, potentially as it includes both testosterone and an IR related factor (SHBG). Androgen assays in women are also challenging, and as we transition to more accurate methods, greater insights may be gained into the relationship between androgens, AMH and ovarian function in PCOS. Yet, previous studies show a positive correlation between follicular number and testosterone[12,29] potentially related to activation of androgen-binding receptors in pre-antral follicles inhibiting follicular growth and maturation,[29,30] increasing AMH production. Consistently, androgen administration in female to male transsexuals or female rhesus monkeys induces PCO-like morphology independent of gonadotrophin effects.[31,32] Exposure to testosterone, either intrauterine or during adulthood, could potentially increase antral follicle number,[32,33]increasing AMH production. Conversely, testosterone may play a mechanistic role in inhibiting AMH production in granulosa cells, as[34] *in vitro* studies have shown lower AMH production with increases in testosterone in women with PCO.[34] However, AMH concentrations were not altered when testosterone levels are at normal physiologic levels. Furthermore, 6-month administration of low-dose dexamethasone and metformin failed to influence AMH levels, even with a decline in testosterone levels.[35] This highlights the complex relationship between hyperandrogenism and ovarian function.

Regarding a potential role for AMH in PCOS diagnosis, our data is consistent with previous studies reporting a two to three-fold higher AMH in PCOS compared with controls with similar

BMI.[10,26] This is consistent with increased immature pre-antral and antral ovarian follicles and increased rate of follicular AMH production in PCOS.[9,11] The relationship between AMH and BMI remains contentious, with few studies including lean women with PCOS. Obesity is proposed to have an inhibitory effect on gonadotrophin release through suppression of LH, reducing consequent dominant follicle development and selection[14] leading to altered AMH through an accumulation of pre-antral and antral follicles. Our finding of no differences in AMH between lean and overweight women both in the PCOS group and in the control group and no relationship between AMH and adiposity (android or abdominal VA:SA ratio) is consistent with some[10,26] but not all prior literature.[11] We extend these previous studies to provide a comprehensive assessment of anthropometry using the gold standard CT. Our CT data suggests that increased AMH in PCOS is a function of PCOS status and not related to differences in body composition.

Prior research has proposed a primary role of gonadotrophins in regulating AMH in PCOS, with LH independently predicting AMH levels more so than hyperandrogenism.[11] This is in contrast to our current findings where a relationship between AMH and LH was not confirmed. However, there is a proposed role of insulin or androgens in regulating gonadotrophins. Hyperinsulinaemia causes an increase in LH receptor expression and premature release of the follicle, which combine to cause follicular arrest and subfertility or infertility.[13] The role of androgens in altered gonadotrophin regulation is supported by prenatally androgenised animal studies where female offspring have LH hypersecretion, potentially due to altered programming of the hypothalamus and increased sensitivity of the pituitary to gonadotrophin releasing hormones.[36] As increased androgens can increase AMH through augmenting follicular growth, high AMH may also negatively feedback on either FSH or oestradiol with consequent effects on FSH production.[36] However, based on our current data, we suggest that prior associations between AMH and gonadotrophins may reflect an indirect regulatory role, with hyperandrogenism playing a primary direct role. Further research is needed to explore these relationships.

The limitations of this study include the lack of complete data on PCO morphology acknowledging that severity of PCO morphology on ultrasound is correlated with increase AMH in PCOS.[23,27] However, this did not impact on the primary aim of the study. Here, a BMI of 27 kg/m^2 was used to classify overweight and obesity, rather than the World Health Organisation criteria as we have previously published that 27 kg/m^2 is the inflection point in the relationship between IR and BMI in PCOS.[6,20] However, AMH and androgen results between groups did not differ regardless of whether the BMI cut-off of 25 kg/m^2 or 27 kg/m^2 was applied. Immunoassays were used for the measurement of steroid sex hormones in this study, which may not be as sensitive as mass spectrometry to detect androgen levels in women. The strengths of this study include the use of gold standard methodology such as insulin sensitivity assessed by hyperinsulinaemic-euglycaemic clamps and visceral and subcutaneous adiposity by CT. Sample size within each group was small; however, with gold standard end-points, we were adequately powered to explore the relative contribution of intrinsic factors (hyperandrogenism, gonadotrophins and IR) or environmental factors (visceral obesity) to ovarian status, as measured by AMH levels.

