Polycystic Ovary Syndrome and Insulin Resistance: Dysregulation of Transforming Growth Factor Beta Signalling and the Effects of Exercise Training

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By

Luke C. McIlvenna
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College of Sport and Exercise Science
Institute for Health and Sport (IHES)
Victoria University - Melbourne, Australia

ABSTRACT

Polycystic Ovary Syndrome (PCOS) is primarily thought of as a reproductive condition, although it has profound effects on metabolic health, with 38-80% of women with PCOS presenting with insulin resistance. This underlying insulin resistance occurs independently of obesity and has negative effects on other features of the condition. The identification of peripheral insulin resistance in PCOS occurred in 1989, and despite several advances in this area, the molecular mechanisms responsible for peripheral insulin resistance in women with PCOS remain unclear. In addition, interventional studies from women with PCOS have identified impaired responses to insulin-sensitizing therapies. A possible explanation for the underlying insulin resistance and poor response to insulin-sensitizing treatments may be the dysregulation of Transforming Growth Factor-beta (TGF-beta) signalling. The dysregulation of TGF-beta signalling is responsible for remodelling in the ovarian tissues and reproductive dysfunction. Due to the systemic action of TGF-beta signalling, it may be conceivable that these effects extend beyond the reproductive tissues to the peripheral tissues. This presents a possible mechanism that could contribute to the development of insulin resistance in women with PCOS. Understanding the underlying mechanisms of insulin resistance will allow for improvements in diagnosis and for targeted therapies to be developed, which are currently lacking for women with PCOS. The overall aim of this thesis was to determine if dysregulated TGF-beta signalling plays a role in skeletal muscle insulin resistance and can influence metabolic responses to exercise in women with PCOS. This was achieved through a combination of *in vivo* and *in vitro* studies focusing on skeletal muscle metabolism.

Study 1 explored the role of TGF-beta ligands, TGF-beta 1 and Anti-müllerian hormone (AMH), on glucose uptake and insulin signalling in myotubes from women with PCOS and healthy controls. In line with previous studies, we showed that *in vivo* metabolic phenotype was not accurately retained in cultured myotubes from women with PCOS, suggesting that a combination of the *in vivo* environmental factors and intrinsic defects lead to the development of insulin resistance. TGF-beta 1 and AMH had distinct metabolic effects. TGF-beta 1 increased basal and insulin-stimulated glucose uptake, while AMH decreased glucose uptake and PI3K-p110 expression, which was accompanied by an increase in inhibitory IRS-1$_{ser312}$ phosphorylation.

Study 2 assessed the response of myotubes from women with PCOS and healthy controls to an *in vitro* model of contraction with and without TGF-beta 1 or AMH. It was found that myotubes from women with PCOS and healthy controls display minimal differences in exercise-induced signalling transduction. Myotubes from healthy women showed an increase in the phosphorylation of p38 MAPK and CREB, which appeared to be absent in the myotubes from women with PCOS. The TGF-beta ligands AMH and TGF-beta 1 do not appear to influence *in vitro* skeletal muscle exercise-like signalling responses.

In Study 3, a cross-sectional approach was used to assess skeletal muscle TGF-beta and insulin signalling in women with PCOS compared to overweight and lean controls. Insulin sensitivity, as determined by euglycemic–hyperinsulinemic clamp, confirmed previous findings that women with PCOS have a significant reduction in insulin sensitivity compared to controls. This insulin resistance occurred in the absence of any identifiable defects in skeletal muscle insulin signalling and did not appear to be related to TGF-beta signalling. Women with PCOS had greater levels of basal phosphorylation of p38 MAPK, suggesting that excessive reactive oxygen species and/or inflammation may contribute to insulin resistance.

In Study 4, overweight women with PCOS participated in a 12-week exercise training intervention. We aimed to determine if changes in insulin sensitivity following 12 weeks of moderate or high-intensity exercise training were related to aberrant TGF-beta signalling and collagen deposition in the skeletal muscle. Exercise training of high and moderate intensities resulted in improvements in insulin sensitivity and cardiorespiratory fitness, with the metabolic benefits being more noticeable following the high-intensity intervention. Improvements in insulin sensitivity occurred independently of changes in body composition, TGF-beta signalling and other clinical measures.

Collectively, the results from these studies demonstrate the role of TGF-beta ligands and signalling dysregulation in skeletal muscle metabolism *in vitro*. However, these findings do not appear to translate *in vivo* where insulin sensitivity was not related to TGF-beta signalling in women with or without PCOS. There was no apparent evidence of dysregulation of TGF-beta signalling or insulin signalling in women with PCOS. Furthermore, improvements in insulin sensitivity following 12 weeks of high- or moderate-intensity exercise training occurred independently of changes in TGF-beta signalling.

Declaration

"I, **Luke C. McIlvenna** declare that the PhD thesis entitled 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".

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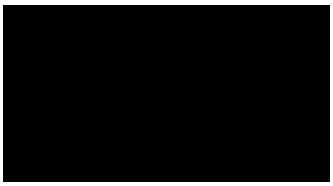
VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

INSTITUTE FOR HEALTH AND SPORT

This thesis was completed under the guidance of Professor Nigel Keith Stepto and Dr Alba Moreno-Asso from the Women's Health Group at the Institute for Health and Sport, Victoria University

Prof Nigel K. Stepto

Dr Alba Moreno-Asso



In memory of Professor Nigel Keith Stepto

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ACC – Acetyl-CoA carboxylase

ALT - Alanine aminotransferase

AMH - Anti-müllerian hormone

AMPK - Adenosine monophosphate-activated protein kinase

AST - Aspartate aminotransferase

BMI – Body mass index

CAMKII - Ca2+/calmodulin-dependent protein kinase

CREB - cAMP response element-binding protein

DCN - Decorin

DHEA - Dehydroepiandrosterone

DHT - Dihydrotestosterone

EPS – Electrical pulse stimulation

E2 – Estradiol

ECM – Extracellular matrix

ERK - Extracellular-signal-regulated kinase

GIR – Glucose infusion rate

GS - Glycogen synthase

GXT – Graded exercise test

HDL - High-density lipoprotein

HIIT – High-intensity interval training

HK – Hexokinase

HRR – Heart rate reserve

IRS – Insulin receptor substrate

IGFBP-1 - Insulin-like growth factor-binding protein 1

IGF1 – Insulin-like growth factor 1

LBM – Lean body mass

LDL - Low-density lipoprotein

LOX - Lysyl oxidase

MACS – Magnetic activated cell sorting

MAPK – Mitogen-activated protein kinase

MET - Metabolic equivalent

MICT – Moderate-intensity continuous exercise

PCOS – Polycystic ovary syndrome

ROS – Reactive oxygen species

SHBG - Sex hormone-binding globulin

SMAD - Sma and Mad proteins from Caenorhabditis elegans and Drosophila

TGF-Beta – Transforming growth factor beta

TGFBRI - Transforming growth factor beta receptor 1

TGFBRII Transforming growth factor beta receptor 2

1 General Introduction

1.1 Polycystic ovary syndrome

Polycystic Ovary Syndrome is a multifaceted condition that occurs in women of reproductive age and can be characterized by androgen excess, ovulatory dysfunction and polycystic ovaries (Ehrmann, 2005). The condition affects up to 8-13% of women of a reproductive age worldwide (Bozdag et al., 2016) depending on the diagnostic criteria applied, with the prevalence in Australia being up to 20% (March et al., 2010; Varanasi et al., 2017). The condition is associated with a number of morbidities including subfertility, pregnancy complications, insulin resistance (Cassar et al., 2016a; Stepto et al., 2013), type 2 diabetes mellitus (T2DM) (Moran et al., 2010), psychological disorders (depression and anxiety (Cooney et al., 2017), and increased risk of cardiovascular disease (Kakoly et al., 2019). The aetiology of PCOS to date remains to be established. However, there are a number of proposed contributing factors and hypothesis such as environmental factors, genetics and in-utero developmental programming.

Traditionally PCOS has been considered a reproductive condition (based upon the diagnostic criteria); however, there is also a strong metabolic component with insulin resistance and compensatory hyperinsulinemia being present in a majority of women with PCOS. Building upon initial findings that women with PCOS have a marked decrease in insulin sensitivity (Dunaif et al., 1989; Lachelin et al., 1979), it has been established that approximately 38-80% of women with PCOS have insulin resistance that occurs independent of body mass index (BMI) but is exacerbated by BMI (DeUgarte et al., 2005; Stepto et al., 2013). This appears to be a distinguishing feature of PCOS indicating differing insulin resistance mechanisms from other metabolic conditions. Furthermore the prevalence of insulin resistance in women with PCOS may have been underestimated given the poor relationship between surrogate markers of insulin sensitivity often utilised by clinicians and more direct measures such as euglycemic -hyperinsulinemic clamp (Cassar et al., 2016a; Tosi et al., 2017). The preference of surrogate markers in a clinical setting can be attributed to cost and practicability. It is important to note that insulin resistance has an adverse effect on the reproductive features of PCOS with hyperinsulinemia leading to an increase in the bioavailability of androgens via different pathways (Micic et al., 1988; Nestler et al., 1991, 1989), creating a vicious cycle. Despite over

two decades of research the mechanisms of peripheral insulin resistance remain unclear. This is probably in part due to the complex nature of PCOS and the variability of the pathogenesis. Women with PCOS appear to have a distinct insulin resistance from that observed in individuals with type 2 diabetes. However, it is still not clear which mechanisms are involved, with a lack of consistent findings between studies (Stepto et al., 2019). This makes the current findings difficult to interpret and creates a challenge for the development of effective therapies for the treatment and management of PCOS.

1.2 Treatment and management

To date treatment approaches for women with PCOS have varied due to a lack of general consensus and knowledge among clinicians and practitioners. In response to this problem a group of international experts on PCOS have put together international evidence-based guidelines for the assessment, management and treatment of PCOS (Costello et al., 2019; Teede et al., 2018b). In regards to lifestyle modifications and specifically exercise, the current evidence base for women with PCOS is of a low quality with blanket approaches and general health guidelines often being applied (Stepto et al., 2019b). A personalized or phenotype specific approach to treatment may be needed due to the heterogeneity of the condition. Just as pharmacotherapies undergo extensive investigation to understand their effects and to be optimized for a given condition or patient, it could be argued that exercise research should take a similar approach (Buford et al., 2013). Allowing for the establishment of condition and patient specific exercise programming.

Exercise is one of the key treatments for improving peripheral insulin sensitivity, given that skeletal muscle accounts for around 85% of whole-body glucose uptake following exercise or hyperinsulinemia (Defronzo et al., 1981). This makes skeletal muscle a therapeutic target in the treatment of PCOS given that there are apparent defects in peripheral insulin signalling (Diamanti-Kandarakis and Dunaif, 2012). The effects of exercise training and other therapeutic approaches on insulin resistance in women with PCOS have been sub-optimal. Several studies have demonstrated the ability of exercise training to improve but not to normalize insulin sensitivity (Harrison et al., 2012; Hutchison et al., 2012). Additionally, the traditional-approach of using insulin sensitizers such as metformin and pioglitazone have been ineffective in

resolving insulin resistance in women with PCOS (Glintborg et al., 2008; Vedtofte et al., 2019). Combined these findings highlight the current limitations of treatment options being used to improve metabolic health in women with PCOS. To date a variety of treatment and management approaches have been recommended for women with PCOS, although very few approaches have been effective in improving aspects of the condition. It should be noted that no specific pharmaceutics have been designed/produced for women with PCOS. This may be related to the lack of understanding regarding the specific mechanism by which PCOS occurs, and the heterogeneous nature of the condition making a one-size-fits all approach unfeasible. This highlights the need for more mechanistic research to identify underlying factors that regulate the severity of the condition.

1.3 Transforming Growth Factor-beta and Polycystic Ovary Syndrome

A possible explanation for the poor response to treatment may be related to the pathophysiology of PCOS. The Transforming Growth Factor-beta (TGF-beta) superfamily is a group of cytokines involved in a variety of physiological processes including but not limited to; cell proliferation, differentiation, apoptosis, angiogenesis and immune system regulation (Chin et al., 2004; Piek et al., 1999). The synthesis of TGF-beta ligands is not restricted to a particular cell type with the majority of cells in the body expressing TGF-beta receptors and being capable of responding to TGF-beta ligands. In relation to PCOS, it has been observed that there is an association between TGF-beta dysregulation with a genetic variant of the fibrillin-3, an extracellular matrix (ECM) protein that regulates the activity of TGF-beta ligands (Raja-Khan et al., 2010). The presence of this genetic variant has been associated with increased resting plasma insulin levels and reduced insulin sensitivity in women with PCOS (Urbanek et al. 2007), suggesting that TGF-beta dysregulation may be responsible for the reproductive and metabolic dysfunction observed in PCOS.

One of the consequences of this dysregulation is the development of fibrosis of the ovarian tissues caused by excess deposition of collagens (Hatzirodos et al., 2011; Pozzer et al., 2017). In support of the role of abnormal TGF-beta signalling in the aetiology of PCOS, there are a number of superfamily ligands expressed in the ovary including the anti-müllerian hormone (Garg and Tal, 2016), activin (Teede et al., 2013; Welt et al., 2005), inhibin, and bone

morphogenetic proteins (Glister et al., 2005) that may be involved in hyperandrogenism and follicular development irregularities associated with PCOS. The presence of a specific fibrillin-3 genetic variant in D19S884 allele 8 has been associated with metabolic features of PCOS with individuals having elevations in resting plasma insulin and being less insulin sensitive (Urbanek et al., 2007). Given the systemic action of TGF-beta ligands it could by hypothesized that these effects are present in multiple tissues and organs in women with PCOS.

Alterations in the TGF-beta signalling pathway in skeletal muscle can result in remodelling of the ECM leading to increase deposition of collagens in skeletal muscle (Berria et al., 2006; Williams et al., 2015). This remodelling has been shown to act as a physical barrier to glucose and insulin as well as impairing insulin signalling (Berria et al., 2006; Williams et al., 2015). The impact of TGF-beta dysregulation and subsequent tissue remodelling may attenuate the benefits of insulin sensitizing therapies such as exercise. Interestingly, in humans, elevations in plasma TGF-beta 1 correlate with poor metabolic homeostasis and is inversely related to exercise capacity (Yadav and Rane, 2012). These findings have been further emphasized in individuals with type 2 diabetes, with failure to improve insulin sensitivity following exercise training due to increase in TGF-beta 1 in the skeletal muscle (Böhm et al., 2016). This resulted in subsequent suppression of key mitochondrial regulators; PGC1α and AMPKα2 (Böhm et al., 2016). These effects have yet to be investigated in women with PCOS but may contribute to the aetiology, linking abnormalities in metabolic and ovarian function.

2 Literature Review

2.1 What is polycystic ovary syndrome?

Polycystic ovary syndrome is the most common endocrine condition that affects women of reproductive age, with the effects being evident throughout the lifespan (Teede et al., 2010). The primary features for diagnosis include hyperandrogenism, anovulation, and polycystic ovaries with two out of three features required for diagnosis (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). Other common features not included in the diagnostic criteria are insulin resistance and hyperinsulinemia, infertility, psychological disorders, weight gain, acne and hirsutism (Goodarzi et al., 2011). Collectively, this range of clinical features has a marked impact on the quality of life and health of women with PCOS. This highlights the complexity and heterogeneity of PCOS, which makes the diagnosis and management challenging for health professionals.

The prevalence of PCOS is estimated to be 8-13%, dependent upon the diagnostic criteria applied (Bozdag et al., 2016). To date, the progress and treatment for women with PCOS have been disadvantaged by the changes in diagnostic criteria. PCOS was first recognised by Stein and Leventhal in 1935 when they presented seven cases where women had an absence of menstruation coupled with bilateral cystic ovaries and hormonal imbalances (Stein and Leventhal, 1935).

2.1.1 Diagnostic criteria

It took until 1990 before the first of three different diagnostic criteria was developed for PCOS **(outlined in Table 1)**. The 1990 NIH diagnostic criteria identified only the most severe form of PCOS, but the criteria have been subsequently made broader to include different combinations of all the features of PCOS via the Rotterdam criteria in 2003 (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004), which as of 2012 it became recognised as the internationally accepted criteria. The severity of PCOS varies widely and can be classified by different phenotypes, depending on the features present. The Rotterdam criteria outline four phenotypes ranging from full-blown PCOS (phenotype A) to less severe with reproductive features in the absence of hyperandrogenism (phenotype D)

(Table 2.). Regardless of phenotype classification, women with PCOS experience a reduction in quality of life (Deeks et al., 2010; Teede et al., 2010).

Health professionals have widely adopted the Rotterdam criteria; however, it has received criticism for being based upon expert consensus rather than prognostic studies and randomized control trials as with previous diagnostic criteria (Wang and Mol, 2017). This has ultimately influenced the research funding and hampered the advances in knowledge. This is evident with PCOS related grant submission resulting in sustainably less funding than conditions with a similar or a lesser prevalence (Brakta et al., 2017; Rodgers et al., 2019). This has also caused inconsistent approaches in clinical practice influencing diagnosis and management, leading to women with PCOS being frustrated with the care they receive (Cree-Green, 2017; Gibson-Helm et al., 2017).

Table 1 Diagnostic Criteria for Polycystic Ovary Syndrome

National Institutes of Health (1990)	Rotterdam (2003)	Androgen Excess and PCOS Society (2006)
Hyperandrogenism Ovulatory dysfunction	Hyperandrogenism Ovulatory dysfunction Polycystic ovary morphology	Hyperandrogenism And Ovulatory dysfunction or Polycystic ovary morphology

Confirmation of Diagnosis		
Both Criteria	Two out of Three	Hyperandrogenism with one of the other criteria

Table 2 Phenotypes of PCOS as defined by Rotterdam Criteria (2003)

PCOS Phenotype	Hyperandrogenism	Ovulatory dysfunction	Polycystic ovary morphology	Prevalence of Insulin resistance (%)
A	X	X	X	80
B	X	X		80
C	X		X	65
D		X	X	38

2.1.2 Origins of PCOS

The development and origins of PCOS remain elusive. To date, it is thought that a combination of genetic, epigenetic, and environmental factors is responsible for the development of this syndrome. Although the PCOS phenotype may be present from birth, the diagnosis may not occur until during later stages of puberty or after when symptoms appear to develop or become more prominent (Kent et al., 2008; Welt and Carmina, 2013). It has been suggested that the in utero environmental exposure of the foetus to high levels of hormones, such as androgens and anti-müllerian hormone, results in a developmental re-programming (Sir-Petermann et al., 2002; Tata et al., 2018). In turn, this leads to the predisposition of PCOS in daughters from women with PCOS and also causing apparent effects on male offspring (Coviello et al., 2009). Due to these effects occurring in utero, it makes the prevention of PCOS difficult. Other potential contributing environmental factors such as diet, exercise, and overall lifestyle may be easier to modify but still present with challenges in implementation (Blackshaw et al., 2019). In support of the genetic predisposition of PCOS, there is a familial cluster with the relatives of those affected presenting with metabolic dysfunction, hyperandrogenism and polycystic ovary morphology (Franks et al., 2008; Kent et al., 2008; Kobaly et al., 2014). The epigenetic contributions to the development and severity of PCOS are evident with alterations in DNA methylation that lead to aberrant gene function in processes related to hormone homeostasis, inflammation, and glucose and lipid metabolism (Vázquez-Martínez et al., 2019). This could suggest that a mix of environmental, genetic, and epigenetic factors may lead to the development of a PCOS phenotype.

Despite the high prevalence of insulin resistance in women with PCOS, it has yet to be included in the diagnostic criteria. Insulin resistance can be defined as a reduction in response to insulin with a reduction in glucose uptake in specific tissues such as skeletal muscle, adipocytes or liver (DeFronzo and Tripathy, 2009). The prevalence of insulin resistance in women with PCOS ranges from 38-80% based upon studies using the gold-standard euglycemic–hyperinsulinemic clamp to measure insulin sensitivity (Moghetti et al., 2013; Stepto et al., 2013). The phenotype of PCOS influences this prevalence, with more severe phenotypes (A+B) being more likely to have insulin resistance. The insulin resistance observed in women with PCOS occurs independently of but is exacerbated by obesity and adiposity (Stepto et al., 2013). This is demonstrated by lean women with PCOS presenting with insulin resistance and other associated metabolic defects (Ciaraldi et al., 2009; Dunaif et al., 1989; Stepto et al., 2013). This underlying reduction in insulin sensitivity may go undetected due to the preference for oral glucose tolerance test or fasting blood samples for diagnosis of metabolic issues by clinicians, making early detection and intervention difficult (Tosi et al., 2017). The recent international guidelines highlighted this point, stating that PCOS is recognised as an insulin-resistant condition. However the lack of sensitivity and accuracy in testing measures for insulin resistance is a limiting factor for its inclusion in the diagnostic criteria (Teede et al., 2018a). This underlines the importance of using a gold-standard euglycemic-hyperinsulinemic clamp in determining peripheral tissue insulin sensitivity in women with PCOS. However, due to the challenges of implementing this technique in the clinical environment, new alternative testing methods are needed to guarantee an accurate measurement of insulin sensitivity. For the purpose of this thesis, we define insulin resistance as a diminished response to insulin in the peripheral tissues, whereby higher levels of insulin are required to stimulate glucose uptake. This physiological response is determined by a euglycemic-hyperinsulinemic clamp.

2.2 The role of insulin resistance and hyperandrogenism in PCOS

Insulin Resistance can be seen as a primary driver of the symptoms of PCOS, leading to hyperinsulinemia, which plays a role in the regulation of androgen production. The role of insulin in the regulation of androgen production has been demonstrated when women with PCOS who have been prescribed insulin sensitizers for short-term interventions such as metformin, and results in a concomitant improvement in insulin sensitivity and decrease in

androgen production (Dunaif et al., 1996; La Marca et al., 2000; Velazquez et al., 1994). A six-month randomized control trial assessing the long-term effects of metformin on multiple clinical features of PCOS showed improvements in insulin sensitivity and reductions in hyperandrogenism with subsequent improvement in menstrual cycle regularity (Moghetti et al., 2000). Understanding the role of hyperinsulinemia in androgen production is challenging. It is not feasible to administer insulin to healthy individuals for an extended amount of time to observe a true physiological effect. Several studies have utilised short term-infusion of insulin (Fox et al., 1993; Micic et al., 1988). The short-term effect of insulin on androgen production appears to occur in a dose-dependent manner in women with PCOS, with a significant increase in androgen production when insulin levels reach those present in insulin resistance state (Micic et al., 1988). Another study showed that a 2-hour insulin infusion caused an increase in androgen levels, whereas a short term postprandial hyperinsulinemia did not alter androgen levels (Fox et al., 1993). Taken together, this suggests that sustained insulin elevation is necessary to stimulate androgen production.

On the other hand, the effect of hyperandrogenism on insulin resistance in women with PCOS remains poorly understood. It has been observed that treatment with anti-androgens results in a reduced androgen secretion but only partially improved insulin sensitivity (Moghetti et al., 1996), indicating that androgen levels effect insulin sensitivity in PCOS. Moreover, trials with anti-androgens such as GnRH analogues lasting greater than six months have demonstrated more robust improvements in insulin sensitivity as measured by a euglycemic-hyperinsulinemic clamp (Dahlgren et al., 1998; Krotkiewski et al., 2003). This lack of influence of hyperandrogenism on insulin sensitivity can be seen when androgen levels are restored to a normal range, but hepatic and peripheral insulin resistance were still present (Dunaif et al., 1990). On the contrary, in healthy non-obese women, short-term androgen administration reduced whole-body insulin sensitivity, with hepatic insulin sensitivity remaining unaltered (Diamond et al., 1998). Therefore, it could be suggested that the role of androgens on insulin sensitivity appears to be less significant than the effect of excess insulin (hyperinsulinemia) as an extra ovarian modulator of androgen homeostasis, increasing its production. It should be noted that hyperandrogenism can contribute to insulin resistance. However, in PCOS, hyperinsulinemia may cause hyperandrogenism rather than the converse. Taken together, the role of hyperandrogenism is still not clear, given that studies to date have used a variety of

different treatment approaches (e.g., drug choice and length of treatment) and have employed different measures of insulin sensitivity.