We report that AMH levels were increased in community-recruited lean and overweight women diagnosed with PCOS based on the Rotterdam criteria, compared with controls. Increased AMH was related to hyperandrogenism rather than to IR, obesity or gonadotrophins. This highlights the direct interaction with androgens and potential indirect role of IR in the pathophysiology of reproductive dysfunction in PCOS. Here, we suggest that AMH reflects PCOS severity and confirms previous reports of the potential utility of AMH as a diagnostic tool for PCOS. An international standard for AMH assays is still needed, as are further studies to determine the optimal cut-offs and define the role that AMH will play in assisting PCOS diagnosis.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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Chapter 7 Discussion and Conclusion

7.0 Discussion and Conclusions

Relevant discussion of the work presented in the body of this thesis has been presented in each of the manuscript chapters. I will conclude with a synopsis of the original contribution my work has made to the knowledge and understanding of PCOS in the areas of aetiology and assessment of IR. I have included integrative discussion, future directions, limitations and overall conclusions.

This thesis comprised a series of related studies aimed at enhancing understanding of IR in PCOS including the relationship between intrinsic and extrinsic factors. I was able to provide comprehensive insights into IR, which is intrinsic to PCOS and exacerbated by obesity, with minimal impact of age or PCOS phenotype (Chapter 2). I have made contributions in original research using a systematic review and meta-analysis approach. In this work the insulin resistant PCOS women provided a useful human model of IR without T2DM, allowing examination of IR independent of hyperglycemia.

Given the prominent role of IR in PCOS, the systematic review and meta-analysis (described in detail in Chapter 2) also addressed the question of the relationship between IR, SHBG and key reproductive markers (testosterone, LH and FSH). IR is strongly inversely related to SHBG, while androgens have a linear relationship with IR in PCOS. The interesting finding of the relationship between androgens and insulin sensitivity is contradictory to conventional thinking in PCOS. But it does present an

intriguing paradigm and as noted in the Chapter 2, larger studies using more sensitive measures of testosterone are needed to further investigate this relationship. Furthermore, commercially available androgen immunoassays in women have proven to be inaccurate, imprecise and have poorly based reference ranges (Handelsman and Wartofsky, 2013) and there is a need for more sensitive techniques such as mass spectrometry to be used in large studies.

In an extension to the extensive meta-analysis work, my cross-sectional work allowed me to investigate intrinsic and extrinsic IR in PCOS across a range of BMI's and different phenotypes using the gold standard technique of euglycaemic-hyperinsulinaemic clamp. The meta-analysis was unable to provide this information with the included papers as phenotypes were not previously identified in the literature. I report here (Chapter 3) that 75% of lean and 95% of obese women with PCOS were affected by IR, whilst 70% milder phenotypes and 80% of more severe phenotypes were affected. Both these studies extend knowledge in the area by highlighting that even lean women with a milder phenotype of PCOS have some degree of IR and would benefit from metabolic profile monitoring and encouragement to maintain a healthy body weight to prevent PCOS complications.

The assessment of IR based on the current gold standard methods used in research is not convenient in a clinical setting and clinicians would benefit from easier and reliable measures of IR. I pursued novel potential measures of IR, ghrelin, resistin, visfatin, GLP-1, leptin, PAI-1, GIP and C-Peptide, and used the PCOS model to investigate my theory. Although other conditions associated with IR, including obesity and diabetes, have adverse biomarker profiles, with impaired GIP and GLP

activity and elevated PAI-1, visfatin, leptin and resistin (Antuna-Puente et al., 2008), the majority these studies have measured the biomarkers in isolation. This limits the understanding of the relative interaction and detailed pathways contributing to metabolic dysfunction in PCOS. In my biomarker study (Chapter 4), C-peptide, ghrelin and leptin were different between overweight and lean women regardless of PCOS status, suggesting that these potential predictors of IR are associated primarily with adiposity rather than IR, given that the lean PCOS women were insulin resistant. Furthermore, I was able to demonstrate that while a number of biomarkers were related to IR in controls (leptin, ghrelin), PAI-1 was the only biomarker that was related to IR in women with PCOS. This paper offers clinically important novel data and comprehensively addresses the area of biomarkers and IR in women with PCOS. Specific strengths of the study are the use of a single assay and serum sample to measure all biomarkers, which has not been previously reported in PCOS and the inclusion of both lean and overweight women with and without PCOS who had IR measured through euglycaemic hyperinsulimic clamps. The other important and novel factor here is the study population who are insulin resistant yet have normal fasting glucose levels. Other measures of IR are urgently needed for both clinical and research purposes. Also greater insight is needed into the role of adipose tissue as endocrine organ with potential roles in IR in PCOS.