In women with PCOS, the ovaries can account for ~60% of androgen production, with the adrenals contributing ~40% (Cedars et al., 1992). It has been observed that hyperandrogenism can cause a preferential deposition of intra-abdominal fat and an increase in small sub-cutaneous adipocytes (Dumesic et al., 2016). This can ultimately contribute to metabolic dysfunction via lipotoxicity (Dumesic et al., 2016). Hyperandrogenism can also contribute to beta-cell dysfunction, as seen in women with PCOS with insufficient insulin secretion to compensate insulin resistance or an augmented early insulin response to glucose (Dunaif and Finegood, 1996; O'Meara et al., 1993; Vrbikova et al., 2002). These metabolic defects cannot be accounted for by insulin resistance but contribute to metabolic dysfunction and are closely related to hyperandrogenism (Goodarzi et al., 2005).

In ovarian theca cells, insulin binds to a cognate receptor, causing an increase in luteinizing hormone-stimulated androgen production. Insulin can inhibit the hepatic production and secretion of sex-hormone-binding globulin (SHBG) contributing to an increase in serum androgens and heightens luteinizing hormone-stimulated ovarian steroidogenesis (Bremer and Miller, 2008; Diamanti-Kandarakis et al., 2008). Insulin inhibits insulin-like growth factor-binding protein I (IGFBP-1) synthesis, which leads to an increase in the bioavailability of insulin-like growth factor I (IGF-1) (De Leo et al., 2003). This occurs in the liver and the ovaries, with IGF-1 reducing SHBG and increasing androgens (Duleba et al., 1998; Homburg et al., 1992). Although it is now well established that women with PCOS have insulin resistance in the peripheral and ovarian tissues, the activation of ovarian steroidogenesis appears to remain insulin sensitive (Diamanti-Kandarakis et al., 2008; Poretsky, 2006) and therefore, susceptible to the hyperinsulinemia present in these women. This phenomenon is evident in the excess production of androgens in response to insulin, influencing reproductive function (Munir et al., 2004; Poretsky et al., 1999; Willis et al., 1996; Willis and Franks, 1995). In line with that, the reduction of insulin secretion in lean women with PCOS with normal insulin sensitivity results in a reduction of androgen levels, emphasising increased insulin sensitivity of androgen secretion pathways (Baillargeon and Carpentier, 2007). On the whole, these findings indicate that the relationship between insulin resistance and hyperandrogenism play a

key role in the pathogenesis of PCOS. The excess insulin levels drive hyperandrogenism and, subsequently, other features of PCOS.

2.2.1 Hyperinsulinemia and androgens *in vivo*

Women with PCOS display a reduced metabolic clearance of insulin, which contributes to the generation of hyperinsulinemia, with serum androgens being a strong predictor of insulin clearance impairment (Tosi et al., 2020). Although the underlying mechanisms for this are unclear, it could be suggested that androgens play a role in insulin degradation. Androgen receptors have been shown to interact with insulin degradation enzyme, linking steroid hormone and insulin production (Kupfer et al., 1994). Increased androgen production in the ovaries and adrenal glands is augmented by high levels of plasma insulin (Ehrmann et al., 1992; Puurunen et al., 2009). Furthermore, insulin can cause the activation of the cytochrome of P450c17 in thecal cells, which can be attributed to an increase in the synthesis of androgens (La Marca et al., 2000). This would suggest that androgens could be in part responsible for hyperinsulinemia present in PCOS.

Few studies have investigated the direct role of androgens on skeletal muscle insulin resistance. In a study where cultured myotubes from patients with PCOS and healthy controls were treated with testosterone (100 nmol/l), there was no change in insulin sensitivity despite increased gene expression of aromatase and androgen receptor (Eriksen et al., 2014). This could indicate that other factors may be inducing the metabolic phenotype in women with PCOS. On the other hand, mouse models of PCOS treated with DHEA display skeletal insulin resistance as characterized by reduced insulin-stimulated phosphorylation of AKT, elevated HOMA-IR and fasting insulin compared to control mice (Song et al., 2018). This whole-body and skeletal muscle insulin resistance resulted from increases in p-mTOR, p-s6, and abnormal mitochondria, with decreases in ATP production and insulin-stimulated GLUT4. In order to understand and confirm this mechanism, C2C12 myotubes were treated with testosterone (500 nmol/l) along with mTORC1 inhibitor rapamycin (1uM) (Song et al., 2018). They identified that in testosterone-treated cells, rapamycin could restore AKT phosphorylation, GLUT4 expression and glucose uptake, accompanied by a reduction in p-mTOR and p-s6 ribosomal protein expression. This suggests that androgens may contribute to peripheral insulin resistance in PCOS by affecting the mTOR pathway and presents a potential therapeutic target. However,

to date, molecular studies do not allow us to conclude whether androgens directly contribute to the development of insulin resistance in PCOS and whether other factors are also involved in to the reduction in skeletal muscle insulin sensitivity. Further studies are needed to understand better the role of androgens on insulin resistance in PCOS.

2.3 Mechanisms of insulin resistance in women with PCOS

Of primary interest for this thesis is the role of skeletal muscle metabolism in PCOS. The skeletal muscle has a high abundance of mitochondria and plays a key role in glucose homeostasis, accounting for around 85% of whole-body glucose uptake following exercise or hyperinsulinemia (Defronzo et al., 1981). Skeletal muscle insulin resistance is one of the earliest detectable metabolic abnormalities to develop in individuals predisposed to metabolic diseases. This has been demonstrated in lean children with a normal glucose tolerance whose parents have T2DM having mild to severe skeletal muscle insulin resistance (Jallut et al., 1990; Perseghin et al., 1997; Vaag et al., 1992). This emphasises the potential for the early detection of metabolic dysfunction and highlights the need for interventions targeting skeletal muscle metabolism for preventing and managing metabolic diseases. The mechanism by which insulin resistance develops in women with PCOS differs from individuals with T2DM, where acquired defects are related to insulin receptor phosphorylation and/or glucose transport (Morino et al., 2006; Schinner et al., 2005). These defects can be normalized by the control of hyperglycaemia, hyperinsulinemia, and a reduction in body weight (Lean et al., 2018). Distinctly, in PCOS, there appear to be intrinsic defects in the post-binding receptor signalling, which are present in the absence of obesity or reduced glucose tolerance. Peripheral insulin resistance is a well-established clinical feature in women with PCOS; however, the mechanisms by which it develops are less clear.

2.3.1 *In vitro* identification of mechanisms of insulin resistance

Early work in this area utilised skin fibroblasts to investigate the mechanism of insulin resistance and signalling defects in women with PCOS (Carol Beth Book and Dunaif, 1999; Ciaraldi et al., 1998; Dunaif et al., 1995; Li et al., 2002). It was identified that there was an increase in insulin receptor serine phosphorylation, coupled with a decrease in insulin receptor tyrosine activity. However, this defect was only present in around 50% of fibroblasts from

women with PCOS despite *in vivo* insulin resistance (Dunaif et al., 1995). A follow-up study showed that the use of serine kinase activity inhibitor could restore the insulin-stimulated phosphorylation of the insulin receptor (Li et al., 2002). Furthermore, they were able to isolate insulin receptors from fibroblasts. Alternatively, others failed to observe any defects in insulin receptor binding or reductions in insulin sensitivity in fibroblasts obtained from women with PCOS (Carol Beth Book and Dunaif, 1999; Ciaraldi et al., 1998). Collectively, these findings suggest a lack of universal causation or defect in the development of insulin resistance in PCOS. A limitation with the use of skin fibroblasts for the assessment of peripheral insulin resistance is that, although they play a role in the whole muscle insulin sensitivity, fibroblasts are not the main insulin targeted cells making the translation and application of these findings challenging.

Several studies have reported that human primary myotubes from women with PCOS are no longer insulin resistant, suggesting that phenotype is not maintained (Ciaraldi et al., 2009; Corbould et al., 2005; Eriksen et al., 2010, 2014). The work from Corbould et al. (2005) found that glucose uptake was increased in PCOS under basal and insulin-stimulated state when compared to control myotubes. On the other hand, they noted that myotubes from women with PCOS had increased phosphorylation of the inhibitory IRS-1_{ser312} leading to a reduction in downstream PI3-K activity and IRS-1 degradation (Corbould *et al.*, 2005), consistent with the findings in the whole muscle (Corbould et al., 2006). They also noted that IRS-2 signalling was impaired in myotubes from women with PCOS under basal and insulin-stimulated conditions despite having a similar abundance as controls. These findings suggest that there are intrinsic defects in insulin signalling that may be exacerbated by *in vivo* circulating factors as the insulin-stimulated glucose uptake was not reduced in myotubes from women with PCOS.

Contrary to these findings, Ciaraldi et al. (2009) noted that under basal and insulin-stimulated conditions, myotubes from women with PCOS had decreased glucose uptake (~50%) compared to controls despite similar GLUT4 content and no apparent insulin signalling defects, representing impairment in insulin responsiveness (Ciaraldi et al., 2009). However, they showed that PCOS and healthy controls normal insulin sensitivity via insulin-dose response curve normalized to % of basal glucose uptake. Adding to these findings, Eriksen *et al.* (2010 and 2014) have shown a lack of insulin resistance in myotubes from women with PCOS and found no intrinsic defects in glucose or lipid metabolism. In addition, these myotubes also

appeared to exhibit normal mitochondrial function and content contrary to *in vivo* findings (Eriksen et al., 2011; Konopka et al., 2015; Skov et al., 2007). Taken together, this suggests that acquired environmental factors rather than intrinsic defects or traits may play a major role in the development of PCOS-specific insulin resistance. This will be the focus of **Study 1,** where we assess the defects in insulin signalling and environmental contributors using human primary myotubes from women with and without PCOS.

2.3.2 *In vivo* identification of the mechanisms of insulin resistance

A post binding defect in the early step of insulin signalling transduction was first detected in the skeletal muscle of women with PCOS (Diamanti-Kandarakis and Papavassiliou, 2006; Dunaif et al., 2001, 1995; Li et al., 2002). β-subunit of the insulin receptor contains a tyrosine kinase that its activity is enhanced by autophosphorylation of the tyrosine residues and inhibited by serine phosphorylation. The activate tyrosine kinase phosphorylates substrates (Insulin receptor substrate (IRS) -1 and -2) inside the cell and initiate signal transduction (White, 1998; Zierath and Wallberg-Henriksson, 2002). IRS-1 is a major docking protein in the skeletal muscle and activates phosphatidylinositol 3-kinase (PI3K) to stimulate glucose uptake. Serine phosphorylation of IRS-1 leads to a reduction in tyrosine phosphorylation, which causes degradation of the receptor and reduces PI3K and Akt activity (Draznin, 2006; Hançer et al., 2014; Pederson et al., 2001). This results in a reduction in insulin-stimulated glucose uptake (Björnholm et al., 1997; Goodyear et al., 1995). The chronic stimulus of this pathway, by obesogenic and inflammatory factors such as TNFα, free fatty acids, and excessive insulin, are thought to lead to the development of insulin resistance (Gual et al., 2005). Several studies have attempted to identify the insulin signalling defects in women with PCOS by obtaining muscle biopsies during the euglycemic-hyperinsulinemic clamp (Ciaraldi et al., 2009; Dunaif et al., 2001a; Glintborg et al., 2008; Hojlund et al., 2008; Rajkhowa et al., 2009). It was found that the IRS-1-associated PI3K activity was significantly decreased, with the abundance of IRS-1 and p85 subunit remaining unchanged in comparison to women without PCOS (Dunaif et al., 2001). This was accompanied by attenuated insulin-mediated glucose uptake and a compensatory increase in the abundance of IRS-2 (Dunaif et al., 2001). These defects in IRS-1 serine dominant phosphorylation have been observed downstream of PI3K with phosphorylation of Akt and AS160 being reduced (Hojlund et al., 2008). In opposition to these findings, it was shown that Akt phosphorylation at ser473 and Thr308 were similar to

healthy controls following insulin stimulation (Ciaraldi et al., 2009). These findings may be accounted for in different insulin infusion rates employed to reach steady-state during the euglycemic-hyperinsulinemic clamps. Glintborg and colleagues further exemplified this, highlighting a reduced insulin activation of glycogen synthase and inhibition of GS kinase-3 (Glintborg et al., 2008). In contrast to previous findings, they noted no differences in the expression and insulin activation of IRS-1 (Rajkhowa et al., 2009). More recently, it has been identified as a defect in the anabolic nutrient sensor mTOR in overweight women with PCOS (N. Stepto et al., 2020). This could potentially have a negative effect on insulin-stimulated glucose transport and the activation of Akt (Takano et al., 2001). In order to separate the effects of obesity and PCOS, others have explored skeletal muscle metabolic defects in lean women with PCOS. It was observed that insulin resistance in lean women with PCOS was not related to changes in the insulin signalling cascade but rather reduced expression and phosphorylation of AMPK (modulator of insulin sensitivity) and dysregulation of pyruvate dehydrogenase (Hansen *et al.*, 2019). This was also accompanied by an increase in skeletal muscle lipid accumulation and low levels of plasma adiponectin. This was associated with low levels of adiponectin (Hansen, *et al.*, 2019), which has previously been linked to adipose dysfunction in women with PCOS (Mannerås-Holm et al., 2011). These studies highlight a potential cross-talk between muscle and adipose tissue in women with PCOS in the development of insulin resistance. This could result in a reduction in insulin sensitivity and glucose uptake via adjusting fuel oxidation.

Intriguingly, insulin resistance in women with PCOS is thought to be selective, affecting only the metabolic pathway but no other aspects of insulin actions in a variety of different cell types: skin fibroblasts (C B Book and Dunaif, 1999) adipocytes (Corbould and Dunaif, 2007) and ovarian granulosa cells (Rice et al., 2005). However, insulin activation of mitogenic signalling in skeletal muscle of women with PCOS is defective (Rajkhowa 2009; Corbould 2006). Rajkhowa et al. (2009) identified an increase in phosphorylation of extracellular signal-regulated kinases (ERK) 1/2 and reduced insulin stimulation of the mitogenic pathway (Rajkhowa et al., 2009). They also observed a reduction in ERK activity *in vivo* following exposure to a dose of insulin. Contrary, Corbould et al. 2006., found that MAPK-ERK1/2 signalling pathway to be constitutively activated, and suggested that this activation may be responsible for increased serine phosphorylation and thus, decreased IRS-1-associated PI3-K activity (Corbould et al., 2006). Although this pathway has been demonstrated to be defective,

this has not been consistently observed in all studies that explore skeletal muscle insulin signalling in women with PCOS. When taken together, these findings provide more confusion than clarity with a lack of agreement on the insulin signalling defects (**Figure1**). This will be the focus of **Study 3,** where we will assess skeletal muscle insulin signalling in biopsy samples collected at rest and during the euglycemic-hyperinsulinemic clamp.

2.3.3 Environmental factors and intrinsic defects

It has been suggested that skeletal muscle insulin resistance occurs *in vivo* due to both environmental factors and intrinsic defects. Both (Corbould *et al.*, 2005) and (Eriksen et al., 2010) found that metabolic defects in cultured human myotubes from women with PCOS were not maintained. In contrast, others have demonstrated that reductions in insulin responsiveness remained in human myotubes from overweight women with PCOS (Ciaraldi *et al.*, 2009). These conflicting results could be due to the severity of PCOS phenotypes selected, differences in patient characteristics such as basal insulin and testosterone levels, and methodological approaches. This presents an opportunity to investigate the environmental factors that lead to the acquired defects in insulin signalling and subsequent reduction in glucose uptake in women with PCOS. This will be explored in **Study 1**.

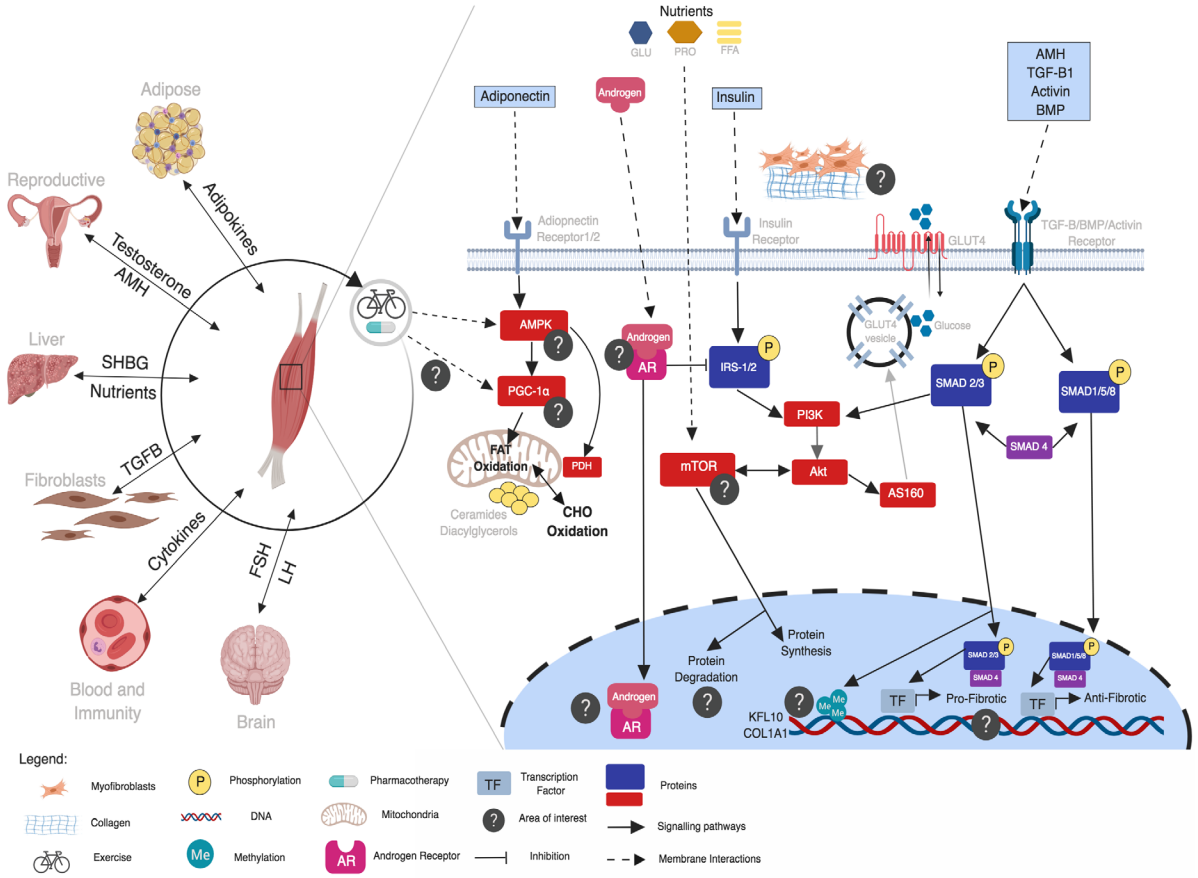

Figure 1 Mechanisms of skeletal muscle insulin resistance

This Schematic diagram outlines the potential molecular mechanisms of skeletal muscle insulin resistance in women with PCOS based upon the research to date. The schematic incorporates organ system cross-talk, genetic and epigenetic programming, signalling pathway dysfunction, all contributing as potential molecular mechanisms of reduced glucose uptake into this vital metabolic tissue. Indicating that not only are the metabolic origins of PCOS complex but most likely, there are multiple contributing factors. The identified defects highlighted with question marks are potential therapeutic targets and areas that require further research, while many defects have been identified, many studies present conflicting findings with regards to the identified defects. This is most likely due to the heterogeneity of women with PCOS and studies with women with different phenotypes of PCOS. The schematic highlights potential external factors that are contributing to the metabolic defects in the skeletal muscle such as adipokines, cytokines, hormones and nutrients. The consequences of disrupted homeostasis in the aforementioned factors result in impaired insulin signalling, reduced PDH activation and subsequent metabolic flexibility, mitochondrial function, tissue fibrosis, and reduced protein synthesis created with Biorender.com, from (Stepto *et al.*, 2019; created by Luke McIlvenna).

2.4 Mechanisms and effects of exercise-induced improvements in insulin sensitivity

Exercise is considered an effective lifestyle therapy for improving peripheral insulin sensitivity with acute and chronic effects. As previously mentioned, skeletal muscle insulin resistance is one of the earliest detectable metabolic abnormalities demonstrating its highly adaptable nature. Similarly, in response to exercise, improved insulin sensitivity can be observed after a single bout of exercise (Mikines et al., 1988; Richter et al., 1989). These beneficial effects are related to an increase in glycogen synthesis to restore glycogen levels post-exercise. This is further exemplified following exercise, where increases in insulin sensitivity may be specific to the exercised muscle groups (Steenberg et al., 2020). This effect was demonstrated using a one-legged dynamic knee extension exercise model, isolating muscle activation to the quadriceps (Andersen et al., 1985). The exercise was performed for 2.5hours or until local muscle exhaustion, resulting in an increase in insulin-stimulated glucose uptake in the exercised limb (~140%) and, decrease in whole-body glucose disposal rate (~18%) and in the non-exercised limb (~37%) (Steenberg et al., 2020). It was suggested that this might occur to redirect glucose to the exercised muscles in order to restore glycogen. Although the mechanism responsible for this response are still not clear, they may be related to PI3K-Akt signalling. The role of improved insulin sensitivity to restore muscle glycogen is made more apparent by the fact that carbohydrate refeeding that occurs immediately after exercise can replete glycogen and eliminate/inhibit an increase in insulin sensitivity (Bogardus et al., 1983). In support of these findings, it has been shown that maintaining a carbohydrate deficit overnight following exercise may be required for improvements in insulin sensitivity and postprandial glycaemic control (Taylor et al., 2018). Elevated muscle glycogen levels have an inhibitory effect on glucose uptake and glycogen synthesis in a basal or insulin-stimulated state (Jensen et al., 2006). Essentially, these findings highlight a relationship between glycogen availability and insulin sensitivity at rest and following acute exercise. The mechanism responsible for this has been partly attributed to AMPK acting as a glycogen sensor. This can be seen following contraction with and without insulin stimulation under low glycogen conditions, where there is a significant increase in AMPKα2 activity and phosphorylation of AMPKα thr^{172} (Lai et al., 2010). A series of studies have identified AMPK and downstream signalling in TBC1D1 and TBC1D4 to be responsible for post-exercise increases in glucose uptake, regulating skeletal muscle insulin sensitivity (Kjøbsted et al., 2019b, 2019a, 2017; Sjøberg et al., 2017). This

process appears to be intact in individuals with type 2 diabetes (Kjøbsted et al., 2016). In animal models, this effect has been shown to occur in a fibre type-specific manner with post-exercise insulin-stimulated phosphorylation of AS160/TBCD14 being greater in type IIA and IIB (glycolytic) fibres than type I or IIX (oxidative) (Pataky et al., 2019). This was complemented by an increase in glucose uptake in type IIA, IIX, IIBX and IIB but not in type I. This may provide a rationale of greater improvements in insulin sensitivity in humans following high-intensity exercise in comparison to moderate-intensity exercise. It has been demonstrated that acute high-intensity interval exercise results in greater phosphorylation of skeletal muscle AMPK, TBC1D4, and ACC in type II fibres compared to continuous/moderate exercise (Kristensen et al., 2015). This occurred in conjunction with a greater reduction in overall muscle glycogen and also in type II fibres. The interaction between AMPK and muscle glycogen in the regulation of insulin sensitivity has been shown following glycogen depleting exercise, with glucose uptake being regulated by increases in the expression of GLUT1, GLUT4, HKI, and HKII proteins (Hingst et al., 2018). This study highlights AMPK being responsible for a post-exercise increase in fatty acid oxidation to reduce glycogen synthesis allowing glycogen levels to be restores. Collectively, this vast body of work highlights the importance of AMPK in acting as a mediator for the health benefits of exercise, particularly insulin sensitivity. Any disruptions to AMPK signalling may influence metabolic exercise adaptations.

Furthermore, exercise promotes GLUT4 translocation, a key step for glucose transport and a determinant of enhanced insulin sensitivity following exercise (Hansen et al., 1998). Exercise training, but not acute exercise, also increases GLUT4 protein expression in skeletal muscle, contributing to improved insulin sensitivity (O'Gorman et al., 2006). Post-exercise increases in insulin-stimulated glucose uptake can occur without changes in GLUT4 abundance, with translocation being the vital step (Cartee et al., 1993; Castorena et al., 2014). Following an acute exercise bout, GLUT4 is recruited to the cell-surface membrane via endocytosis. This results in greater receptiveness to recruitment by insulin, facilitating increases in insulin-stimulated glucose uptake (Geiger et al., 2006). It could be suggested that these effects occur independently of muscle glycogen, given the lack of direct association or interaction of GLUT4 and muscle glycogen at rest or following exercise (Murphy et al., 2018). It has recently been observed that insulin stimulation results in an increase in muscle membrane permeability (McConell et al., 2020), with a 17-fold increase in the rested muscle and a 36-fold increase in

exercised muscle. It may be postulated that this increase in permeability represents an increase in the number of GLUT4 transporters. Due to difficulties when accurately measuring transient GLUT4 translocation following exercise or following *ex vivo*/*in vivo* contraction, further work is required to understand how exercise regulates GLUT4. A recent advance has been made in this field using multiple microscopy techniques to demonstrate that a single bout of exercise causes a redistribution of intramyocellular GLUT4 to GLUT4 storage vesicles and T-tubuli (Knudsen et al., 2020). These responses may be impaired in individuals who have insulin resistance and would lead to a reduction in insulin-stimulated glucose uptake. Indeed, AMPK signalling may be the linking factor between GLUT4 translocation and glycogen depletion in exercise-induced improvements in insulin sensitivity. This may be explained by AS160/TBC1D4 being activated via AMPK, with greater phosphorylation of AS160/TBC1D4 being linked to enhanced glucose transport in skeletal muscle following exercise and insulin stimulation (Cartee, 2015a). It has been demonstrated that individuals with type 2 diabetes have decreased insulin-stimulated phosphorylation in AS160/TBC1D4 at multiple phosphorylation's sites. This can be improved by ten weeks of aerobic endurance training and was linked to improvements in insulin sensitivity (Vind et al., 2011). This demonstrates the role of exercise to improve insulin sensitivity via increases in TBC1D4 phosphorylation, which plays a role in facilitating GLUT4 translocation.

The beneficial effect of exercise for improving insulin sensitivity extends to improved microvascular perfusion with increases in endothelial function facilitated by endothelial nitric oxide synthase. This enables an increase in the delivery and disposal of glucose in the skeletal muscle (Sjøberg et al., 2017). This cross-talk between the endothelium and skeletal muscle has been observed when endothelial insulin signalling is disrupted, and there is a subsequent reduction in skeletal muscle glucose uptake (Kubota et al., 2011). Additionally, there is an increase in circulating factors such as cytokines and myokines, which may play a role in tissue cross-talk and subsequent regulation of whole-body metabolism (Hoffmann and Weigert, 2017; Laurens et al., 2020). Improvements in skeletal muscle and whole-body insulin sensitivity can last up to 48 hours following acute exercise (Cartee et al., 1989). Therefore, regular exercise can lead to sustained improvements in insulin sensitivity. However, these effects appear to be acutely driven, with active individuals having comparable insulin resistance to sedentary individuals after only ten days of sedentary behaviour (Heath et al., 1983). However, insulin sensitivity could be restored to trained levels following a single

exercise bout (Heath et al., 1983), suggesting that the benefits of regular exercise on insulin sensitivity are largely reliant on the most recent exercise session. Others have confirmed these findings showing that short-term reduction in daily activity leads to the development of metabolic dysfunction characterized by worsening of peripheral insulin sensitivity (King et al., 1988; Krogh-Madsen et al., 2010), further highlighting the high level of skeletal muscle plasticity. From these studies, it could be postulated that there is an underlying metabolic memory, which may include sustained low muscle glycogen, increases in insulin-independent AS160/TBC1D4 phosphorylation, and GLUT4 recruitment to the cell surface membrane (Cartee, 2015b). However, further research is required to elucidate whether any of these pathways and proteins are responsible for the retained metabolic benefits of exercise training.

It is well established that exercise training can improve whole-body insulin sensitivity, signified by an increase in insulin-stimulated glucose uptake. Despite acute exercise acting as a potent insulin sensitizer, this effect is dampened by exercise training (Steenberg et al., 2019). The authors suggested this may be due to the upper physiological limit of muscle insulin sensitivity or related to absolute muscle glycogen levels, with a reduction in glycogen degradation following training. While changes in insulin sensitivity may be relatively short-lived following exercise, several long-term adaptations prove beneficial for metabolic health. These include increased capillarization, enhanced substrate utilization capacity, increased basal insulin sensitivity, and increases in protein expression of GLUT4 and mitochondrial proteins (Sylow and Richter, 2019). Exercise intensity appears to be a key determining factor in the extent of improvement in metabolic health observed following exercise training. When exercise intensity increases, there is a matched increase in skeletal muscle glucose uptake (Romijn et al., 1993; Wahren et al., 1978), indicating that high-intensity exercise may be more favourable for improving metabolic health, driven by greater reductions in muscle glycogen.

High-intensity interval exercise has been suggested to produce superior metabolic health benefits in comparison to moderate-intensity exercise in some but not all cases. In those studies reporting distinct metabolic health outcomes between interventions, differences may be purely related to study design, for instance, when interventions are not matched for total workload and training volume. This has been demonstrated in data from the STRRIDE trial, where regardless of the intensity of the exercise performed, the total time had a greater effect on insulin sensitivity (170 min/week compared to 115 min/week) (Fay et al., 2009). Others have shown

that similar improvements in insulin sensitivity occur independent of exercise intensity (Braun et al., 1995; Burgomaster et al., 2008), demonstrating that both moderate- and high- intensity exercise interventions are capable of improving insulin sensitivity despite the mechanism by which they improve insulin sensitivity may differ. In another study, eight months of moderate or vigorous exercise training led to improvements in insulin sensitivity, with moderate leading to greater improvements in disposition index, a marker of beta-cell function, while high-intensity resulted in a greater reduction in acute insulin response to glucose (Slentz et al., 2009). Remarkably, there appears to be a longer-lasting benefit on insulin sensitivity following high-intensity exercise training, as seen following a period of training cessation, which may be accounted for differences in acute responses between exercise intensities (Bajpeyi et al., 2009). However, in cases where volume is matched between moderate-intensity (40-55% VO_{2peak}) and high-intensity training (65-80% VO_{2peak}), moderate-intensity training results in greater improvements in insulin sensitivity index in individuals who were obese and sedentary (Houmard et al., 2004). It should be noted that conflicting results in a number of studies examining exercise interventions using different intensities may be dependent on diet, which can influence the extent of improvements in metabolic health alone. This has been shown by Stocks et al. (2019), whereby exercise was performed in fasted and fed state and observed different responses in post-exercise skeletal muscle signalling, with greater increases in the phosphorylation of AMPK and PDK4 gene expression following exercise in a fasted state (Stocks et al., 2019).

The differences in response between exercise intensities may be explained by changes in energy demand and the activation of intracellular regulators of insulin sensitivity and glucose uptake. Several studies have investigated how exercise intensity influences skeletal muscle metabolism (Barrès et al., 2012; Combes et al., 2015; Egan et al., 2010; Kristensen et al., 2015). These studies show that high-intensity exercise produces greater increases in the gene expression of PGC1α, TFAM, PPARdelta and PDK4, as well as an increase in phosphorylation of AMPK, ACC, CAMKII, P38MPAK, ATF2, helping to facilitate post-exercise glucose uptake. It has been established that there is a link between glucose metabolism and PGC1α expression, with PGC1α causing an increase in GLUT4 gene expression in myotubes and resulting in an increase in basal and insulin-stimulated glucose uptake (Michael et al., 2001). These findings may be related to greater perturbations in homeostasis via metabolic fluctuations and related to phospho-proteins. It is clear that both high- and moderate-intensity

exercise interventions are capable of improving insulin sensitivity via different mechanisms. This may be relevant for different diseases states, where insulin resistance may develop from different defects. In order to make sure exercise interventions are effective and optimal for health benefits, it is important to determine minimal effective dose, which could then in turn, influence physical activity guidelines.

2.4.1 Resistance to metabolic benefits of exercise evidence from individuals with type 2 diabetes

The phenomena of impairments in exercise adaptations have been explored in individuals with type 2 diabetes. Data from the TULIP study identified that individuals who failed to see improvements in glucose homeostasis were severely insulin resistant, had low VO_{2peak}, and increased levels of fat deposits in the liver and abdomen (Stefan et al., 2015, 2006; Thamer et al., 2007; Totsikas et al., 2011). On the other hand, the DEXLIFE study noted that clinical variables were unable to differentiate or predict response to an exercise intervention and stated that additional biomarkers are required (O'Donoghue et al., 2019). A recent comprehensive assessment of transcriptional regulators of metabolic adaptations in skeletal muscle in individuals with type 2 diabetes following acute exercise found similar increases in stress-response genes (NR4A1, NR4A2, NR4A3, ATF3, ERG1, JUNB, SIK1) and upstream regulators (CaMKII, p38 MAPK, Erk1/2, CREB, and AMPK) to matched controls (Sabaratnam et al., 2019). Although responses to acute exercise appear to be intact in individuals with type 2 diabetes, there appears to be a disparity in response to long-term exercise training. Recent observations have noted that exercise interventions are not effective for all individuals with type 2 diabetes with some failing to see improvements in insulin sensitivity and mitochondrial function (Stephens et al., 2018). Individuals who failed to see positive responses were characterized by a unique pre-training epigenetic and transcriptomic pattern in skeletal muscle, with some characteristics maintained in cultured primary skeletal muscle cells, highlighting intrinsic T2DM defects in the skeletal muscle. There was a total of 533 differently methylated CpG sites between those who showed improvements in insulin sensitivity and those who did not, with insulin signalling and mitochondrial pathways being enriched in both DNA methylation and RNAseq data. This highlights the use of primary cell culture as an appropriate model to investigate these defects and presents an opportunity to study the response to exercise in these participants using an *in vitro* physiological approach.

2.4.2 *In vitro* models of exercise: electrical pulse stimulation

Electrical pulse stimulation (EPS) has become a popular *in vitro* model of contraction in human primary and C2C12 myotubes (Nikolić et al., 2017). A comparison of endurance exercise *in vivo* (voluntary wheel running) and EPS *in vitro* (contraction), showed that similar to *in vivo* exercise, EPS caused an increase in gene expression of Myh7 and Myh2, and increases in contraction-induced signal transduction proteins p38MAPK, CaMKII, AMPK, PGC1α, and ATF2 (Son et al., 2019). An application for EPS studies is showing how different donor groups respond to *in vitro* exercise and exploring how intrinsic and environmental factors influence EPS-induced responses. For instance, Park et al. (2019) detected differences in EPS-induced signalling between myotubes from lean individuals (BMI ~23) and severely obese individuals (BMI ~ 44). They demonstrated differences in the phosphorylation of AMPK and ACC immediately after 24hrs of EPS, with both groups seeing an increase but myotubes from severely obese having a significant reduction in response (Park et al., 2019). However, others have found normal activation of AMPK and increase in IL6 following EPS in myotubes from individuals with type 2 diabetes, with impairments in insulin-stimulated glucose uptake and phosphorylation of $AS160_{thr642}$ following EPS (Al-bayati et al., 2019). Both studies add to evidence that individuals with metabolic diseases may not achieve the same insulin-sensitizing benefits achieved by muscle contraction as healthy individuals. Interestingly, metabolic changes in skeletal muscle *in vivo* following exercise training, such as improvements in fatty acid and glucose oxidation, are preserved in cultured myotubes from healthy sedentary men (Lund et al., 2017). However, to date, no one has assessed contractile responses to EPS in myotubes from women with PCOS. This *in vitro* model will be applied in **Study 2** to compare exercise-induced signalling in myotubes from women with and without PCOS.

2.5 Effect of exercise in women with PCOS: insulin sensitivity and resistance to the beneficial effects

2.5.1 Acute responses to exercise in skeletal muscle metabolism and insulin signalling

Lifestyle modification is the recommended first-line therapy for women with PCOS who have insulin resistance or are overweight. A limited number of studies have attempted to understand how women with PCOS respond to acute exercise and chronic exercise training in terms of changes in insulin signalling and insulin sensitivity. Preliminary evidence suggests that women with PCOS who are overweight have lesser improvements in peripheral insulin sensitivity following exercise training than healthy controls (Harrison et al., 2012). Defects in skeletal muscle insulin signalling observed in women with PCOS may be responsible for this divergent response (Stepto et al., 2019). Differences would be apparent in response to an acute bout of exercise, with the differences in response determining the effectiveness of exercise training. In response to an acute bout of exercise, women with PCOS have a similar ability to increase GLUT4 translocation (a key step required for exercise-induced increases in glucose uptake and insulin sensitivity) as healthy controls (Dantas et al., 2015). Despite similar GLUT4 translocation, women with PCOS had a lack of increase in PI3K-p85 activity and phosphorylation in AS160, resulting in a compensatory increase in the phosphorylation of Akt and AMPK. These compensatory mechanisms may increase skeletal muscle glucose uptake despite underlying defects in insulin signalling transduction. One common defect in the insulin signalling pathway in PCOS is an increase in phosphorylation of IRS-1_{ser312} (Corbould et al., 2006, 2005; Diamanti-Kandarakis and Dunaif, 2012). Following exercise, these defects were no longer apparent with levels similar to those observed in healthy controls (Dantas et al., 2015), demonstrating that exercise is an effective mean for women with PCOS to protect against insulin resistance and improve their insulin sensitivity. Although there were differences in molecular responses to acute exercise between women with PCOS and healthy women, signalling responsible for glucose uptake appeared to be intact. Women with PCOS also show irregular molecular responses to exercise with upregulation of genes related to inflammation in skeletal muscle, SOCS3, IL-8, and NAMPT compared to overweight controls, and increased NF-κB expression at rest (Dantas et al., 2017). Another notable difference was the lack of an increase in PGC1α mRNA following exercise in skeletal muscle of women with PCOS.

PGC1α is a key regulator of mitochondrial biogenesis and metabolism. Of interest, PGC1α has been shown to regulate GLUT4 gene expression and mitochondrial biogenesis and angiogenesis following exercise (Baar et al., 2002; Chinsomboon et al., 2009). This may suggest that mitochondrial dysfunction plays a role in developing insulin resistance and atypical responses to exercise in women with PCOS. This response has been observed in adults with early onset of type 2 diabetes with a lack of increase in PGC1α following acute exercise in contrast to a 4-fold increase seen in adults who were obese (Hernández-Alvarez et al., 2010). Dantas and Nilsson have demonstrated elevations in gene expression of NAMPT at rest and following exercise in skeletal muscle of women with PCOS (Dantas et al., 2017; Nilsson et al., 2018). NAMPT is a key regulator of NAD+ biosynthesis, regulates sirtuins activity, and is believed to function as both a cytokine and adipokine (Garten et al., 2015). The overexpression of NAMPT has been shown to improve whole-body insulin sensitivity in rats via IRS-1 tyrosine phosphorylation in the liver and muscle (Sun et al., 2009). This could act as a protective effect to help mitigate the effects of insulin resistance. On the other hand, NAMPT has been shown to play a role in the regulation of the ECM, with NAMPT capable of increasing matrix metalloproteinases and subsequent activation of NF-κB and MAPK signalling (Dahl et al., 2012; Oita et al., 2010). This could lead to an inflammatory response and potentially remodel the ECM. The contrasting outcomes for NAMPT may be related to discrepancies in its function within the cell and the extracellular space. These differences in oxidative stress and reactive oxygen species production would indicate that PCOS is a pro-inflammatory condition. The role of NAD and its metabolism in women with PCOS requires further investigation.

2.5.2 Effect of acute exercise on inflammation in women with PCOS

To follow up on these findings, Dantas and colleagues explored the effects of acute exercise on skeletal muscle inflammation in PCOS and matched controls (Dantas et al., 2019). Similar to previous findings regarding the insulin signalling defects, they noted baseline differences in inflammatory markers, with elevated levels of pro-inflammatory cytokine TNF-alpha and protein expression of IKKα/β and JNK in skeletal muscle. Both IKKα/β and JNK can inhibit insulin signalling, contributing to the development of insulin resistance. It has been proposed that this occurs via a reduction in the phosphorylation of the insulin receptor and AKT, which is accompanied by an increase in the phosphorylation of IRS-1$_{ser307/312}$ (Aguirre et al., 2000;

Hirosumi et al., 2002; Solinas and Becattini, 2017). Following a single exercise session, levels of TNF-alpha and protein expression of IKKα/β and JNK were restored to levels similar to controls. The protective effects of acute exercise in these studies were measured one hour following the cessation of exercise. However, it is not clear if these benefits would last beyond this period. Altogether, current literature supports that acute responses to exercise are beneficial for insulin signalling and the inflammatory state of the skeletal muscle of women with PCOS, but it is yet to be fully established whether underlying defects in skeletal muscle metabolism in women with PCOS can be resolved with long-term exercise training.

2.5.3 Effects of exercise training on insulin sensitivity and skeletal muscle insulin signalling

Harrisson, Stepto, and colleagues proposed the initial idea that exercise-induced improvements in peripheral insulin sensitivity may be reduced or blunted in women with PCOS. They found that their cohort of overweight and obese women with PCOS (n=20) were 46% less insulin sensitive as measured by the euglycemic-hyperinsulinemic clamp compared to controls (n=13) at baseline (Harrison et al., 2012). However, it should be noted that the level of reduction in insulin sensitivity appears to be highly cohort dependant. Following exercise training (3 days per week for 12 weeks, moderate-high-intensity) women with PCOS improved insulin sensitivity by 16.37%, whereas healthy controls improved by 23.75%. Both groups had comparable adherence and compliance with training and increases in aerobic capacity (VO_{2peak}). Given that women with PCOS had lower insulin sensitivity before training, it would be expected they would have had enormous scope for improvement. Overall, women with PCOS tend to be around 27% less insulin sensitive independent of BMI (Cassar et al., 2016b). Follow-up work by Hutchison et al. attempted to determine if increased lipid content and compromised mitochondrial function were determining factors for insulin sensitivity and changes following exercise training in women with PCOS (Hutchison et al., 2012). They highlighted that differences in insulin sensitivity between women with PCOS and healthy controls could not be explained by mitochondrial function and skeletal muscle lipid content.
Furthermore, following a period of exercise training, improvements in peripheral insulin sensitivity as measured by the euglycemic-hyperinsulinemic clamp, occurred independently of

changes in mitochondrial function (Hutchison et al., 2012). Others have confirmed these findings, suggesting that mitochondrial function is likely not the primary candidate in skeletal muscle insulin resistance and effects of exercise training on metabolic parameters (Eriksen et al., 2011; Rabol et al., 2011; Konopka et al., 2015). It is important to note that most measures of mitochondria are carried out using permeabilized muscle fibres or isolated mitochondria *ex vivo*. To overcome these methodological limitations, Cree-Green et al. assessed mitochondria function *in vivo* of lean women with PCOS using MRI during exercise. They noted that women with PCOS had a slower rate of PCR recovery and more significant ADP depletion despite exercising lower intensity than healthy women, indicating skeletal muscle mitochondrial dysfunction during exercise (Cree-green et al., 2017). Further studies are needed to investigate whether these findings may be explained by differences in mitochondrial content.

In a further attempt to understand how peripheral insulin sensitivity and insulin signalling is influenced by exercise training, Stepto, Hiam et al. (2020) assessed insulin-stimulated skeletal muscle biopsies following twelve weeks of treadmill-based exercise intervention incorporating moderate and high-intensity exercise sessions. Before training, they identified a reduced expression in insulin-stimulated phosphorylation of mTOR, which was not normalized following the exercise intervention. This may have implications for phosphorylation of IRS1 and downstream PI3K/Akt pathway with subsequent impact on intracellular glucose handling and GLUT4 translocation, but further studies are needed to elucidate the role of mTOR Complexes and relevant downstream signalling in PCOS skeletal muscle metabolism. This may have important implications for glucose metabolism given AMPK-mTORC2 has been shown to suppress hepatic glucose production in the liver and contrastingly, improves glucose uptake in response to energetic stress in muscle and fat (Kazyken et al., 2019). Any alterations in this pathway could, in theory, alter this response and result in ineffective glucose handling, which could, in turn, influence the beneficial effects of exercise on insulin sensitivity. This will be the focus of **Study 4,** where women with and without PCOS took part in a twelve week exercise intervention.

2.5.4 The hypothesis of the mechanisms of impaired metabolic benefits to exercise training

Stepto et al., also noted that women with PCOS had altered gene expression of TGF-beta ligands and components of the extracellular matrix, which was still present after 12 weeks of exercise training (N. Stepto et al., 2020). A significant limitation of this study is the lack of measures of skeletal muscle morphology and protein expression to support the notion that tissue fibrosis plays a role in PCOS-specific skeletal muscle peripheral insulin resistance. This presents the hypothesis that TGF-beta ligands and ECM-remodelling may play a role in the PCOS-specific skeletal muscle insulin resistance, and this may influence the metabolic adaptations to exercise.In line with these findings suggesting that women with PCOS may be resistant to the beneficial effects of exercise training, Hansen et al. (2018) found that skeletal muscle GLUT4 and hexokinase II protein and mRNA content were unaltered by training in lean women with PCOS in contrast to fitness and BMI matched control group. Also, insulin-stimulated AS160/TBCD14 and Akt were not altered by training. However, they were unable to identify the specific reason for this lack of response in key proteins following training. In **Study 4,** we explore the potential underlying factors that may be negatively modulating the beneficial effects of exercise training on insulin sensitivity in women with PCOS.

A limitation with the current exercise training studies in women with PCOS is that none have assessed the acute response of skeletal muscle to exercise following training to understand further the effect of exercise training on insulin signalling and metabolic function. . To be able to address that, it would require a larger number of samples from women with PCOS across the different phenotypes. For this to be feasible, considering the challenges in long-term studies (e.g., participant retention and engagement) and invasive techniques such as biopsies, a multi-centre trial across multiple countries would need to occur.

2.6 The role of transforming growth factor-beta superfamily ligands in PCOS

2.6.1 Overview of TGF-beta signalling

Most tissues have an abundance of latent TGF-beta ligands within the extracellular environment with the regulation of its function being dependent on activation. This activation takes place by environmental stressors, tissue injury, changes in pH, growth factors and mechanical activation (K. K. Kim et al., 2018). Once activated, TGF-beta ligands bind to co-receptors TGFBRI and TGFBRII and results in phosphorylation of SMAD 2/3 and binds to SMAD4 before being translocated to the nucleus. This signalling results in the activation of ECM, collagen and matrix metalloproteases genes (**Figure 2)** (Derynck and Budi, 2019). Other members of the TGF-beta family, namely BMP's, activate the SMAD1/5/8 pathway, which is seen as an antifibrotic pathway (Walton et al., 2017). TGF-beta signalling and receptors can activate non-SMAD signalling pathways, including MAPK (Erk1/2, JNK1/2/3 and p38) and PI3K-AKT-mTOR (Zhang, 2009). The interaction of TGF-beta signalling with these other pathways leads to the release of other cytokines and growth factors (Guo and Wang, 2009). Collectively, the function of TGF-beta signalling is diverse, playing a vital role in tissue regeneration and repair, although dysregulation of this signalling by factors such as inflammation and reactive oxygen species may promote tissue fibrosis and tumour growth (Ábrigo et al., 2018; David and Massagué, 2018; Jain et al., 2013). The diverse role of TGF-beta signalling and its interaction with other signalling pathways confer a possible role in altering metabolism and tissue morphology.

2.6.2 TGF-beta signalling in the pathogenesis of PCOS

The seminal work from Rodgers and colleagues on ovarian tissue fibrosis in women with PCOS has highlighted that PCOS may be a fibrotic condition that may help to explain a number of the symptoms that develop. This raises the question, do the effects seen in ovarian tissue have a systemic impact (**Figure 3**). This seems plausible given that women with PCOS often develop non-alcoholic fatty liver disease, which is defined as a profibrotic condition (Vassilatou et al., 2010), increased tissue density of the skeletal muscle (Hutchison et al., 2012), and the upregulation of TGF-beta signalling gene expression in adipose tissue (Kokosar et al., 2016).