With regards to potential mechanisms of intrinsic IR in PCOS (Chapter 5), I investigated the role of mitochondrial function and expression, measured by various techniques including western blots and respiration, as mitochondrial dysfunction has been proposed as a contributor to IR in other conditions. I report that there was no significant differences in mitochondrial function or expression between well-matched

lean women with and without PCOS, despite clear differences in IR. In this setting it appears the intrinsic IR observed in women with PCOS cannot be explained by mitochondrial dysfunction in skeletal muscle. Overall, this study contributes to the evolving literature on the role of mitochondrial function in IR with the advantage of the insulin resistant lean PCOS cohort studied providing a useful human model of IR without T2DM. This enabled me to examine the impact of IR independent of either obesity or hyperglycemia. I was able to conclude that mitochondrial dysfunction was not associated with intrinsic IR in PCOS. In the case of IR aetiology in PCOS, multiple mechanisms are likely involved and our group is currently further exploring this issue by investigating insulin signalling pathways in skeletal muscle. Together, these aetiological studies are an important reminder that despite positive associations between factors in cross-sectional studies, complex relationships often exist and interventional studies need to be performed to further explore whether a true causal relationship exists or whether these factors are merely markers of IR.

PCOS is a metabolic and reproductive syndrome, yet links between reproduction, IR, androgen excess and metabolic features remain unclear. In Chapter 6 I report that AMH, a marker of reproductive status in PCOS, is related to androgen excess and not IR. I also add to the literature suggesting that AMH provides a potentially useful reproductive diagnostic marker in PCOS.

7.1 Limitations

Whilst my studies have made important contributions to the literature, it must be acknowledged that the results presented are from cross sectional studies, rather than longitudinal studies, therefore caution needs to be taken when interpreting the results.

Large, adequately powered, long-term prospective studies are required to track the link between biomarkers, sex steroids, SHBG, adiposity and gonadotropins and IR. Furthermore, androgen immunoassays were used to measure testosterone in all of my cross sectional studies. Recently, a leading clinical endocrinology journal (Handelsman and Wartofsky, 2013) has recommended mass spectrometry sex steroid assays. At the time of this PhD mass spectrometry was expensive and unavailable for clinical research use in our laboratories. Moving forward we would benefit from establishment of reliable androgen assays for women and defined normal ranges with national implementation across pathology services.

7.2 Key Future Research Goals

Although the findings of thesis have improved our knowledge in the area, it has also raised more questions and areas where future research should focus. These include:

- A clearer understanding of the origins, aetiology and pathology of PCOS, including the use of epigenetics
- Causes and implications of IR, including longitudinal studies, with clarity of natural history across the phenotypes
- Insights into mechanisms and role of adipose tissue and bone as endocrine organs having a direct effect on IR
- Effective IR treatment including structured exercise interventions and programs that are community orientated given that women with PCOS are likely to be sedentary (Moran et al., 2013a) and insulin sensitisers that are safe and effective (Misso and Teede, 2014)
- Future investigation into the relationship between insulin and hepatic SHBG production to clarify mechanisms through which SHBG is linked to metabolic

disturbances providing physiologic constructs for other disorders of IR including GDM and NAFLD. Also, the use for SHBG as a screening tool.

- Future investigation into the relationship between testosterone and IR using the best available techniques to measure both variables in longitudinal studies.

7.3 Overall Conclusion

Given the remarkably high prevalence (up to 1 in 5 women) of PCOS and the key central role of IR in the aetiology and metabolic consequences of PCOS, our knowledge around IR in PCOS is surprisingly limited. The findings presented in this thesis have made a significant contribution to the understanding of IR in PCOS, where there is a strong and ongoing need for accurate assessment and understanding of the mechanisms of IR in order to alleviate the increasing clinical and economic burden of PCOS.

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