It has been established that ovarian hormones can influence skeletal muscle metabolism and function (Boisseau et al., 2012; Livingstone and Collison, 2002). Given that women with PCOS have significant alterations in ovarian hormones and morphology, these factors may play a role in the development of insulin resistance have systemic effects. Rodgers and colleagues (Bastian et al., 2016; Hatzirodos et al., 2011; Raja-Khan et al., 2014) have shown that thickening of the ovarian capsule and stroma are caused by an increase in collagen deposition and subsequent formation of fibrotic tissue. This effect is related to the dysregulation of TGF-beta signalling. The TGF-beta superfamily ligands are a group of cytokines involved in various physiological processes, including cell proliferation, differentiation, apoptosis, angiogenesis and immune system regulation (Chin et al., 2004; Piek et al., 1999). Alterations in the TGF-beta signalling pathway can result in remodelling of the ECM leading to increased deposition of collagen in skeletal muscle and other tissues (Berria et al., 2006; Williams et al., 2015). The ECM is responsible for tissue structure and mediator of intracellular signalling. Insulin resistance is suggested to be directly related to ECM remodelling with an increased synthesis of fibronectin, laminin and collagen (Berria et al., 2006), which is accompanied by a decrease in phosphorylation of IRS-1 tyrosine in skeletal muscle (Berria et al., 2006). Given the lack of temporal data on the development of insulin resistance, it is not clear which occurs first, collagen deposition/ECM remodelling or peripheral insulin resistance. This dysregulated remodelling has been shown to act as a physical barrier to glucose and insulin as well as impairing their signalling cascade (Berria *et al.*, 2006; Williams, Kang and Wasserman, 2015). In line with this, subcutaneous abdominal adipose tissue insulin resistance in lean women with PCOS has been linked to TGF-beta signalling. Approximately 25% of differentially expressed genes were regulated by TGF beta 1 (Dumesic et al., 2019). The TGF-beta ligands SMAD2/3 have been shown to inhibit the actions of key insulin signalling regulators, Akt and mTOR (Trendelenburg et al., 2009). This will be explored *in vivo* and *in vitro* in **Study 1** and **Study 3**.

2.6.3 TGF-beta signalling and exercise

This presents a possible explanation for the development of the aforementioned metabolic defects present in the skeletal muscle of women with PCOS. Exercise is a cornerstone therapy in the management of PCOS, and it has been demonstrated in preclinical models that exercise can attenuate the effects of obesity and diabetes on skeletal muscle ECM (Lehti et al., 2006;

Pincu et al., 2015). Additionally, there is evidence to suggest that the skeletal muscle ECM is a mediator of exercise adaptations (Böhm et al., 2016; Rullman et al., 2009). It has been identified that increases in skeletal muscle TGF-beta 1 activity can suppress key mitochondrial regulators (PGC1α and AMPK) and mitigate the beneficial effects of exercise training on insulin sensitivity in individuals with type 2 diabetes (Böhm et al., 2016). In support of the role of TGF-beta signalling playing a vital role in exercise adaptations, it was identified that JNK regulates SMAD2/myostatin signalling acting as a molecular switch modulating between muscle hypertrophy and endurance adaptations (Lessard et al., 2018). TGF-beta 2 acts as an exercise inducible adipokine that is responsible for improvements in insulin sensitivity, with exercise training resulting in an increased expression of TGF-beta 2 in subcutaneous adipose tissue (Takahashi et al., 2019). The increase in TGF-beta 2 was modulated by lactate, presenting another role of TGF-beta ligands in the beneficial effects of exercise. In support of aberrant response to exercise in individuals with metabolic disease, it has shown that there are abnormal elevations in JNK activity (Hirosumi et al., 2002). These findings have been supported by Nikooie *et al.* (2020) that resistance training can reduce TGF-beta 1 expression, causing satellite cell activation and increase protein synthesis, leading to muscle hypertrophy through alterations in SMAD signalling. Other studies in mice have shown that a high-fat diet leads to an increase in TGF-beta1 expression, increased SMAD3 activation and collagen deposition that these negative effects could not be attenuated by moderate-intensity exercise training. Additionally, in mice during recovery from high-intensity exercise, elevations in TGF-beta1 were responsible for fatty acid oxidation (Nikooie and Samaneh, 2016). These findings provide a basis by which alterations in TGF-beta signalling could influence response to exercise training interventions. However, it is unclear how the skeletal muscle ECM of women with PCOS is affected by TGF-beta ligands and exercise training. This will be explored in **Study 2** and **Study 4**.

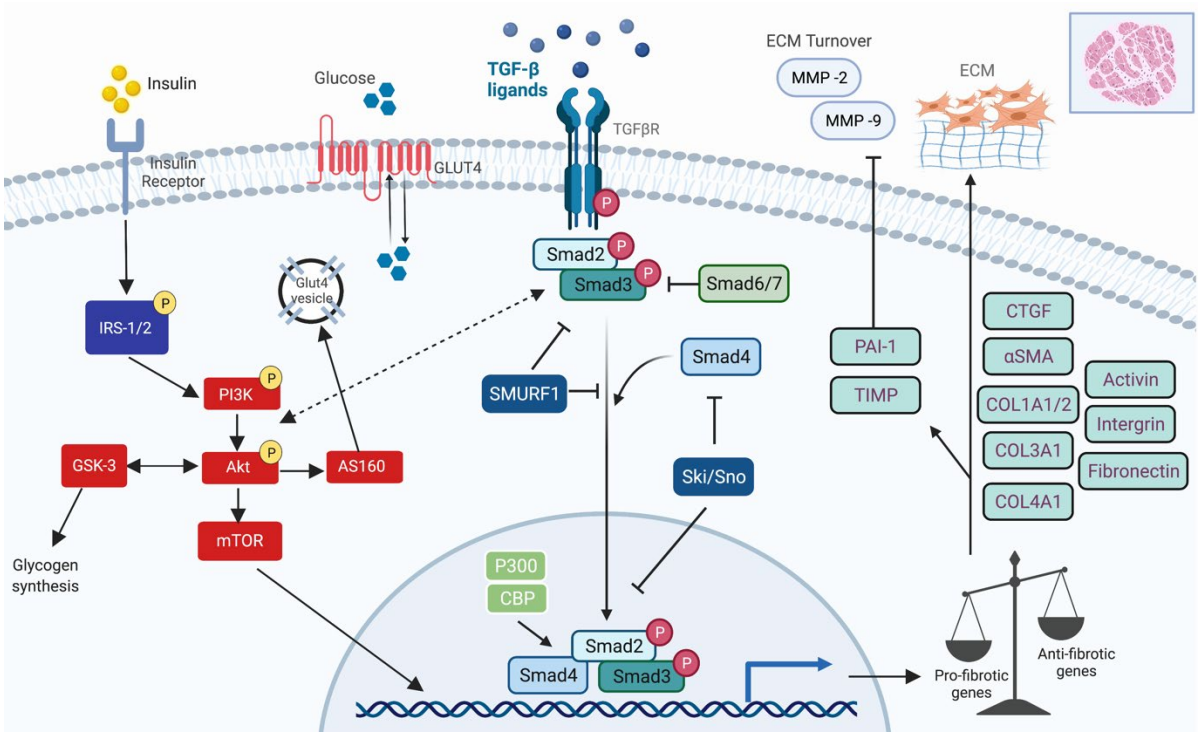

Figure 2 TGF-beta signalling via SMAD2/3 promotes fibrosis
TGF-beta ligands are activated in response to stress or tissue damage. The activation of latent TGF-beta results in the upregulation of SMAD2/3 phosphorylation and in conjunction with SMAD4 is translocated to the nucleus activating ECM enzymes and collagens genes. This process is a delicate balance between tissue repair and tissue fibrosis. Created with Biorender.com.

2.7 Gaps in the research – discovering the mechanisms of insulin resistance in women with PCOS

To date, a variety of factors have been identified as the potential cause of insulin resistance in women with PCOS. Despite a significant number of studies, there has been a lack of consistency in these findings. The primary reason for conflicting or differing findings may be the heterogeneity of PCOS with four different phenotypes and variances in the severity within each of them. This may suggest that insulin resistance could develop differently depending on the phenotype. Alternatively, these findings may be explained by the variances in experimental approaches applied, and there may be a common physiological response linking defects in insulin signalling. This leaves researchers with a great opportunity to identify an underlying factor that could be responsible for multiple defects observed. From previous research, we hypothesise that TGF-beta ligands may play a role in skeletal muscle insulin resistance, with

tissue remodelling occurring and creating a physical barrier for insulin signalling. This could also explain why women with PCOS may have ineffective adaptive responses to exercise. Therefore, further investigation is required to understand the molecular mechanisms regulating insulin resistance in women with PCOS and how they respond to exercise, both acutely and long-term, which may ultimately improve their metabolic abnormalities.

2.8 Aims and Hypothesis

Based upon gaps in the current literature as identified in the literature review, this thesis attempted to address the following aims:

- Aim 1: To determine if the TGF-beta ligands, TGF-beta 1 or AMH, play a role in the development of peripheral insulin resistance via the induction of aberrant insulin signalling in myotubes from women with and without PCOS. It was hypothesized that TGF-beta 1 and AMH would promote insulin signalling defects and decrease glucose uptake. (Chapter 4/Study 1)
- Aim 2: To determine if myotubes from women with PCOS retain their *in vivo* metabolic characteristics/phenotype. It was hypothesized that women with PCOS would not retain metabolic the donor's characteristics, indicating that skeletal muscle insulin resistance is acquired rather than intrinsic. Confirming the role of environmental and circulating factors in PCOS-specific insulin resistance. (Chapter 4/Study 1)
- Aim 3: To determine if myotubes from women with PCOS display a resistance to the beneficial effects of exercise by assessing exercise specific signalling following *in vitro* electrical pulse stimulation (EPS). It was hypothesized that myotubes from women with PCOS would have impaired exercise-induced signalling following *in vitro* contractions, consistent with the lack of training-induced improvements in metabolism observed *in vivo.* (Chapter 5/Study 2)
- Aim 4: To assess if TGF-beta 1 or AMH influences exercise-specific signalling in myotubes following *in vitro* electrical pulse stimulation. It was hypothesized that these TGF-beta ligands would further augment the impairment in exercise-induced signalling. (Chapter 5/Study 2)

- Aim 5: To assess insulin sensitivity and identify insulin signalling defects as previously observed in the skeletal muscle of women with PCOS. It was hypothesized that women with PCOS would have a greater degree of insulin resistancethan women who were lean or overweight. Also, that insulin resistance would be linked to defects in proximal insulin signalling (Chapter 6/Study 3).
- Aim 6: To establish if TGF-beta signalling is dysregulated in the skeletal muscle of women with PCOS and could be related to insulin resistance. It was hypothesized that the skeletal muscle of women with PCOS would have an increased expression of collagens and TGF-beta 1, resulting in tissue fibrosis. This profibrotic phenotype extending beyond the reproductive tissues could contribute to the development of skeletal muscle insulin resistance (Chapter 6/Study 3).
- Aim 7: To determine if 12 weeks of high-intensity and moderate-intensity exercise training could improve VO_{2peak} and insulin sensitivity in women with PCOS. It was hypothesized that greater exercise intensities would be required to see improvements in VO_{2peak} and insulin sensitivity (Chapter 7/Study 4).
- Aim 8: To assess the effects of 12 weeks of high-intensity and moderate-intensity exercise training on the TGF-beta signalling pathway in the skeletal muscle of women with PCOS. It was hypothesized that exercise training would result in a down-regulation of the expression of the profibrotic factors TGF-beta 1 and SMAD3 and increased expression of the adipokine TGF-beta 2 (Chapter 7/Study 4).

3 Methodology

3.1 Participants

Twenty-nine overweight women with PCOS, six overweight and ten lean healthy control women from the local community participated in the study (see **figure 3** – Consort diagram). Participants were recruited from the local community through social media groups and community advertising (posters, leaflets and word of mouth). Women who took part in this study were primarily Caucasian (80%), but the cohort also included European (10%) and Asian/Indian (10%) backgrounds. These participants and related samples from the TGF-beta-PCOS trial (ACTRN12618000155291) and the IHITPCOS trial (ACTRN12615000242527). The participants in study 1, study 2 and study 4 were a subset of participants from the main cross-sectional study - study 3. Women with PCOS were premenopausal women aged between 18-45yr. PCOS was diagnosed using the Rotterdam Criteria (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004), with the diagnosis being confirmed by an endocrinologist. Rotterdam criteria required confirmation of two of the following: (i) oligo- or anovulation; (ii) clinical (hirsutism and acne) and/or biochemical hyperandrogenism; (iii) polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism. The healthy control group consisted of premenopausal women aged between 18-45yr without any features of PCOS. The exclusion criteria included menopause, secondary causes of menstrual disturbance and hyperandrogenism, pregnancy, smoking, Type 1 diabetes, Type 2 diabetes mellitus, uncontrolled hypertension (>160/100mm/Hg), cardiac ischemia, established cardiovascular disease, renal impairment and malignancy, and use of medications that interfere with endpoints (e.g., contraception, metformin, anti-androgens, and progestins anti-hypertensives, and lipid-lowering agents). Ethical approval was obtained from the Victoria University Human Research Ethics Committee (Reference - HRE17-232), and all participants provided written informed consent prior to participation in the study.

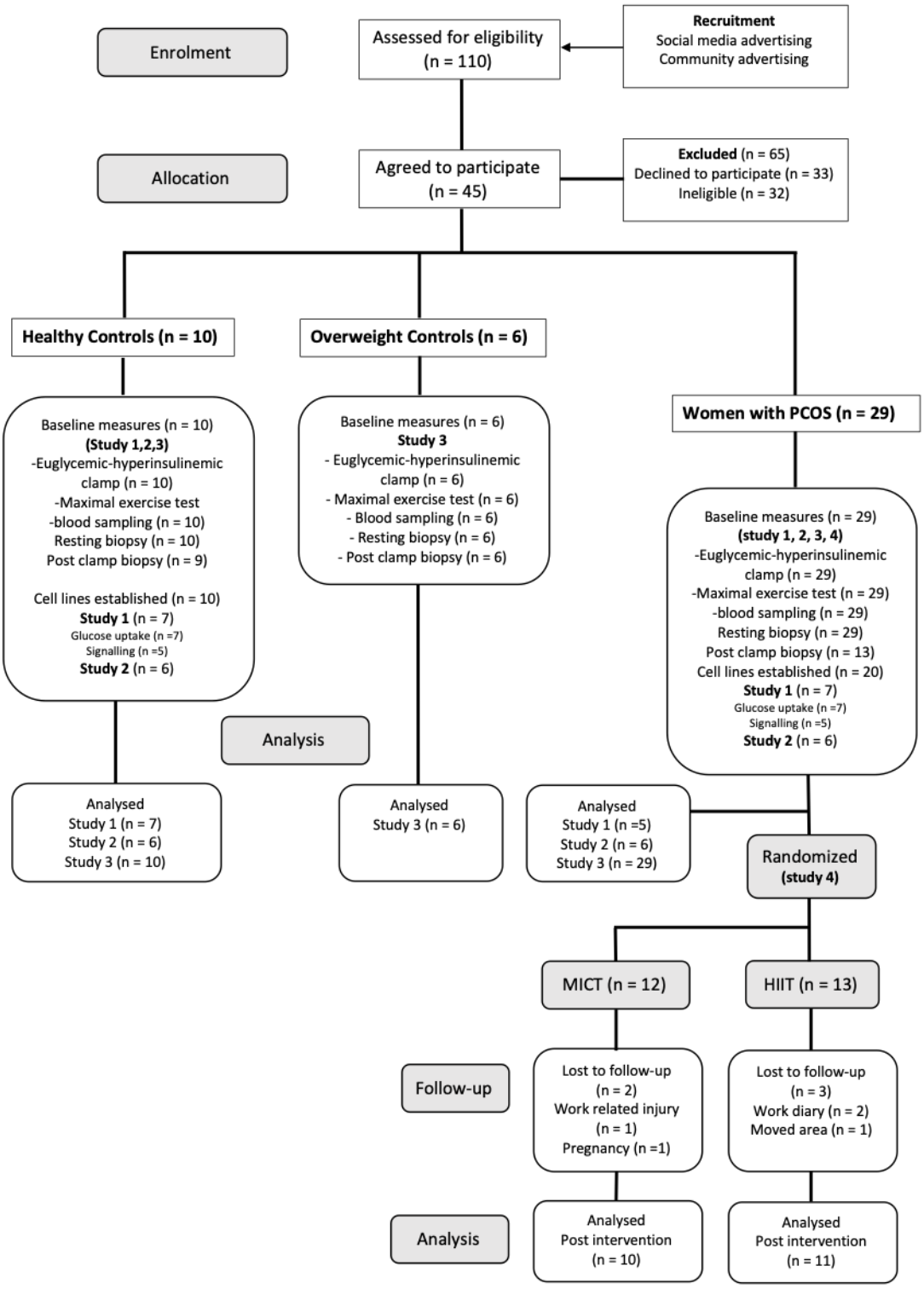

Figure 3 Diagram: detailing the sample sizes and which samples were used for each of the experimental studies (study 1-4).

3.2 Metabolic and body composition assessment

Participants reported to the lab between 6 am-8 am following an overnight fast. A whole-body dual x-ray absorptiometry (DXA; GE Lunar iDXA) scan was performed to assess total body fat mass and lean mass. Participants were asked to avoid intense or strenuous physical activity for 48 hours prior to the testing visit as it is well established that the effects of an acute bout of exercise or short-term exercise training (e.g. 7days) on insulin sensitivity can still be observed up to 48 hours following exercise cessation (Mikines et al., 1988; Ortega et al., 2014). *In vivo* insulin sensitivity was determined using a euglycemic -hyperinsulinemic clamp (DeFronzo et al., 1979) with a constant infusion of human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) at an infusion rate of 40 mU/min/m^2 for 120-180mins. Plasma glucose was modulated using a variable infusion of 25% Glucose (Viaflex Bag I.V. Solution 25% Glucose 1000ml, Baxter), to maintain the target level of ~5 mmol/l. To reduce the risk of hypokalaemia, participants were given a single dose (600 mg) of slow-release potassium before the commencement of the insulin clamp. An intravenous catheter was inserted in the antecubital vein of both arms, one for the infusion of insulin and glucose and the other for blood sampling. The arm which was used for blood sampling was covered with a heat blanket (Beurer personal heating pad, HKCOMFORT 4211125273262, Germany) at 50°C to arterialize the venous blood. Baseline blood samples were collected in tubes for either serum or plasma, plasma and serum separation were achieved via centrifugation at 3500 rpm, 4°C for 10mins. Samples were aliquoted into 1.5ml Eppendorf tubes and snap-frozen on liquid nitrogen before being transferred to -80°C for later analysis. Approximately 1-2ml of the arterialized blood was obtained every 5mins to measure blood glucose with a Yellow Springs Instruments 2300 STAT Glucose Analyzer (Yellow Springs Instruments Inc., Yellow Springs, OH). Blood samples were obtained every 30mins for later analysis of insulin. Glucose infusion rates were calculated for the steady-state period during the final 30mins of the clamp. As skeletal muscle is responsible for a large percentage of insulin-stimulated glucose uptake, the glucose infusion rate was expressed as glucose (mg) per lean body mass (kg) (lbmkg) per min. In addition, prior to the commencement of the clamp and during the steady-state phase of the clamp, indirect calorimetry was carried out to determine substrate utilization and metabolic rate. This was performed using a metabolic cart (Quark RMR, COSMED, Australia) and a ventilated canopy hood. (COSMED, Australia). The system was calibrated before conducting each test using a volumetric syringe (Hans Rudolph, Shawnee, KS, USA) and known references gases for O_2

and CO_2. Lean, healthy controls followed this procedure only at baseline while women with PCOS were assessed prior to and after the exercise training intervention.

3.3 Muscle biopsy

A muscle biopsy was obtained from the *vastus lateralis* using the modified Bergstrom technique (Bergström, 1975; Shanely et al., 2014) prior and during steady-state phase of the euglycemic-hyperinsulinemic clamp at baseline for both controls and PCOS, and also after the 12-week exercise training for the women with PCOS. Local anaesthetic (1% Xylocaine) was injected into the skin and fascia at the biopsy site, and a small incision was made with a scalpel blade. A sample of ~100-200mg was obtained using Bergstrom needle with suction. The sample was then processed on dental wax with any visible connective tissue or adipose tissue being removed then the sample was divided for immunoblotting and cell culture. The sample for immunoblotting was snap-frozen in liquid nitrogen and then stored at -80°C until later analysis.

3.4 Assessment of exercise capacity

Participants completed two symptoms-limited graded exercise tests (GXT) with seven days between each test. The first GXT served as a familiarisation of the protocol, and the later test was used to determine VO_{2peak} and maximal power output in watts (W). For the women with PCOS, this data was used to prescribe initial training intensities. The test was carried out on electronically braked cycle ergometer (Excalibur, V2.0; Lode, Groningen, the Netherlands), the test consisted of two phases- sub-maximal and step protocol. The submaximal phase consisted of cycling at the following workloads: 25w, 50w, and 75w for 3minutes. Upon completion of this phase, the test continued into step protocol with 25W increase every minute until exhaustion or an RPE of 20 (Borg scale) was reached. In cases where clinical signs and symptoms of metabolic or cardiovascular abnormalities the test was stopped. During the testing, participants were monitored by 12-lead ECG. Expired respiratory gases were collected breath by breath for VO_2, VCO_2 and ventilation using a metabolic cart (Quark RMR, COSMED, Australia). Following collection, the VO_2, data was smoothed to remove values that were less than or greater than rolling seven breath mean ± two standard deviations.

Subsequently, this data was then averaged over a rolling seven breath mean, and the largest value recorded was determined as the $VO_{2peak.}$ This testing was repeated following the 12-week exercise intervention

3.5 Exercise training intervention

Following the completion of baseline testing, overweight women with PCOS were randomly allocated to one of two 12-week supervised exercise training interventions; supervised standard exercise (MICT) or high-intensity intermittent training (HIIT). All exercise session were conducted at Victoria University Footscray Park campus under the supervision of exercise physiologists. The MICT intervention was designed to meet the minimum physical activity guidelines of 150 min each week, this made up of three sessions per week, and consisted of 50mins of moderate-intensity cycling at 50-60% of heart rate reserve. The HIIT group was designed to meet the minimum vigorous physical activity guidelines of 75 min each week; again, this consisted of three sessions per week. Two different HIIT protocols were used based on collective evidence for improvements in metabolic health (Cassidy et al., 2017). This consisted of two weekly sessions of 12 x 1 min at >85% of HRR with 1 min active recovery and one weekly session of 6-8 x 4 min at >85% of HRR with 2 min of active recovery **(Figure 4)**. The initial concepts for these training interventions come from seminal work from Stepto *et al.*, 1999. All training was carried out on electronically-braked cycle ergometer (Excalibur, V2.0; Lode, Groningen, the Netherlands) allowing exercise intensity to be pre-programmed and individualized for each session. Both exercise interventions were matched for training volume as determined by metabolic equivalent task (MET).min/week. The training interventions were progressive starting at 312 MET.min/week at week one and progressing to 530 MET.min/week in weeks 6-12. The protocols for the exercise intervention has been published for further details see: (Hiam et al., 2019).

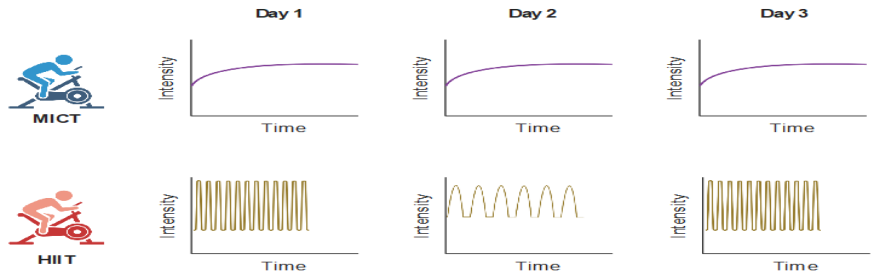

Figure 4. Overview of weekly exercise sessions for MICT and HIIT created with Biorender.com.

3.6 Bloods pathology and analysis

Stored blood samples were batch analysed by Monash pathology for the following measures: HBA1c, testosterone, sex hormone-binding globulin (SHBG), alanine aminotransferase (ALT), aspartate aminotransferase (AST) cholesterol, triglycerides, high-density lipoprotein (HDL), Low-density lipoprotein (LDL), Dihydrotestosterone (DHT), Estradiol, (E2), and Androgens. The serum steroids were determined using mass spectrometry, and other measures were determined by clinical-grade ELISAs. Additional analysis for AMH and insulin were conducted at Victoria University. Serum AMH was concentration was determined using a commercially available ELISA kit (Ultra-Sensitive AMH/MIS ELISA, AL-105, Anash Labs). Plasma insulin levels at rest and for the major time points of the euglycemic -hyperinsulinemic clamp were determined via Radio-Immuno Assay kit from Millipore (Human Insulin-Specific RIA, HI-14K, Millipore).

3.7 Human primary culture of myotubes

Primary human cell lines were established using approximately 40-50 mg of muscle sample obtained from the biopsy. The sample was immediately transferred into a 15 ml falcon tube containing 10 ml of ice-cold alpha MEM (Life Technologies Australia, Gibco cat no. 12571-063). Following collection, the sample was washed 3x with ice-cold PBS to ensure there was no blood or debris on the sample, before transferring it to a petri dish and removing any visible connective or adipose tissue. The muscle tissue was then minced into small pieces (<1-2mm^3)

using a scalpel blade and 3 ml of 0.05% Trypsin-EDTA. Then, the samples were transferred to a 100ml sterile conical flask containing 12 ml of 0.05% Trypsin-EDTA placed on an orbital shaker at 60-70rpm for 20 min at RTºC to disassociate the cells enzymatically. The supernatant was removed and transferred to 50ml Falcon tube containing 5mL of FBS on ice to inactivate the trypsin-EDTA immediately and avoid over-digestion of the tissue. This process was repeated two more times with total end volume of 45ml of cell suspension and 5ml of FBS. The cell suspension was filtered through 100um cell strainer to remove any undigested tissue and then centrifuged for 10 min at 1500rpm. Cells were resuspended in 5 ml of growth medium (low-glucose alpha MEM supplemented with 10% fetal bovine serum, 0.5% Penicillin-streptomycin and 0.5% Amphotericin B), then pre-plated in an uncoated flask to reduce the population of fibroblasts and incubated for 20 min at 37°C and 5% CO_2 before transferring the supernatant to a fresh ECM-coated T-25 flask and cultured at 37°C and 5% CO_2 **(Figure 5)**. All cells were cultured in the growth medium, with medium changed after 24 hours and then every second day thereafter. For step by step video of the procedures and further justification of this method see (Agley et al., 2015; Cornall et al., 2012). All chemicals and culture media were obtained from Sigma-Aldrich/life tech.

3.7.1 Purification of satellite cells

Satellite cells were selected from the mixed cell population, including remaining fibroblasts, following the method by Agley *et al.*, (2013). Magnetic activated cell sorting (MACS) with anti-CD56 microbeads (MACS Miltenyi Biotec #130-050-401) was used in order to achieve a fraction of enriched myogenic cells. CD56 is considered to be the gold-standard surface marker for the identification of human satellite cells. After reaching 70-80% confluence, primary cultured cells in T-25 flask were washed with PBS before being detached with 1.5ml of 0.05% Trypsin-EDTA and incubated for 3min at 37°C and 5% CO2. Trypsinization was stopped by adding 3.5ml of the growth medium when the majority of the cells had detached from the surface of the flask. All medium and cells were transferred to a 15ml falcon tube and were centrifuged at 300g for 10 min. Then, the pellet of cells was resuspended with 80 ul of autoMACS™ running buffer (MACS Miltenyi Biotec #130-091-221) and 20 ul of CD56 magnetic microbeads (MACS Miltenyi Biotec #130-050-401) and incubated for 15 min at 4°C. Then cells were washed with 2 ml of the running buffer and re-pelleted. Then the supernatant

was removed, and the cells were resuspended in 500 ul of autoMACS™ running buffer before being separated using miniMACS™ separator combined with MS column (MACS Miltenyi Biotec #130-091-632). The column was rinsed with 500 ul of the running buffer before loading the cell suspension. When the cell suspension passes through the column, the $CD56^+$ cell fraction (previously magnetically labelled) binds to the column, whilst the $CD56^-$ cell fraction pass freely through the column. To ensure only the $CD56^+$ cells remained, the column was rinsed 3 x 500ul of the running buffer. To collect the $CD56^+$ cells, the column was removed from the separator and placed over a 15ml falcon tube. Next, 1 ml of running buffer was added to the column, and the cells were flushed out into the falcon tube using the plunger. Finally, 9 ml of growth medium was added to the purified fraction and centrifuged for 10min at 300g. The cell pellet was resuspended in growth medium before plating in four ECM-coated T75 flasks. After growing a pure population of myoblasts and reaching 80-90% confluence, a proportion was plated for experiments, and the rest of the cells were frozen in cryovials using freezing media (Growth medium and 10% DMSO). Cryovials were placed in a freezing box (Corning™ CoolCell™ LX Cell Freezing) and stored at -80ºC, before being transferred to liquid nitrogen storage.

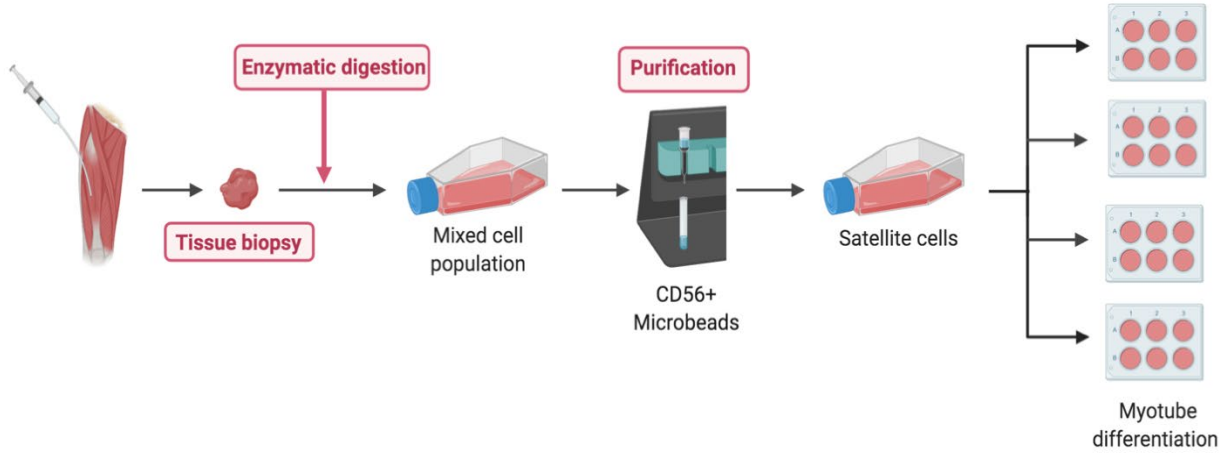

Figure 5 Schematic experimental procedure for myotube culture experiments.
Muscle tissue was obtained from the *vastus lateralis* using Bergstrom needle biopsy technique and enzymatically digested using trypsin. Cell purification was carried out using anti-CD56 microbeads to select satellite cells. Cells were then plated and differentiated for experiments, created with Biorender.com.

3.8 TGF-beta and AMH myotube experiments

Cells were plated (passage 3) into 6-well plates for protein expression and 12-well plates for glucose uptake. Growth medium was changed every second day until cells reached 80-90% confluence, then differentiation was started. Prior to differentiation, cells were washed twice with PBS, and differentiation medium (low-glucose alpha MEM supplemented with, 2% horse serum, 0.5% Penicillin-streptomycin and 0.5% Amphotericin B) was added to the cells and changed every day for five days. On the fifth day the following treatments were applied to the myotubes for 24 hours: TGF-beta 1 (1ng/ml and 5ng/ml) (Transforming Growth Factor-β1 human, T7039, Sigma Aldrich, St Lewis, MO, USA), AMH (5ng/ml, 10ng/ml and 30ng/ml) (AMH (NM_000479) Human Recombinant Protein, amsbio, Cambridge, MA, USA), and a control with no treatment (vehicle) was also included. Treatments and control conditions were prepared in serum-free low glucose media (alpha MEM with 0.1% BSA, 0.5% Penicillin-streptomycin and 0.5% Amphotericin B).

3.9 Electrical pulse stimulation: *in vitro* contraction

Electrical Pulse stimulation (EPS) of human primary muscle cells is an *in vitro* model of skeletal muscle contraction. The use of EPS produces effects similar to those observed following *in vivo* exercise (Nikolić et al., 2017). Following five days of differentiation, human primary myotubes were stimulated with EPS using the C-Pace EP multichannel Culture Pacer (IonOptix, MA) **(Figure 6)**. A chronic low-frequency stimulation protocol of 11.5 V, 2 ms, 1Hz for 6 hours in serum-free low glucose media (alpha MEM) was selected to represent an exercise training like stimulus. Previous studies utilising this or similar protocols have observed activation of exercise-mediated signal transduction (AMPK, MAPK, Ca^{2+} handling), improved glucose uptake and insulin sensitivity, increased myokine release and structural changes (e.g., Hypertrophy) (Al-bayati et al., 2019; Lambernd et al., 2012; Nikolić et al., 2012; Scheler et al., 2013; Son et al., 2019). It should be noted that during pilot work, a variety of different published stimulus was applied, with higher voltages ≥ 30 volts resulting in cells dethatching from the surface of the plate.

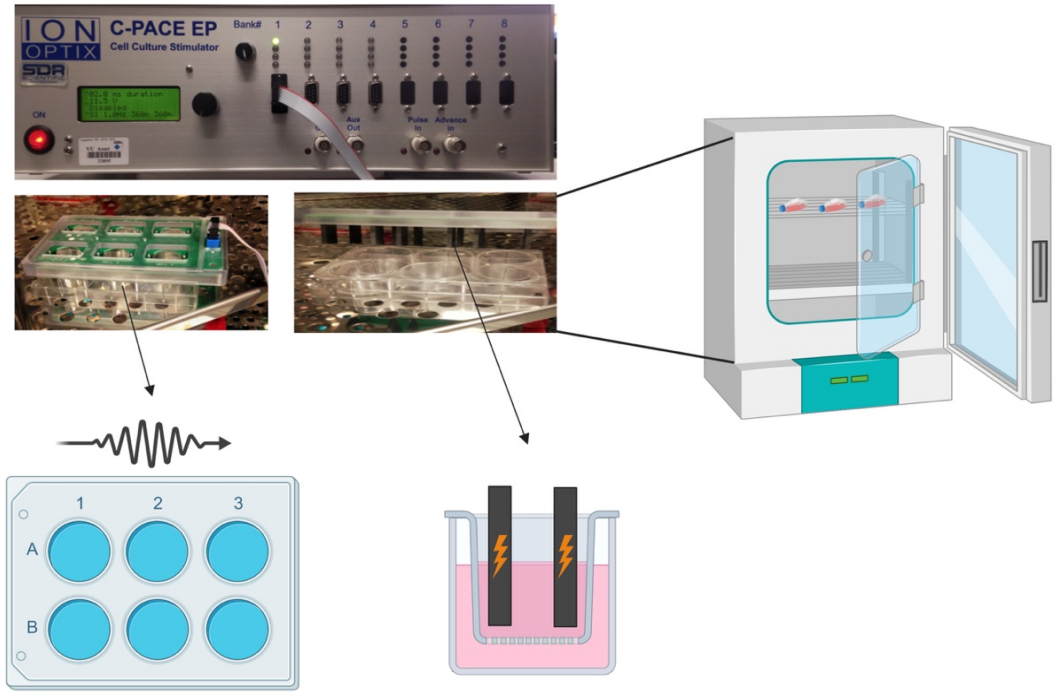

Figure 6. Electrical pulse stimulation experimental setup

Specially designed carbon electrodes are able to sit close to the wall of the well in the cell culture dish. Electrodes are connected to cell stimulator that allows manipulation of voltage and currency applied to the cells (ION OPTIX C-PACE EP, Cell Culture Stimulator). 6-well plate with electrodes is designed to work in the incubator, created with Biorender.com.

On day 5 of differentiation, media of the cells was changed to a serum-free medium, with or without the respective treatment (TGF-beta 1 or AMH) for 16 hours overnight. On day 6, the EPS protocol was applied, and following stimulation, cells were rinsed twice with PBS and lysed in RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with fresh phosphatase and protease inhibitors (1:100) to extract protein for immunoblotting or in Trizol to extract RNA for qPCR. Samples were then stored at -80ºC for later analysis.

3.10 Glucose uptake assay

A radio-activity based assay [2-^{3}H]Deoxy-D-glucose ([2-^{3} H]DG) uptake was used to measure glucose uptake. Myotubes were pre-incubated overnight (16 hours) in serum-free medium with and without the previously described treatments of TGF-beta and AMH. The medium was then removed, and cells were washed three-times and pre-incubated with Krebs buffer (10 mM HEPES, 2.5 mM NaH_2PO_4, 150 NaCl, 5 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 0.1% BSA) with and without insulin (100 nM) for 30mins. To assess glucose uptake, 10µM 2-deoxy-D-glucose (2-DG) at 1µCi/mL/well ([2-^{3} H]DG) was added for exactly 15 min at 37 °C. The cells were then rinsed twice with cold PBS and lysed in 500ul of 0.2 M NaOH. 400ul of the lysate was transferred to a scintillation vial, and 100ul was kept for total protein quantification. Glucose uptake was determined using liquid scintillation counting on β-spectrometer (Perkin-Elmer), unit of measurement in picomoles of [2-^{3} H]DG taken up per minute per milligram normalized to total protein.

3.11 Western blot analysis

Protein expression of TGF-beta and insulin signalling pathways, as well as of markers of tissue fibrosis were determined via western blotting. Cell lysates were prepared using ice-cold RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Product No.89900, ThermoFisher Sci, USA) with the addition of phosphates and protease inhibitor cocktail at 1:100 (Halt™ Phosphatase Inhibitor Cocktail Product No.78440, ThermoFisher Sci, USA). Following treatment, the cells were then rinsed twice with ice-cold PBS, and 100ul of the RIPA buffer with inhibitor cocktail was added per well. A cell scraper was used to lyse the cells into the lysis buffer before transferring the lysate into an Eppendorf tube. The lysate was then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a new Eppendorf tube and stored at -80°C until later analysis. The total protein concentration for each sample was determined using Red 660 protein assay (cat no. 786-676, G-Biosciences, USA) with SDS Neutralizer (cat no. 786-673, G-Biosciences, USA) for cell culture work and with Pierce™ BCA Protein Assay Kit (cat no. 23225, Thermofisher) for whole muscle lysate. Samples were prepared in 4x Laemmli buffer with beta-mercaptoethanol and boiled for 5min at 95°C. Samples were loaded into 10% Criterion™ TGX Stain-Free™ protein gels (Biorad, #5678034). Fifty micrograms for the cell culture samples and nine to

eighteen micrograms for the whole muscle samples, and different concentrations of the internal standards (pooled sample from different lysates) was separated via electrophoresis (Criterion™ Vertical Electrophoresis Cell, Biorad, #1656001) for 90 min at 200V. Afterwards, the gel was transferred on to a nitrocellulose membrane (Biorad, #1704271) or PVDF low fluorescence, depending on the target. The transfer was performed using Trans-Blot® Turbo™ Transfer System (Biorad, #1704150) using the following protocol: 2.5 A, 25 V, for 7min. The membrane was then imaged for total protein using the stain-free protocol on ChemiDoc™ XRS+ System (Biorad #1708265). All membranes were blocked for 1 hour in 5% skimmed milk or 5% bovine serum albumin in Tris-buffered saline (TBS) plus 0.1% Tween 20 (TBS-T). Membranes were then washed for 3 x 5 min in TBS-T, then incubated overnight on a rocking platform at 4°C in primary antibody solution containing selected antibody (**Table 3**) at 1:1000 dilution, 5% bovine serum albumin, 0.02% sodium azide and 15ml of TBS. The next day, membranes were washed for 3 x 5 min in TBS-T then incubated in appropriate secondary horseradish peroxidase-conjugated antibody (1:10,000) for 90 min. Following 3 x 5 min wash with TBS-T, proteins were visualized by ultra-sensitive enhanced chemiluminescence (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Scientific, cat no. 34094) or ECL substrate (clarity western ECL substrate, #1705060, Biorad) and were quantified by densitometry. Images of membranes for total protein and target proteins were analysed using Biorad Image Lab 6.0.1 to determine band density. The band density data was normalized to total protein content **(Figure 7)** for each lane and to the internal standard loaded in each gel.

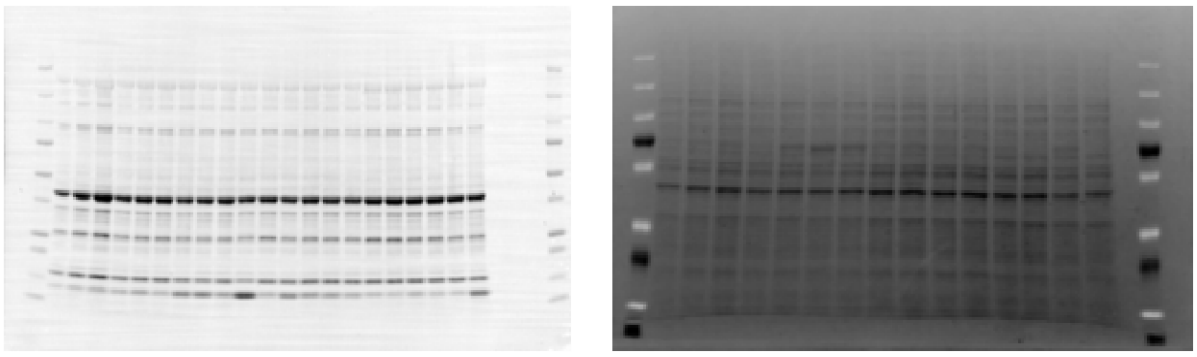

Figure 7. Total protein content used for normalization on Low-Flow PVDF membrane (left) and Nitrocellulose membrane (right).

Table 3 Antibody list

Protein	Abbreviation	Company	Concentration
Phospho-Smad3 (Ser423/425) (C25A9) Rabbit mAb 9520S	pSMAD 3	Cell signalling Technology	1:1000
Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad9 Ser465/467) (D5B10) Rabbit mAb 13820S	pSMAD 1/5/9	Cell signalling Technology	1:1000
Smad3 (C67H9) Rabbit mAb 9523S	SMAD 3	Cell signalling Technology	1:1000
Smad5 (D4G2) Rabbit mAb 12534S	SMAD 5	Cell signalling Technology	1:1000
Smad4 (D3M6U) Rabbit mAb 38454S	SMAD4	Cell signalling Technology	1:1000
Anti-IRS1 (Phospho s312) Rabbit pAB ab4865	pIRS-1	Abcam	1:1000
IRS-1 (D23G12) Rabbit mAb 3407S	IRS-1	Cell signalling Technology	1:1000
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb 4060	pAkt	Cell signalling Technology	1:2000
Akt (pan) (C67E7) Rabbit mAb 4691	Akt	Cell signalling Technology	1:1000
PI3 Kinase p85-alpha (6G10) Mouse mAb 13666S	PI3K-p85	Cell signalling Technology	1:1000
PI3 Kinase p110-alpha (C73F8) Rabbit mAb 4249S	PI3K-p110	Cell signalling Technology	1:1000
mTOR Rabbit AB 2972S	mTOR	Cell signalling Technology	1:1000
Phospho-mTOR (Ser2448) 2971S	pmTOR	Cell signalling Technology	1:1000
Raptor (24C12) Rabbit mAb 2280S	RAPTOR	Cell signalling Technology	1:1000
Phospho-Raptor (Ser792) 2083S	pRAPTOR	Cell signalling Technology	1:1000
Rictor (53A2) Rabbit mAb 2114S	RICTOR	Cell signalling Technology	1:1000
Phospho-Rictor (Thr1135) (D30A3) Rabbit mAb 3806S	pRICTOR	Cell signalling Technology	1:1000
PhosphoPlus(R) CREB (Ser133) Antibody Duet 8212	pCREB	Cell signalling Technology	1:1000
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb 4695	ERK1/2	Cell signalling Technology	1:1000

Phospho-p44 42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb 4370	pERK1/2	Cell signalling Technology	1:1000
NOR1/TEC ab155535	NOR1	Abcam	1:2000
NUR77 ab109180	NUR77	Abcam	1:2000
Glucose transporter 4 ab654	GLUT4	Abcam	1:4000
Collagen I ab34710	COL1A1/2	Abcam	1:1000
Collagen III ab184993	COL3A1	Abcam	1:1000
Collagen IV ab6586	COLIV	Abcam	1:1000
Collagen I ab34710	COLI	Abcam	1:1000
Collagen III ab184993	COLIII	Abcam	1:1000
TFG-beta 1 ab92486	TGFbeta1	Abcam	1:1000
TGF-beta 2 ab36495	TGFbeta2	Abcam	1:1000
TGF-beta receptor 1 ab31013	TGFBR-1	Abcam	1:1000
TGF-beta receptor 2 ab61213	TGFBR-2	Abcam	1:1000
p38 MAPK 9212	p38MAPK	Cell signalling Technology	1:1000
phospho-p38 MAPK (Thr180/Tyr182) 9211	P-p38MAPK	Cell signalling Technology	1:1000
AMPKα Rabbit mAb 2603	AMPK	Cell signalling Technology	1:1000
p-AMPKα (Thr172) Rabbit mAb 2535	pAMPK	Cell signalling Technology	1:1000
Anti-PGC-1α Mouse mAb ST1202	PGC1α	Merck	1:1000
GSK-3B Rabbit mAB 9315	GSK3B	Cell signalling Technology	1:1000
phosphoGSK-3α/β (Ser21/9) 9331	pGSK3	Cell signalling Technology	1:1000
Total OXPHOS Rodent WB antibody cocktail ab110413	OXPHOS	abcam	1:2000
Acetyl-CoA Carboxylase Rabbit mAb 3676	Acc	Cell signalling Technology	1:1000
Phospho-Acetyl-CoA Carboxylase (Ser79) rabbit mAb 11818	pAcc	Cell signalling Technology	1:1000
phospho-AS160 (Thr642) 4288	pAS160	Cell signalling Technology	1:1000
Phospho-FOXO1 (ser256) Rabbit mAb 84192S	pFOXO1	Cell signalling Technology	1:1000
Secondary Anti-rabbit IgG, HRP-linked Antibody 7074S	Rab IgG	Cell signalling Technology	1:10000
Secondary Pierce Anti-Mouse IgG (Goat) - HRP-Labeled #31430	Ms IgG	Thermofisher	1:10000

3.12 Statistical analysis

All analyses and graphing were carried out using GraphPad Prism Version 8 (GraphPad Software Inc., San Diego, USA). All data are reported as mean ± standard deviation (SD) unless stated otherwise, and statistical significance was declared when $P < 0.05$. Where statistical significance was detected, data were presented as absolute change or percentage of relative change ± SD, 95% CI, P-value. Specific analysis performed is outlined in the methodology of each study.

4 **Study 1:** The Effects of Anti-Müllerian Hormone and Transforming Growth Factor Beta 1 on Glucose Uptake and Insulin Signalling in Human Primary Myotubes from Women with and Without Polycystic Ovary Syndrome

4.1 Introduction

Insulin resistance appears to be a key feature and a driver of the symptoms of PCOS, with approximately 38-80% of women with PCOS being insulin resistant when measured by euglycemic–hyperinsulinemic clamp (Stepto et al., 2013; Tosi et al., 2017). The insulin resistance and subsequent hyperinsulinemia appear to contribute to hyperandrogenism, ovulatory dysfunction and subfertility, highlighting the importance of understanding the mechanisms by which insulin resistance develops in women with PCOS. To date, several studies have attempted to identify the mechanisms responsible for the development of insulin resistance and the aberrations in the insulin signalling cascade. Despite significant progress in this area, there is still a lack of consensus and understanding; this presents a significant challenge for developing new therapeutics. The prominent peripheral insulin resistance in women with PCOS was first identified by Dunaif et al. (1989). It was noted that women with PCOS had insulin resistance that occurred independently of obesity or impaired glucose tolerance, with unique abnormalities in insulin action (Dunaif et al., 1989). The skeletal muscle plays a key role in whole-body glucose homeostasis. It is the primary site for peripheral glucose disposal, accounting for ~85% whole-body insulin-stimulated glucose uptake (Defronzo et al., 1981), making it a key therapeutic target for improving whole-body metabolism. Although a variety of different defects have been observed, which can be attributed to the heterogeneity of PCOS, there has been little progress in understanding the contributing or causative factors in the development of insulin resistance in women with PCOS.

Human primary myotubes appear to retain the key characteristics of skeletal muscle, such as fibre type composition and glucose metabolism (Aas *et al.*, 2013). The use of human primary myotubes creates the opportunity to differentiate between intrinsic and environmental

contributions in the aetiology of a condition and, in particular metabolic features such as insulin resistance. The assessment of insulin-mediated glucose uptake and the subsequent activation of insulin signalling cascade has been used to determine insulin sensitivity in cultured myotubes. In primary skeletal muscle myotube cultures established from individuals with type 2 diabetes, there is a reduction in insulin-stimulated glucose uptake when compared to myotubes from healthy controls (Ciaraldi et al., 1995; Henry et al., 1995; McIntyre et al., 2004a). However, the relationship and direct measures of comparison between the *in vivo* phenotype and the response of the *in vitro* culture is not often assessed (Gaster, 2019). For instance, the comparison of glucose disposal rate obtained from the euglycemic-hyperinsulinemic clamp and insulin-stimulated 2-deoxy-glucose uptake in myotubes from the same donor. This makes it difficult to interpret the extent of the phenotype retained and the contribution of the *in vivo* environment. Several studies have reported that human primary myotubes from women with PCOS are no longer insulin resistant, suggesting that phenotype is not maintained (Ciaraldi et al., 2009; Corbould et al., 2005; Eriksen et al., 2010, 2014). Adding to these findings, Eriksen *et al.* (2010 and 2014) have shown a lack of insulin resistance in myotubes from women with PCOS and found no intrinsic defects in glucose or lipid metabolism. In addition, these myotubes also appeared to exhibit normal mitochondrial function and content contrary to *in vivo* findings (Eriksen et al., 2011; Konopka et al., 2015; Skov et al., 2007). Taken together, this suggests that acquired environmental factors rather than intrinsic defects or traits may play a significant role in the development of PCOS-specific insulin resistance.

Given the heterogeneity of PCOS, there may be multiple environmental factors that could lead to the development of insulin resistance. A possible candidate for the development of metabolic abnormalities in women with PCOS is the TGF-beta superfamily ligands. To date, several of the TGF-beta ligands have been demonstrated to play a role in the pathophysiology of PCOS. These TGF-beta ligands have been shown to be responsible for alterations in ovarian hormones and morphology, with the thickening of the ovarian capsule and stroma caused by an increase in collagen deposition and fibrotic tissue related to dysregulation of TGF-beta superfamily ligands (Bastian et al., 2016; Hatzirodos et al., 2011; Raja-Khan et al., 2014). Women with PCOS have elevated levels of serum TGF-beta 1 (Irani et al., 2015; Raja-Khan et al., 2010; Tal et al., 2013) and AMH (Cook et al., 2002; Seifer and MacLaughlin, 2007). The role of AMH in PCOS has been primarily outlined in regulating reproductive function (Bhide et al., 2015;

Maciel et al., 2004; Pellatt et al., 2011). Although the direct role of AMH in insulin resistance has not been determined, it has been shown to be positively associated with HOMA-IR across all phenotypes (Wiweko et al., 2018). The relationship and ratio between advanced glycation end products and AMH levels are directly linked to follicle count and anovulation (Diamanti-Kandarakis et al., 2009). This links insulin resistance to ovulatory dysfunction but does not imply that ovulatory dysfunction and AMH influence insulin resistance. The interaction of AMH with other hormones is evident, with hyperandrogenism and levels of AMH being linked (Cassar et al., 2014). Despite AMH being associated with insulin resistance, the mechanisms of this relationship remain unexplored. It is clear that AMH has a central role in reproductive function, but its systemic effects have not been established.

It remains to be determined if the TGF beta-driven tissue remodelling of reproductive tissues is also occurring in the peripheral tissues and subsequently influencing insulin signalling via alterations in ECM and tissue morphology. It has been demonstrated that the gene expression of key components of the TGF-beta signalling pathway is altered in the skeletal muscle of women with PCOS (Skov *et al.*, 2007, Stepto et al., 2020). The role of TGF-beta signalling in glucose homeostasis is becoming more apparent. An increase in glucose and insulin leads to rapid translocation of intracellular TGF-beta receptor 1 (TGFBRI) and 2 (TGFBRII) to the cell surface in a variety of cell types (Budi et al., 2015; Wu and Derynck, 2009). This occurs via the activation of Akt and is regulated by subsequent activation of AS160 to enhance TGF-beta responsiveness (Budi et al., 2015). This translocation of TGF-beta receptors causes an amplification of TGF-beta signalling through SMAD activation (Duan and Derynck, 2019). The interaction between insulin and TGF-beta signalling may be particularly pertinent in relation to women with PCOS, given that many presents with hyperinsulinemia. Chronic stimulation of TGFBR and subsequent SMAD2/3 and other TGF-beta related pathways could promote tissue fibrosis. Together, these findings highlight the potential crosstalk between TGF-beta and insulin signalling. This may be a possible mechanism by where TGF-beta could influence insulin signalling directly or indirectly via tissue remodelling.

4.1.1 Aims and hypothesis

Therefore, the aim of this study was to determine if TGF-beta ligands, TGF-beta 1 or AMH, play a role in the development of peripheral insulin resistance via the induction of aberrant insulin signalling. It was hypothesized that AMH and TGF-beta1 would both promote insulin signalling defects and decrease glucose uptake in human primary myotubes from women with PCOS and healthy lean controls. A secondary aim was to determine if myotubes from women with PCOS retained their *in vivo* metabolic characteristics. It was hypothesized that myotubes from women with PCOS would not retain the donor's metabolic characteristics, indicating that skeletal muscle insulin resistance is acquired rather than intrinsic. This would confirm the role of environmental and circulating factors in PCOS-specific insulin resistance.

4.2 Methods

4.2.1 Participants

The myotube cultures established from biopsy samples for seven women with PCOS who were overweight, and seven healthy women were used for this study (total n =14) (For further details see Methodology section 3.1)

4.2.2 Muscle biopsy

Biopsy samples obtained at baseline before the euglycemic-hyperinsulinemic clamp were used for this study. For more information, see Methodology section 3.3.

4.2.3 Human primary culture of myotubes

See Methodology section 3.7

4.2.4 Purification of satellite cells

See Methodology section 3.7.1

4.2.5 TGF-beta 1 and AMH treatment of myotubes

Differentiated myotubes were exposed for 24 hours to TGF-beta 1 (1ng/ml and 5ng/ml) and AMH (5ng/ml, 10ng/ml and 30ng/ml). A control with no treatment was also included (See more details in Methodology section 3.9).

4.2.6 Glucose uptake assay

The sample size for this outcome was 7 per group (for more details, see Methodology section 3.11).

4.2.7 Western blot analysis

The sample size for this outcome was 5 per group. Targets quantified in this study were phospho and total: SMAD3, SMAD1/5/8, IRS-1, AKT, MTOR, RAPTOR, RICTOR, ERK1/2, Total: SMAD4, PI3K p85 and PI3K p110 (for more details see Methodology section 3.12).

4.2.8 Statistical analysis

Clinical characteristics were compared with two-tailed unpaired student's t-test. A linear mixed model analysis was used to examine the differences between treatments, groups, and within donor variation. Analysis of the treatments of TGF-beta and AMH were carried out independently. Changes in protein total expression and phosphorylation were assessed using a mixed-effects model (2 groups and 12-time points). Significant effects of interaction (group x time), Time (Basal control vs treatments) and group (Lean Con vs OW PCOS). The distribution of the data was tested using a Shapiro-Wilk test. Post-hoc analysis of significant interaction and main effects was carried out using the Sidak multiple comparisons test. Correlation between *in vivo* and *in vitro* measures of insulin sensitivity were assessed by Spearman's correlation coefficient. Data in text for basal expression and phosphorylation of proteins is presented as relative difference percentage ± SD 95% CI [lower, upper], P-value, unless stated otherwise (For additional details See Methodology section 3.13)

4.3 Results

4.3.1 Clinical characteristics

Clinical characteristics of participants involved in this study, overweight women with PCOS and healthy lean controls, are summarised in **Table 4**.

Table 4 Clinical Characteristics of participants

	PCOS	**Healthy controls**	**p-value**
Number of participants	7	7	
Age	30 ± 6	26 ± 6	0.175
Bodyweight (kg)	99 ± 18	65 ± 14	<0.001
BMI	36.7 ± 6.7	22.1 ± 2.8	<0.001
Lean mass (%)	49 ± 6	68 ± 5	<0.001
Fasting glucose (mM)	4.7 ± 0.4	4.4 ± 0.3	0.240
Fasting insulin (uIU/ml)	15.6 ± 5.9	9.9 ± 2.6	0.069
Serum AMH (pmol/l)	53.5 ± 19.3	76.73 ± 43.5	0.236
GIR (mg/lbmkg/min)	6.75 ± 2.89	16.36 ± 5.1	0.001
*Insulin sensitivity index ((GIR/LBM)/SS INS)*100*	7.0 ± 2.9	14.97 ± 4.6	0.003

4.3.2 Relationship between *in vivo* insulin sensitivity and *in vitro* insulin-stimulated 2-deoxyglucose uptake in myotubes.

There was a significant difference between women with PCOS and healthy women in whole-body insulin sensitivity as derived from the euglycemic-hyperinsulinemic clamp, with healthy controls being more insulin sensitive (PCOS: 6.75 ± 2.89 mg/lbmkg/min, CI [4.07, 9.43], Healthy: 16.36 ± 5.13 mg/lbmkg/min, CI [11.61, 21.11] P = 0.001) **(Figure 8A)**. In Primary myotubes there was no significant differences between groups in the absolute change of insulin-stimulated 2-deoxy glucose uptake (PCOS: 1.12 **±** 3.77 pmol/mg/min, CI [-2.37, 4.61], Healthy: 2.33 **±** 2.28 pmol/mg/min, CI [0.22, 4.44], P = 0.48) (**Figure 8B**). In addition, there was a poor relationship between GIR (*in vivo*) and 2-deoxyglucose uptake (*in vitro*) in both groups (PCOS: r = 0.5467, CI [-0.35, 0.92], P = 0.2041, Healthy: r = -0.3596, CI [-0.87, 0.54], P = 0.4282).

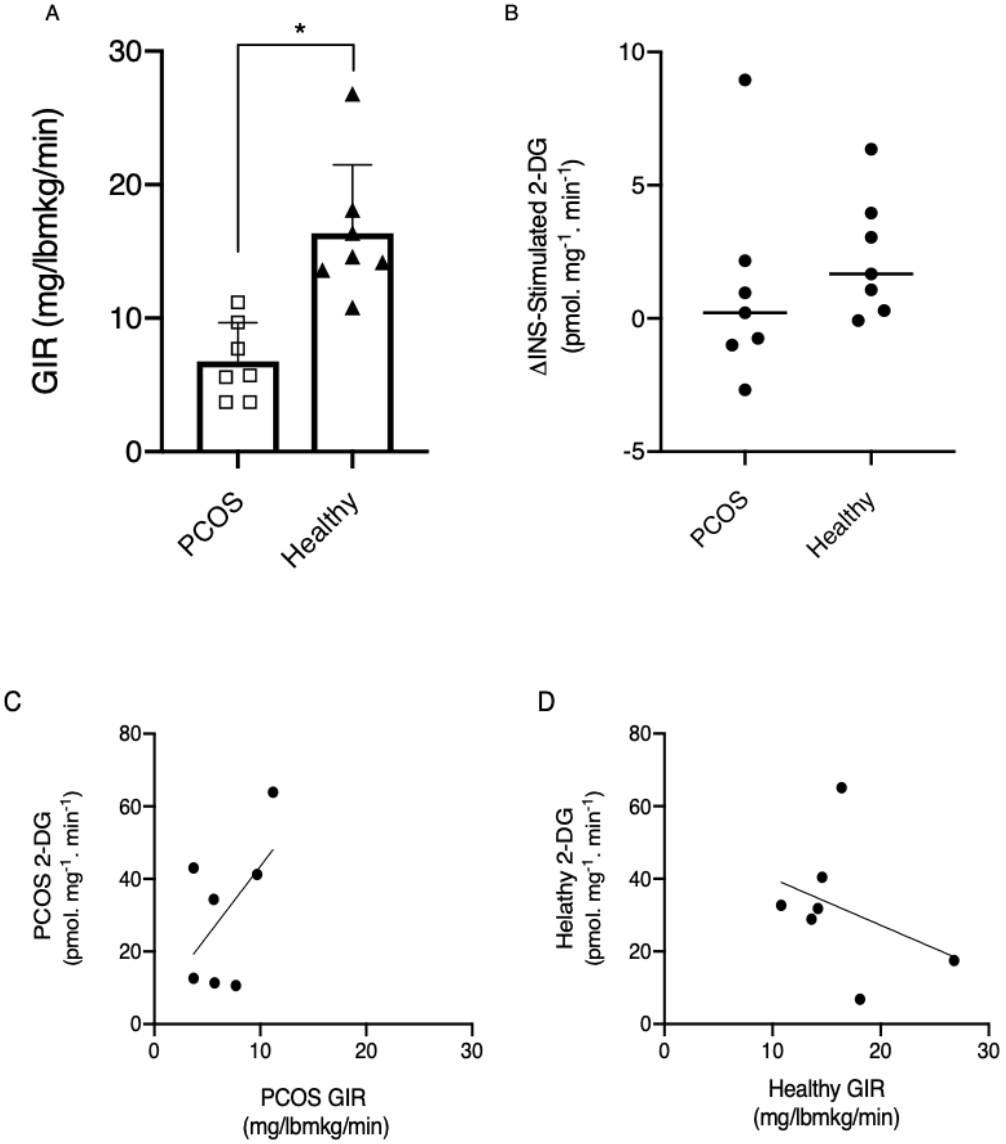

Figure 8: *In vivo* and *in vitro* comparison of insulin sensitivity and their relationship to allow for assessment of phenotype retention in primary myotubes from both women with PCOS and healthy controls

Comparison of glucose infusion rate (mg/lbmkg/min) obtained during the euglycemic-hyperinsulinemic clamp between women with PCOS and healthy controls. B) Comparison of absolute change of insulin-stimulated 2-deoxy glucose uptake (pmol/mg/min) in myotubes from women with PCOS and Healthy controls. C) Relationship between glucose infusion rate (mg/lbmkg/min) *in vivo* and absolute change of insulin-stimulated 2-deoxy glucose uptake (pmol/mg/min) in women with PCOS D) Relationship between glucose infusion rate (mg/lbmkg/min) *in vivo* and absolute change of insulin-stimulated 2-deoxy glucose uptake (pmol/mg/min) in healthy controls. *significant difference between PCOS and Healthy controls, Data reported mean ± SD (PCOS: N= 7, Healthy: N= 7).

4.3.3 2-Dexoxyglucose uptake

4.3.3.1 TGF-beta

Following treatment with TGF-beta 1 there was an increase in glucose uptake in both the PCOS and control myotubes (Group effect, P = 0.88; treatment effect, P < 0.001; Interaction effect, P < 0.001), (mean difference pmol/mg/min ± SD, 95% CI, P value). In PCOS myotubes the increase in glucose uptake occurred following treatment with TGF-beta 1 (5ng/ml) (9.220 ± 2.724, CI [0.88, 17.55], P = 0.01) and TGF-beta 1 (5ng/ml) plus insulin (11.72 ± 2.724, CI [3.39, 20.06], P = 0.001). In control myotubes the increase in glucose uptake occurred following treatment with TGF-beta 1 (1ng/ml) (12.94 ± 2.862, CI [4.18, 21.69], P < 0.001) and TGF-beta 1 (1ng/ml) plus insulin (12.33 ± 2.862, CI [3.57, 21.08], P = 0.001) **(Figure 9A)**.

4.3.3.2 AMH

Following treatment with AMH there was a decrease in glucose uptake in both the PCOS and control myotubes (Group effect, P = 0.94; treatment effect, P < 0.001; Interaction effect, P = 0.82), (mean difference pmol/mg/min ± SD, 95% CI, P value), In the PCOS myotubes there was a trend for decrease in glucose uptake following treatment with AMH (-6.49 ± 3.08 pmol/mg/min), but no significant differences were detected (P > 0.37). In control myotubes the decrease in glucose uptake occurred following treatment with AMH (5ng/ml) (-10.92 ± 3.150, CI [-19.61, -2.23], P = 0.006) **(Figure 9B)**.

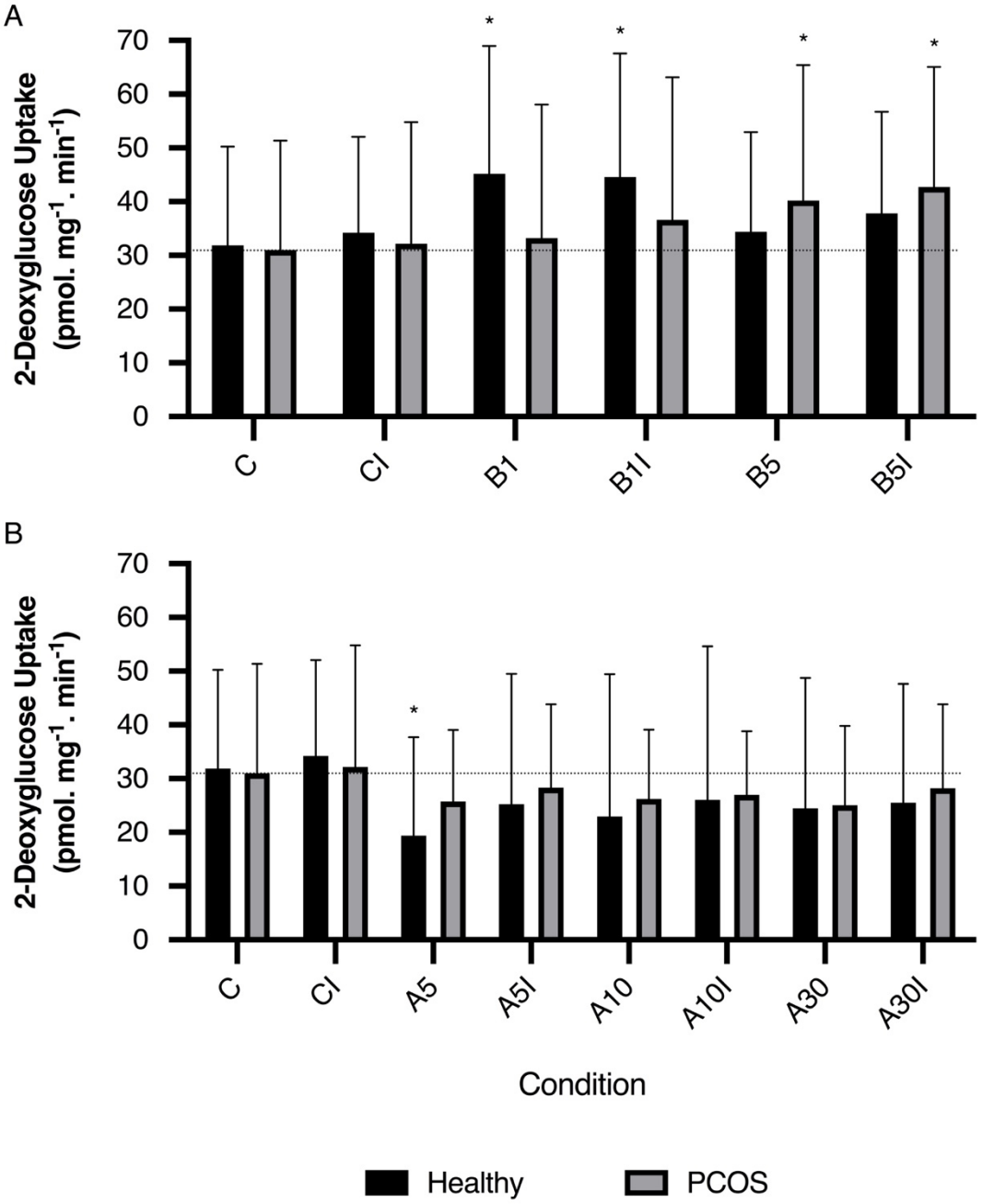

Figure 9 Skeletal muscle myotube 2-Deoxyglucose uptake
Control (C) as no treatment and following 24hour treatment with TGF-beta 1 (1 and 5 ng/ml) (Fig.6A) (B1 and B5) and AMH (5,10,30 ng/ml) (A5, A10, A30) (Fig.6B) in basal and insulin-stimulated states (I). C: control (no treatment), CI: Control + insulin, B1: TGF-beta 1ng/ml, B1I: TGF-beta 1ng/ml + insulin, B5: TGF-beta 5ng/ml, B5I: TGF-beta 5ng/ml + insulin, A5: AMH 5ng/ml + insulin, A5I: AMH 5ng/ml, A10: AMH 10ng/ml, A10I: AMH 10ng/ml + insulin, A30: AMH 30ng/ml, A30I: AMH 30ng/ml + insulin. Black bars = Healthy myotubes Grey bars = PCOS myotubes. Data reported as Mean ± SD. *significantly different from basal control. (Healthy: N= 7, PCOS: N= 7).

4.3.4 TGF-beta/SMAD signalling

4.3.4.1 TGF-beta

There was an increase in SMAD3 phosphorylation following treatment with TGF-beta 1, this increase was similar in both the PCOS and healthy control myotubes (Group effect P = 0.36; treatment effect, P < 0.001; Interaction effect, P = 0.23) (percentage of relative change ± SD, 95% CI, P value). The increase was observed following TGF-beta 1 (1ng/ml and 5ng/ml) with and without insulin in myotubes from both groups (PCOS: (B1: 498 ± 431%, CI [121, 876], P = 0.002, B1I: 511 ± 485%, CI [86, 936], P < 0.001, B5: 774 ± 728%, CI [166, 365], P < 0.001, B5I: 1011 ± 1232%, CI [69, 2090], P < 0.001) **(Figure 10A)** (Healthy: (B1: 1932 ± 1554%, CI [569, 3294], P < 0.001, B1I: 1892 ± 1471%, CI [603, 3181], P < 0.001, B5: 2224 ± 2131%, CI [356, 4092], P = <0.00001, B5I: 2409 ± 2502%, CI [216, 4603], P = <0.001) **(Figure 10A).** There were no detectable differences in SMAD 1/5/8 phosphorylation (Group effect P = 0.91; treatment effect, P = 0.05; Interaction effect, P = 0.28) although there was dose-dependent activation trend following treatment with TGF-beta 1 **(Figure 10B)**. There were no detectable differences in total SMAD4 expression following treatment with TGF beta 1 (Group effect, P = 0.14; treatment effect, P = 0.84; Interaction effect, P = 0.81) **(Figure 10C)**.

4.3.4.2 AMH

There were no detectable differences in for the phosphorylation of SMAD3 (Group effect, P = 0.14; treatment effect, P = 0.08; Interaction effect, P = 0.79) **(Figure 10A)** or SMAD1/5/8 (Group effect P = 0.79; treatment effect, P = 0.64; Interaction effect, P = 0.37) **(Figure 10B)** or for total SMAD4 expression following treatment with AMH in both groups (Group effect, P = 0.18; treatment effect, P = 0.64; Interaction effect, P = 0.41) **(Figure 10C)**.

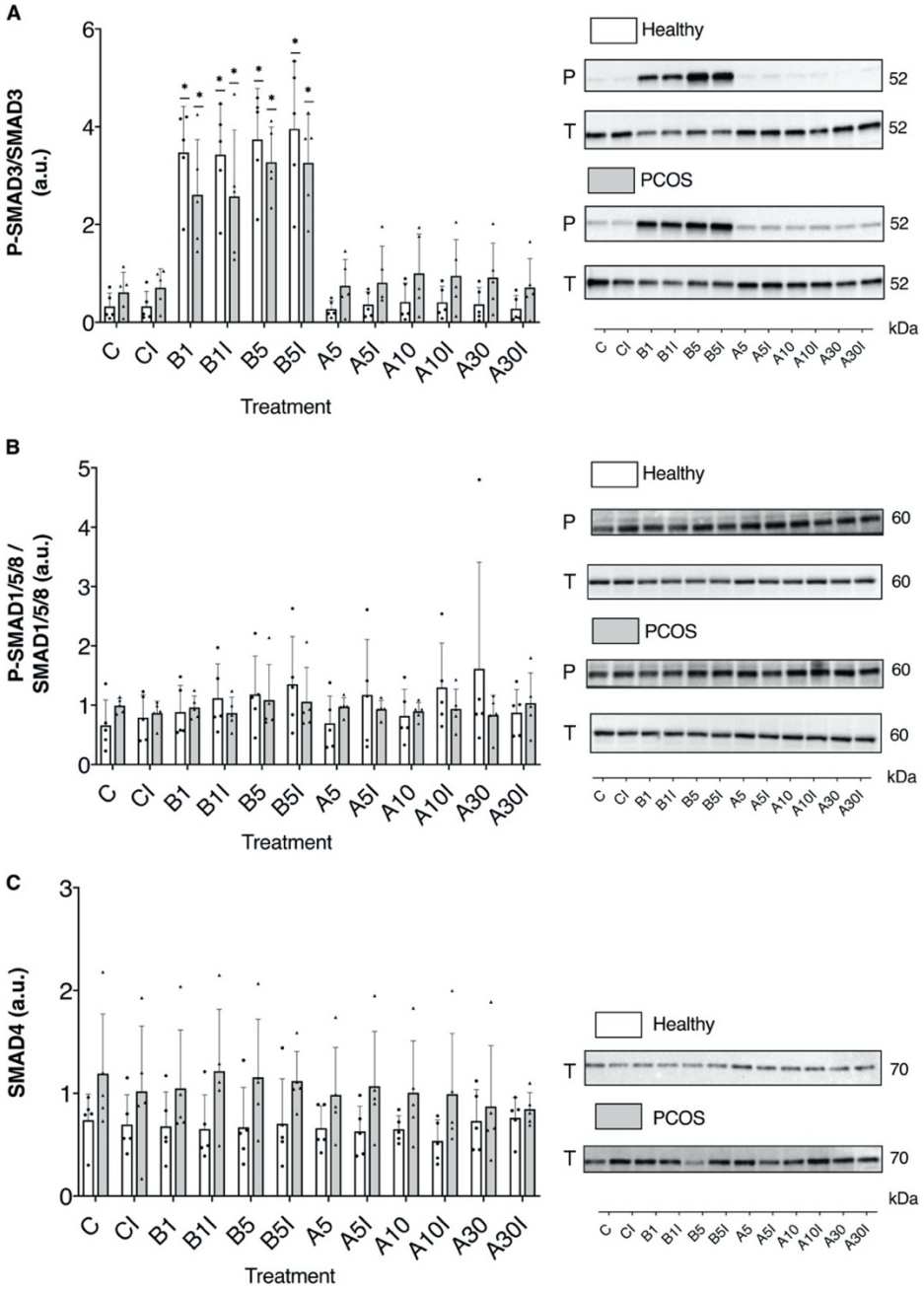

Figure 10. TGF-beta/SMAD signalling.
SMAD3 phosphorylation relative to total SMAD3 expression. B) SMAD1/5/8 phosphorylation relative to total SMAD1/5/8 expression. C) Total SMAD4 expression. Control (C) as no treatment and following 24hour treatment with TGF beta (1 and 5 ng/ml) (B1 and B5) and AMH (5,10,30 ng/ml) (A5, A10, A30) in basal and insulin-stimulated states (I). C: control (no treatment), CI: Control + insulin, B1: TGF-beta 1ng/ml, B1I: TGF-beta 1ng/ml + insulin, B5: TGF-beta 5ng/ml, B5I: TGF-beta 5ng/ml + insulin, A5: AMH 5ng/ml + insulin, A5I: AMH 5ng/ml, A10: AMH 10ng/ml, A10I: AMH 10ng/ml + insulin, A30: AMH 30ng/ml, A30I: AMH 30ng/ml + insulin. White bars = Healthy myotubes, Grey bars = PCOS myotubes. Data reported as Mean ± SD. *significantly different from basal control. Representative images shown for Phosphorylation (P) and total (T) protein expression shown for (Healthy: n = 5, PCOS: n = 5).

4.3.5 Insulin signalling

4.3.5.1 TGF-beta

There were no significant changes in the phosphorylation IRS-1 following treatment with TGF-beta 1 (Group effect P = 0.84; treatment effect, P = 0.17; Interaction effect, P = 0.86), **(Figure 11A).** There was a significant increase in phosphorylation of AKT following treatment with TGF-beta (Group effect, P = 0.06; treatment effect, P < 0.001; Interaction effect, P = 0.002) (percentage of relative change ± SD, 95% CI, P value), significant increases in phosphorylation only occurred in the healthy control group following no treatment with insulin stimulation (3751 ± 4591%, CI [-273,7776], P = 0.001) and TGF-beta 1 (1ng/ml) treatment with insulin 5420 ± 6855%, CI [-589,11428], P < 0.001) **(Figure 11B)**. There were no significant changes in the phosphorylation ERK1/2 following treatment with TGF-beta 1 (Group effect, P = 0.92; treatment effect, P = 0.58; Interaction effect, P = 0.83) **(Figure 11C)**. There were no significant changes in the expression of PI3K-p85 following treatment with TGF-beta 1 (Group effect P = 0.27; treatment effect, P = 0.97; Interaction effect, P = 0.17). Although not statistically significant, PI3K-p85 expression tended to be higher in myotubes from women with PCOS at basal level and with TGF-beta 1 **(Figure 12A)**. There were no significant changes in the expression of PI3K-p110 following treatment with TGF-beta 1 (Group effect, P = 0.27; treatment effect, P = 0.97; Interaction effect, P = 0.17) **(Figure 12B).** There was significant difference between groups or following treatment for ratio of PI3K-p85/PI3K-p110 (P > 0.16) **(Figure 12C)**.

4.3.5.2 AMH

Following treatment with AMH there was a significant difference in phosphorylation of IRS-1 in the healthy control myotubes only, this occurred following treatment with AMH (30ng/ml) and insulin (Group effect P = 0.24; treatment effect, P = 0.11; Interaction effect, P = 0.03) (percentage of relative change ± SD, 95% CI, P value), (A30I: 517 ± 592%, CI [2, 1035], P = 0.006). There was a trend for a dose response activation of IRS-1 phosphorylation in myotubes from the healthy control women following treatment with AMH **(Figure 11A).** There was a significant increase in phosphorylation of AKT following treatment with AMH (Group effect

P = 0.16; treatment effect, P < 0.001; Interaction effect, P = 0.07) (percentage of relative change ± SD, 95% CI, P value), significant increases in phosphorylation only occurred in the healthy control group following treatment with AMH (5ng/ml) with insulin (1487 ± 1100%, CI [523, 2452], P = 0.001) and AMH (10ng/ml) with insulin (844 ± 749%, CI [187, 1501], P = 0.006) **(Figure 11B).** There were no significant changes in the phosphorylation ERK1/2 following treatment with AMH (Group effect P = 0.59; treatment effect, P = 0.14; Interaction effect, P = 0.41) **(Figure 11C)**. Treatment with AMH did not result in any changes in the expression of PI3K-p85 (Group effect P = 0.06; treatment effect, P = 0.54; Interaction effect, P = 0.53), PI3K-p85 expression tended to be higher in myotubes from women with PCOS **(Figure 12A).** Following treatment with AMH there was a reduction in PI3k-p110 expression in healthy control myotubes only (Group effect P = 0.77, treatment effect, P = 0.01, Interaction effect, P = 0.67). This reduction occurred following AMH (30ng/ml) with and without inulin (A30: -48 ± 21 %, CI [-30, -67], P =0.019; A30I: -50 ± 37 %, CI [-18, -82], P = 0.01) **(Figure 12B)**. There was significant difference between groups or following treatment for ratio of PI3K-p85/PI3K-p110 (P > 0.15) **(Figure 12C)**.

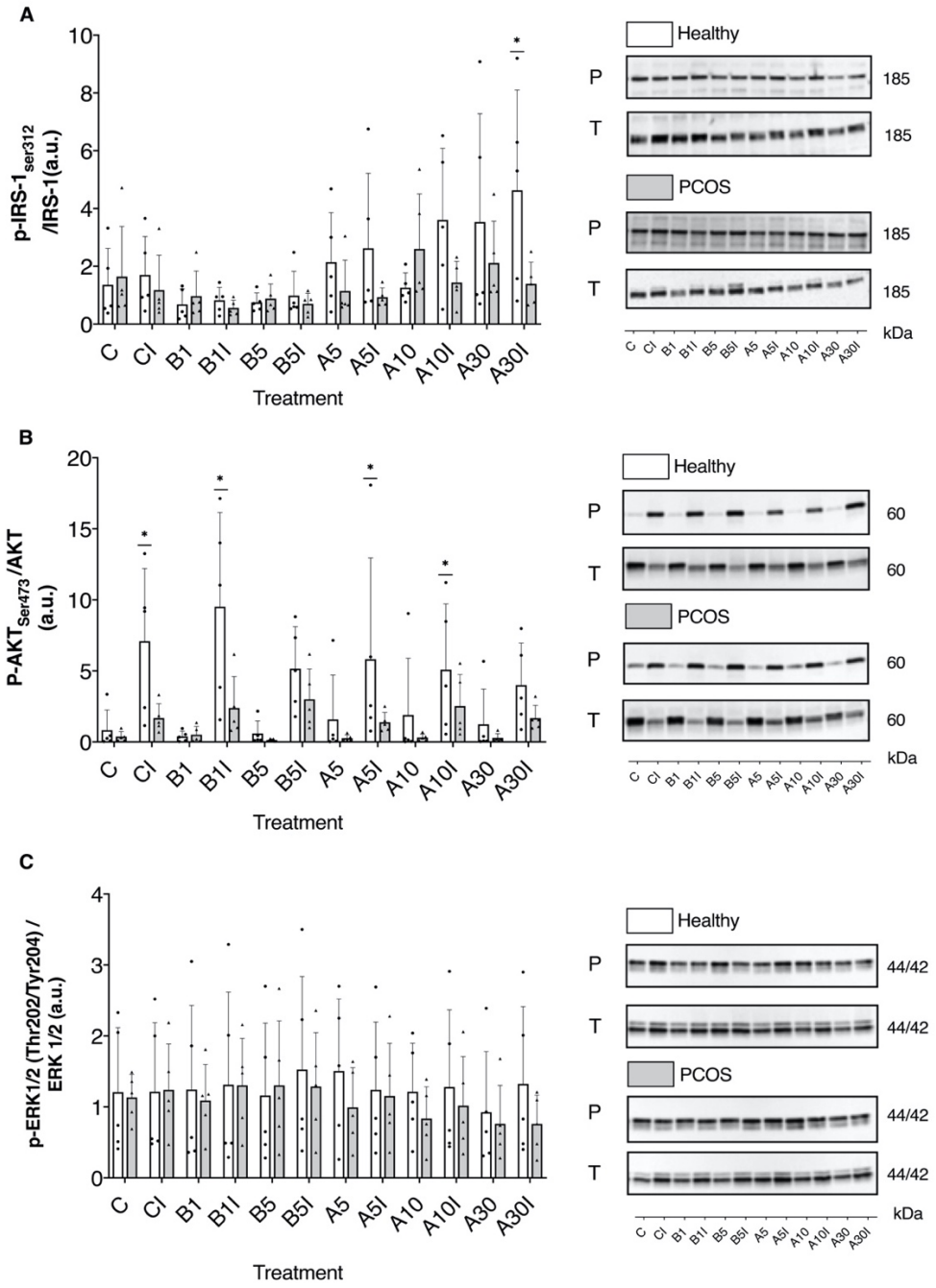

Figure 11. Insulin signalling

A) IRS-1 phosphorylation relative to total IRS-1 expression. B) AKT phosphorylation relative to total AKT expression. C) ERK1/2 phosphorylation relative to total ERK1/2 expression. Control (C) as no treatment and following 24hour treatment with TGF beta (1 and 5 ng/ml) (B1 and B5) and AMH (5,10,30 ng/ml) (A5, A10, A30) in basal and insulin-stimulated states (I). C: control (no treatment), CI: Control + insulin, B1: TGF-beta 1ng/ml, B1I: TGF-beta 1ng/ml + insulin, B5: TGF-beta 5ng/ml, B5I: TGF-beta 5ng/ml + insulin, A5: AMH 5ng/ml + insulin, A5I: AMH 5ng/ml, A10: AMH 10ng/ml, A10I: AMH 10ng/ml + insulin, A30: AMH 30ng/ml, A30I: AMH 30ng/ml + insulin. White bars = Healthy myotubes, Grey bars = PCOS myotubes. Data reported as Mean ± SD. *significantly different from basal control. Representative images shown for Phosphorylation (P) and total (T) protein expression shown for (Healthy: n = 5, PCOS: n = 5).

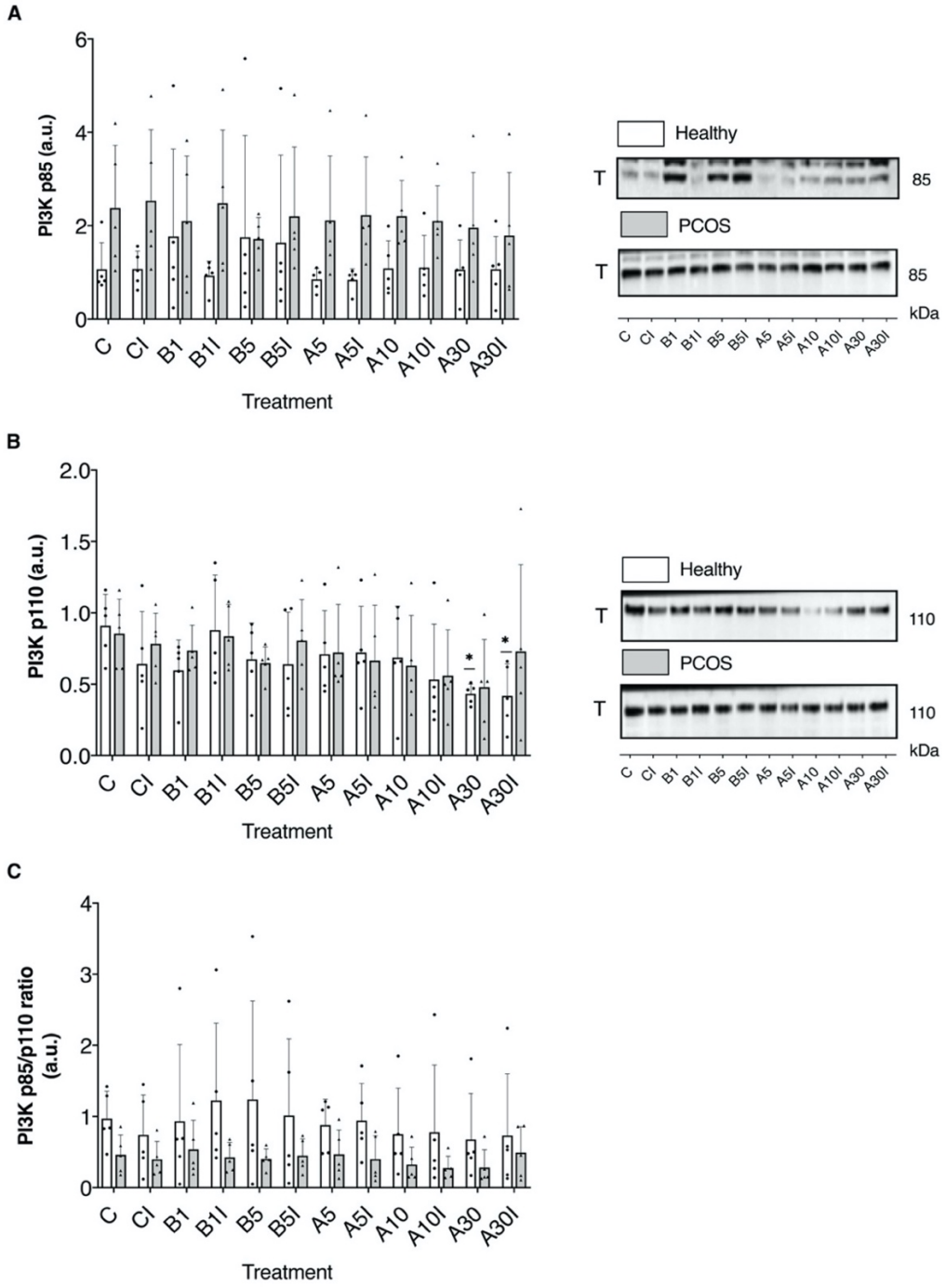

Figure 12. Insulin Signalling (cont.)
A) Total protein expression PI3K-p85. B) Total protein expression PI3K-p110. C) Total protein expression PI3K-p85/PI3K p110 ratio. Control (C) as no treatment and following 24hour treatment with TGF beta (1 and 5 ng/ml) (B1 and B5) and AMH (5,10,30 ng/ml) (A5, A10, A30) in basal and insulin-stimulated states (I). C: control (no treatment), CI: Control + insulin, B1: TGF-beta 1ng/ml, B1I: TGF-beta 1ng/ml + insulin, B5: TGF-beta 5ng/ml, B5I: TGF-beta 5ng/ml + insulin, A5: AMH 5ng/ml + insulin, A5I: AMH 5ng/ml, A10: AMH 10ng/ml, A10I: AMH 10ng/ml + insulin, A30: AMH 30ng/ml, A30I: AMH 30ng/ml + insulin. White bars = Healthy myotubes, Grey bars = PCOS myotubes. Data reported as Mean ± SD. *significantly different from basal control. Representative images shown for total (T) protein expression shown for (Healthy: n = 5, PCOS: n = 5).

4.3.6 mTOR signalling

4.3.6.1 TGF-beta

Following treatment with TGF-beta 1 there was no differences in the phosphorylation of mTOR (Group effect, P = 0.260; treatment effect, P = 0.07; Interaction effect, P = 0.35) **(Figure 13A)**. Following treatment with TGF-beta 1 there were differences between groups in phosphorylation of Raptor (Group effect, P = 0.04; treatment effect, P = 0.06; Interaction effect, P = 0.44. Post-hoc analysis identified no differences (P > 0.12). Although there was trend for healthy control myotubes displaying higher levels of phosphorylation of Raptor compared to myotubes from women with PCOS and for TGF-beta 1 treatment to decrease phosphorylation of Raptor **(Figure 13B)**. Following treatment with TGF-beta 1 there was differences between groups in phosphorylation of Rictor (Group effect, P = 0.89; treatment effect, P = 0.004; Interaction effect, P = 0.94) Post-hoc analysis identified no differences (P > 0.10) **(Figure 13C)**.

4.3.6.2 AMH

Following treatment with AMH there was no differences in the phosphorylation of mTOR (Group effect, P = 0.28; treatment effect, P = 0.11; Interaction effect, P = 0.33) **(Figure 13A)**. Following treatment with AMH there was no differences in the phosphorylation of Raptor (Group effect, P = 0.05; treatment effect, P = 0.49; Interaction effect, P = 0.50) **(Figure 13B)** Following treatment with AMH there was differences in the phosphorylation of Rictor (Group effect, P = 0.91; treatment effect, P = 0.004; Interaction effect, P = 0.99) Post-hoc analysis identified no differences (P > 0.26) **(Figure 13C)**.

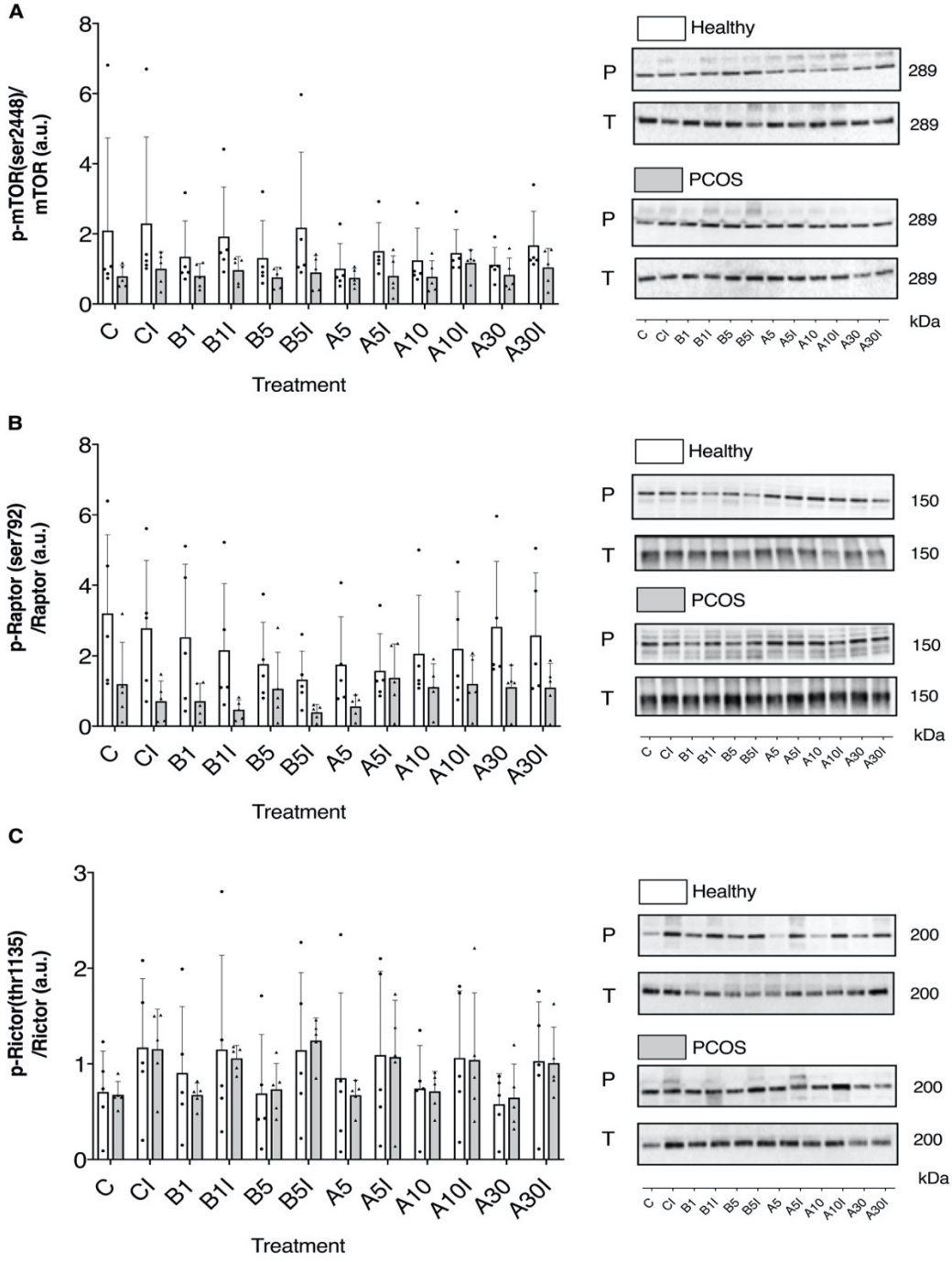

Figure 13. mTOR Signalling.
mTOR phosphorylation relative to total mTOR expression. B) Raptor phosphorylation relative to total Raptor expression. C) Rictor phosphorylation relative to total Rictor expression. Control (C) as no treatment and following 24hour treatment with TGF beta (1 and 5 ng/ml) (B1 and B5) and AMH (5,10,30 ng/ml) (A5, A10, A30) in basal and insulin-stimulated states (I). C: control (no treatment), CI: Control + insulin, B1: TGF-beta 1ng/ml, B1I: TGF-beta 1ng/ml + insulin, B5: TGF-beta 5ng/ml, B5I: TGF-beta 5ng/ml + insulin, A5: AMH 5ng/ml + insulin, A5I: AMH 5ng/ml, A10: AMH 10ng/ml, A10I: AMH 10ng/ml + insulin, A30: AMH 30ng/ml, A30I: AMH 30ng/ml + insulin. White bars = Healthy myotubes, Grey bars = PCOS myotubes. Data reported as Mean ± SD. *significantly different from basal control. Representative images shown for total (T) protein expression shown for (Healthy: n = 5, PCOS: n = 5).

4.4 Discussion

4.4.1 Do myotubes from women with PCOS retain their donor's metabolic characteristics?

We sought to determine if *in vitro* insulin sensitivity measured in the myotubes reflected *in vivo* insulin sensitivity obtained via a euglycemic-hyperinsulinemic clamp. Our results indicate that the metabolic phenotype *in vivo* was not maintained *in vitro* **(Figure 8 C and D)**. Women with PCOS displayed insulin resistance *in vivo,* but this was not reflected *in vitro*. Based on these results, culture myotubes from women with PCOS do not reflect donor characteristics, suggesting a role of environmental factors in the PCOS-specific IR. Despite a lack of retention of donor characteristics in both myotubes from women with PCOS and healthy controls when assessing metabolic characteristics *in vivo* and *in vitro*, we did observe some differences in insulin signalling. Remarkably, the assessment of insulin sensitivity *in vitro* by measuring 2-deoxyglucose uptake does not seem to represent the *in vivo* insulin sensitivity even in healthy controls **(Figure 8. D)**. Although in our study this effect may be due to the sample size used (n=7 per group), this highlights the limitations of this technique and therefore suggests the need of complementary physiological measurement such as glucose oxidation to support the results. Others have found that *in vitro* glucose uptake cannot be predicted by BMI, age, gender or muscle origin of the donor (Aas et al., 2020). This would suggest that under *in vivo* conditions, muscle cells undergo metabolic reprogramming or alterations in metabolic mechanisms compared with their role *in vivo*. Another possibility for the metabolic differences between *in vivo* and *in vitro* may be related to the degree of differentiation of the myotubes. It has previously been observed that myotubes from women with PCOS exhibit a greater degree of differentiation when compared to matched control myotubes as evidenced by higher creatine kinase activity and the abundance of myosin heavy chain protein expression (Corbould et al., 2005). This effect occurred despite cells being cultured side by side under the same conditions, which may suggest a unique characteristic of myotubes from women with PCOS. Although we did not assess the degree of differentiation in our study, this may account for lack of differences in insulin responsiveness between groups. Poor insulin responsiveness was evident in myotubes from both groups for glucose uptake, whereas insulin signalling appeared to be more insulin responsive. In general, myotubes express all the core metabolic proteins of the insulin signalling cascade as observed *in vivo* (Gaster, 2019; Thingholm et al., 2011); however, the

levels of expression can differ. Myotubes have higher protein expression of GLUT1 than GLUT4, in contrast to whole skeletal muscle where the opposite is observed (Deshmukh et al., 2015). Higher amounts of GLUT1 protein leads to an elevation of basal glucose uptake and reduced insulin responsiveness, with less GLUT4 translocation occurring in human primary myotubes in comparison to *in vivo* (Abdelmoez et al., 2019; Gaster, 2019). Another important consideration is the requirement of a supraphysiological dose of insulin to stimulate glucose uptake above basal levels, coupled with modest increases in glucose uptake following insulin stimulation. This may suggest that *in vitro* techniques may be of insufficient sensitivity to detect differences between groups.

The extent to which the donor characteristics are retained in cultured myotubes from women with PCOS has already been debated. Supporting our results, it is thought that defects in insulin responsiveness in skeletal muscle from women with PCOS are a result of a combination of *in vivo* environment and intrinsic defects. Eriksen and collages have demonstrated that neither insulin resistance nor mitochondrial dysfunction is maintained in culture (Eriksen et al., 2010, 2011). Others have also shown that despite displaying insulin resistance *in vivo,* cultured myotubes form women with PCOS had normal insulin sensitivity/responsiveness (Ciaraldi *et al.*, 2009). In contrast to the work from Eriksen, others have claimed there is an intrinsic defect in PCOS myotubes, causing increased phosphorylation of IRS-1_{ser312} and decreased PI3K-activity (Corbould et al., 2005). In a follow-up work, they suggested that ERK1/2 activation was responsible for these alterations by modulating inflammatory factors (Corbould et al., 2006). We found no detectable differences in IRS-1 or ERK1/2 phosphorylation at baseline and during insulin stimulation in PCOS myotubes, suggesting that this defect is not seen in all women with PCOS **(Figure 10.A and C).** Collectively, the work by Eriksen and Corbould suggests that the defects seen *in vivo* may be a combination of acquired and intrinsic defects. This work contributed to our study rationale that circulating factors that are elevated in the serum of women with PCOS may contribute to insulin resistance either by worsening existing defects or lead to the development of defects in insulin signalling.

The extent to which myotubes retain different aspects from their donor characteristics is uncertain. It has been noted that when mammalian cells are in culture, they undergo a rapid and substantial modification of the transcriptome and epigenome (Nestor et al., 2015). This

indicates alteration of the physiological state of the cells, which likely reflects the change in the cellular environment (e.g., two-dimensional and lack of *in vivo* circulating factors). Indeed, when comparing human skeletal muscle and myotubes from healthy young women, there were substantial differences in the transcriptome (Raymond et al., 2010). The main differences observed were related to mitochondrial dysfunction, increased extracellular matrix, and altered glucose metabolism in the myotubes, suggesting that the *in vitro* environment alters the cellular phenotype. In regards to the retention of the metabolic characteristics in our cultured myotubes, we have found that myotubes from women with PCOS and healthy controls no longer reflect the donors' metabolic state when in culture. Others have shown that myotubes from obese and non-obese individuals have comparable insulin-stimulated glucose uptake, despite myotubes from obese individuals having reduced GLUT4 content (Pender et al., 2005). Similarly, lean myotubes from insulin-resistant and insulin-sensitive individuals display similar insulin-stimulated glucose uptake, glycogen synthesis and signalling despite clear *in vivo* differences (Krutzfeldt et al., 2000). In contrast, myotubes derived from skeletal muscle of individuals with type 2 diabetes retain insulin resistant phenotype in culture (Gaster et al., 2002; Henry et al., 1995; McIntyre et al., 2004b; Thingholm et al., 2011). Taken together, this would suggest that only severe or genetic defects are retained in myotubes, allowing for the separation of acquired and intrinsic factors that contribute to skeletal muscle insulin resistance. Caution should be applied when utilising PCOS myotubes when investigating the mechanism of insulin resistance or other phenotypical features observed *in vivo*, given the lack of retention in characteristics shown in our study and others (Eriksen et al., 2010, 2011). This highlights a need to replicate the *in vivo* environment to make this model more relevant to the disease state.

Future studies should consider supplementing the growth medium with the donor's serum to better represent the *in vivo* tissue microenvironment, as circulating factors may be responsible for activating intrinsic signalling defects. For instance, in our study, the donors' PCOS phenotypes were, three from phenotype A, two from phenotype B, and two from phenotype D (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004), and therefore each skeletal muscle is exposed to different levels of circulating factors, which may affect insulin resistance. Thus, it is important to factor in the heterogeneity of PCOS for the differences observed between studies. A limitation of our study is the use of myotubes from lean, healthy women rather than BMI matched comparison group, meaning we cannot fully account for the metabolic impact of obesity. The addition of a BMI-matched control group,

and the inclusion of lean PCOS group would mean that the effect of PCOS and the effect of obesity on insulin signalling and sensitivity could be independently quantified. However, it has been shown that obesity-related insulin resistance is not maintained in primary myotubes (Pender et al., 2005),suggesting that the use of myotubes allows to identify intrinsic versus acquired factors. Another possible influence on the results is the high day-to-day variation from the same donor for glucose uptake and glycogen synthesis in human primary myotubes (Gaster, 2019). These results are also influenced by experimental factors such as cell confluency and the level of differentiation, which is difficult to precisely control.

4.4.1.1 Intrinsic defects in the insulin signalling pathway

Defects in insulin-stimulated Akt phosphorylation at both Thr308 and ser473 despite normal PI3K activity were previously observed in muscle samples from women with PCOS (Hojlund et al., 2008). In our study, the myotubes from women with PCOS had a reduction in insulin-stimulated phosphorylation of Akt and a tendency for greater expression of PI3K-p85 (inhibitor of PI3K activity) than myotubes from lean, healthy controls, which may contribute to the defect in Akt phosphorylation. An increase in expression of PI3K-p85 adaptor subunit has been demonstrated to play a role in insulin resistance in type 2 diabetes via an increase in phosphorylation of IRS-1_{ser312}, ERK1/2, p38MAPK and JNK, to inhibit PI3K activity (Bandyopadhyay et al., 2005; Barbour et al., 2005). However, this does not explain the defects we observed, given the lack of differences in IRS-1_{ser312} and ERK1/2 phosphorylation at baseline and during insulin stimulation. Moreover, defects in AKT are unlikely to fully account for or explain insulin resistance observed in women with PCOS, given that only a small proportion of Akt phosphorylation is required to elicit downstream insulin action (Hoehn et al., 2008; Tan et al., 2012). It has been shown that there is a lack of linearity between insulin signalling phosphorylation and glucose uptake. For example, in L6 myotubes (Hoehn et al., 2008) and adipocytes (Whitehead et al., 2001), Akt phosphorylation of 28% was sufficient to achieve maximal-insulin-stimulated glucose uptake and only 5% to achieve maximal GLUT4 translocation. In addition, Hoehn et al. 2008 investigated a number of different physiological relevant experimental models of insulin resistance; this included palmitate, glucocorticoid, hyperinsulinemia, oxidative stress, chronic low-dose inflammation. This extensive approach allowed them to identify that impaired insulin and platelet-derived growth factor-stimulated

glucose uptake and GLUT4 translocation could occur without any defects in IRS/PI3K/Akt signalling (Hoehn et al., 2008). Additionally, it has been demonstrated a reduction in IRS-1 protein levels of 68% (using short hairpin RNA's) was insufficient to influence glucose disposal and induce insulin resistance *in vivo* (Cleasby et al., 2007). Therefore, changes in proximal insulin signalling would likely have a substantial impact on insulin signalling and would lead to the development of insulin resistance.

It is known that insulin resistance can occur independently of decreases in proximal insulin signalling transduction (Meyer et al., 2002; Tonks et al., 2013; Vind et al., 2011). In myotubes and whole muscle from women with PCOS, it has been demonstrated that reductions in insulin-stimulated glucose uptake occurred independently of changes in insulin-signalling proteins (Ciaraldi *et al.*, 2009). In the case of our study, myotubes from women with PCOS displayed a decrease in the basal mTOR signalling with comparable insulin sensitivity to healthy control myotubes. This does not rule out metabolic defects, such as differences in glycogen synthesis and fatty acid oxidation. Our findings may demonstrate that factors beyond defective insulin signalling could be contributing to the development of insulin resistance. It has been suggested that insulin resistance itself may be responsible for the development of impaired insulin signalling from animal models and cell culture studies (Fazakerley et al., 2019). Hyperinsulinemia has been shown to lead to impairment of insulin-stimulated IRS-1_{tyr612} and Akt_{ser473} phosphorylation in human primary myotubes (Turner et al., 2020), and increases basal phosphorylation serine IRS-1_{ser307} and mTOR in conjunction with a decrease in IRS-1_{tyr612} and PI3k activity in rats (Ueno et al., 2005). This makes sense in the case of our study with the removal of whole muscle from hyperinsulinemia, and defects observed in myotubes in Akt and mTOR expression may be seen as residuals of this environment. This could explain the variety of defects in the insulin signalling cascade observed in PCOS as each individual has different levels of hyperinsulinemia. To fully understand if myotubes obtained from women with PCOS retain donor characteristics and which environmental factors in the physiological milieu are involved in the development of insulin resistance, more advanced sequencing and *omics* studies should be considered.

4.4.2 The effects of TGF-beta 1 on glucose uptake and insulin signalling

The primary aim of this study was to determine if the TGF-beta ligands, TGF-beta 1 and AMH, were in part responsible for the development of insulin resistance and aberrant insulin signalling, previously observed in the skeletal muscle and myotubes of women with PCOS. Contrary to our hypothesis, treatment with TGF-beta 1 resulted in an increase in basal and insulin-stimulated glucose uptake in myotubes from women with PCOS and from healthy women. This increase was observed following a dose of 1ng/ml in the myotubes from healthy women and following the higher dose of 5 ng/ml in the myotubes from women with PCOS. This presents the possibility that myotubes from PCOS are desensitized to the effects of TGF-beta 1 due to already being chronically exposed and therefore need a stronger stimulus to respond. This could be related to the proposed hypothesis that skeletal muscle has an epigenetic memory, whereby the response to environmental stressors can be influenced by prior exposure to the same stressor (Sharples et al., 2016). Likewise, others have shown that metabolic adaptations following 8-12 weeks of exercise training are retained in human primary myotubes (Bourlier et al., 2013; Lund et al., 2017), and that myotubes from trained individuals display protection from fatty acid-induced insulin resistance (Green et al., 2013). The alteration in glucose metabolism following TGF-beta 1 treatment was accompanied by an increase in the phosphorylation of SMAD3 and Akt_{ser473}, while AMH treatment increased $IRS1_{ser312}$ phosphorylation in both groups. This may help to explain the opposing effects of the respective treatments on glucose uptake, with TGF-beta 1 resulting in an increase, and AMH resulting in a decrease in glucose uptake.

The effects of TGF-beta 1 on glucose metabolism and insulin signalling has not been previously explored in myotubes. However, there is a substantial body of evidence for its role in metabolism in other cell types whereby TGF-beta1 leads to profibrotic effects via mTORC1/2. This process itself is physiologically vital; however, when this pathway is chronically stimulated, it can promote fibrosis. Further studies using murine fibroblasts and myofibroblasts have shown that TGF-beta 1 induces glycolysis via SMAD2/3 signalling to increase GLUT1 under normoxia (Andrianifahanana et al., 2016; Selvarajah et al., 2019). It was identified that this elevated GLUT1 expression results in an increased expression of profibrotic genes PAL-1, CTGF, alpha-SMA, leading to increases in collagen I and fibronectin,

with this effect being mediated by ERK1/2 and mTORC1 (Andrianifahanana et al., 2016). In addition, it was demonstrated in myofibroblasts that TGF-beta 1 activates signalling cascade of SMAD3-mTORC1-ATF4-GLUT1 to induce glycolysis to meet the enhanced demands of collagen production (Selvarajah et al., 2019). Therefore, suggesting that TGF-beta 1 may induce fibrosis via GLUT1 upregulation. However, in our study, we did not see any changes in the phosphorylation of ERK or mTOR with treatment with TGF-beta 1. We did observe increases in GLUT1 mRNA but not GLUT4 (Unpublished data Moreno-Asso et al.) following treatment with TGF-beta1 in both groups. We have not yet measured GLUT1 protein expression to confirm this effect in our study, nor changes in GLUT4 translocation, which may be more relevant for glucose uptake than changes in mRNA. Moreover, to fully understand if this fibrotic effect is occurring in PCOS myotubes and whole skeletal muscle, we would need to assess downstream targets such as collagens and profibrotic genes in future studies. Furthermore, it has been described that C2C12 myotubes treated with TGF-beta 1 result in an increase in ROS production which could augment the fibrotic effects, and lead to aberrant insulin signalling (Abrigo *et al.*, 2016; Ábrigo *et al.*, 2018), suggesting that other pathways may be involved in regulating fibrosis in skeletal muscle. Future studies may wish to explore how the responses to treatment with TGF-beta 1 and AMH are influenced by other physiological factors present in women with PCOS, such as hyperandrogenism and oxidative stress.

In line with the increase in GLUT1 mRNA following treatment with TGF-beta 1 in our results, it has been demonstrated that myotubes from women with PCOS had a greater expression of GLUT1 than cells from overweight controls (Corbould et al., 2005). This was also accompanied by an increase in basal glucose uptake and no changes in GLUT4. This may indicate there is a potential metabolic reprogramming via environmental factors such as TGF-beta 1 or other growth factors in PCOS skeletal muscle causing a shift to a preferential glycolytic ATP synthesis as opposed to mitochondrial oxidative phosphorylation under conditions of normoxia. This process is also known as the Warburg effect in oncology and has been linked to diabetes and ageing (Burns and Manda, 2017), increasing cancer predisposition. In support of this theory, metabolomics of plasma samples from women with PCOS showed a significant increase in lactate and glucogenic amino acids with a reduction in glucose, a sign of glycolysis in skeletal muscle (Zhao et al., 2012). However, we did not observe an elevated basal glucose uptake in the myotubes from women with PCOS in our study.

4.4.3 The effects of AMH on glucose uptake and insulin signalling

We observed that treatment with AMH resulted in a trend for a decrease in basal and insulin-stimulated glucose uptake, which was accompanied by an increase in the phosphorylation of IRS-1$_{ser312}$ and a decrease in PI3K-p110 **(Figure 11A and 12B)**, which is associated with insulin resistance. This effect appeared to be more pronounced in the myotubes from healthy women following treatment with 30 ng/ml, and we expected it to occur via SMAD dependent signalling pathways. This dose of AMH (214.3 pmol/l) would be considered as high serum levels. However, there was a lack of activation of SMAD2/3 or SMAD1/5/8 signalling following treatment with AMH. The magnitude of the effect of AMH may be related to the low expression of AMH receptor 2 in the skeletal muscle (Uhlén et al., 2015; Wang et al., 2005), which may be further affected when cells are cultured. Although AMH is capable of acting through Type 1 receptors ALK2 and ALK3, in this case, this seems unlikely given the lack of SMAD activation. The presence of AMH receptor 2 has been difficult to assess to date due to anti-bodies showing cross-reactivity in many tissues (Pankhurst et al., 2016). A limitation of our study is that we did not assess the expression and activation of AMH receptor 2 or other type 1 receptors. Moreover, we were unable to detect mRNA expression of AMH receptor 2 in skeletal muscle or cultured myotubes (Unpublished data Moreno-Asso et al.).

To our knowledge, no other study has assessed the effects of AMH on metabolic function and insulin signalling in myotubes. From our results, we would indicate that AMH results in a decrease in glucose uptake and disrupts insulin signalling in human primary myotubes from women with and without PCOS, suggesting that it may play a role in peripheral insulin resistance in women with PCOS. However, the effect seen would not account for the whole extent of insulin resistance observed. It may be possible that AMH interacts with other factors such as androgens and have a synergistic effect. In addition, insulin has been shown to upregulate AMH expression in granulosa cells from women with PCOS (Liu et al., 2019). This may indicate that hyperinsulinemia drives an increase in AMH, suggesting that AMH action

and expression is influenced by insulin resistance, creating a negative feedback loop where AMH worsens insulin resistance. In fact, the increase in phosphorylation of IRS-1$_{ser312,}$ which is associated with insulin resistance, may have occurred via SMAD independent signalling pathways and was greater with AMH and insulin in comparison to AMH alone. An effect of the increase in IRS-1 serine phosphorylation is a decrease in the expression of PI3K-p110α; this effect has been observed in the skeletal muscle of individuals who are obese and individuals with type 2 diabetes (Bandyopadhyay et al., 2005). In p110α/β knockout mice, there is a decrease in insulin sensitivity and glucose disposal, as well as a reduction in PI3K-P85 expression (Brachmann et al., 2005). This results in an increase in the ratio of p85α to p110α which has been associated with a decline in insulin sensitivity (Draznin, 2006). More recently, in the skeletal muscle of mice, it has been shown that p110α deletion results in impaired insulin signalling, with a decrease in Akt phosphorylation (Li et al., 2019). In our study, the reduction in p110α was accompanied by a trend for a slight reduction in Akt. This presents a possible mechanism by which exposure of peripheral tissues to high levels of AMH may result in aberrant insulin signalling and decreases in glucose uptake but needs to be explored further.

AMH needs to undergo cleavage to be able to bind to AMH receptor 2 and activate SMAD signalling (Papakostas et al., 2010). For our experiments, we treated the myotubes with a cleaved form of AMH, which must be taken into account for the physiological relevance of these results. It has been demonstrated that there are high levels of cleaved AMH in the blood, with most of it remaining inactive as it is not competent for binding to the AMH receptor 2 (Pierre et al., 2016). This indicates that despite high circulating levels in serum of women with PCOS, these measures do not reflect the bioactivity. It also raises the question if it has a role in skeletal muscle metabolism, given the likelihood that *in vivo* skeletal muscle is exposed to minimal concentrations of bioactive AMH. However, our results may indicate that AMH can act through other pathways to cause metabolic dysfunction, despite the lack of SMAD phosphorylation.

4.4.4 Conclusions

In conclusion, myotubes from women with PCOS do not appear to retain their metabolic phenotype, and only insulin-stimulated phosphorylation of Akt and basal mTOR was altered compared to controls. This suggests that external environmental factors may contribute to the development of insulin resistance in combination with underlying intrinsic alterations. We observed that treatment with TGF-beta 1 increases glucose uptake and phosphorylation of SMAD3, which appears to occur independently of changes in the insulin signalling cascade. In comparison, AMH decreased glucose uptake with an increased expression of IRS-1_{ser312} and a reduction in PI3Kp110. The role of TGF-beta ligands, including TGF-beta 1 and AMH, requires further work to understand its role in skeletal muscle insulin resistance in women with PCOS.

4.4.4.1 Key findings

- The *in vivo* metabolic phenotype is not accurately retained in cultured myotubes from women with PCOS or healthy controls.
- TGF-beta 1 treatment increases basal and insulin-stimulated glucose uptake in skeletal muscle myotubes.
- AMH decreased glucose uptake and is accompanied by an increase in IRS-1_{ser312} and a decrease in PI3Kp110. Its mechanism of action is unclear, given that it failed to activate SMAD signalling pathways.

5 **STUDY 2:** The Effects of Electrical Pulse Stimulation in Combination with TGF-beta 1 or Anti-Müllerian Hormone on Exercise-induced Signal Transduction in Skeletal Muscle Myotubes

5.1 Introduction

To date, lifestyle modification is seen as first-line therapy for women with PCOS to manage the clinical features, including metabolic, reproductive and psychological characteristics. A key component of this lifestyle modification is structured exercise (Stepto et al., 2019b; Teede et al., 2018a). There are multiple benefits of exercise training for improving metabolic health. In particular, the following adaptations to skeletal muscle correspond to improvements in insulin sensitivity, increases in muscle mass, capillarization, GLUT4 and hexokinase II protein expression (responsible for glucose transport), and mitochondrial content (Egan and Zierath, 2013; Sylow and Richter, 2019).

5.1.1 Resistance to metabolic benefits of exercise in women with PCOS

A key determinant in the risk of developing metabolic syndrome and type 2 diabetes is cardiorespiratory fitness ($VO_{2peak/max}$). Based on the limited available data, women with PCOS display a marked reduction in cardiorespiratory fitness when compared with matched controls (Dona et al., 2017). This may be attributed to insulin resistance in women with PCOS who are overweight or obese, while in lean patients, it is attributed to hyperandrogenism (Dona et al., 2017) and may depend on the phenotype. It has also been suggested that women with PCOS have resistance to the beneficial effects of exercise on insulin sensitivity. This notion stemmed from the study from Harrisson, Stepto, and colleagues, where they observed that women with PCOS had an impairment in exercise-induced improvements in peripheral insulin sensitivity measured by the euglycemic-hyperinsulinemic clamp technique (Harrison et al., 2012). Women with PCOS improved insulin sensitivity by 16.37%, whereas matched controls improved by 23.75% after 12-week exercise intervention. However, women with PCOS were 46% less insulin sensitive before the exercise intervention (Harrison et al., 2012). In individuals

with type 2 diabetes, the mechanism behind this reduced improvement in insulin sensitivity after exercise training has been attributed to TGF-beta 1 (Böhm et al., 2016). It has been shown that low responders to exercise training have greater TGF-beta 1 activity, resulting in the suppression of AMPKα2 and ATP synthase subunit α, PGC1α, and TFAM (Böhm et al., 2016). These factors are responsible for the regulation of post-exercise metabolism. A similar role of TGF-beta signalling has been shown in women with PCOS, whereby intensified exercise training did not fully restore insulin sensitivity to levels comparable to overweight controls (N. Stepto et al., 2020). This was accompanied by a profibrotic gene expression profile (COL1A2, COL3A1, LOX, Decorin, TGFBR2), which was augmented following exercise training.

5.1.2 Aims and hypothesis

Therefore, we aimed to determine if myotubes from women with PCOS display a resistance to the beneficial effects of exercise by assessing exercise-specific signalling following *in vitro* electrical pulse stimulation (EPS), compared with myotubes from healthy controls. It was hypothesized that myotubes from women PCOS would display impaired exercise-induced signalling following *in vitro* contraction, consistent with the lack of training-induced improvements in metabolism observed *in vivo*. In addition, we wanted to assess if TGF-beta 1 or AMH influence exercise-specific signalling in myotubes following *in vitro* EPS. It was hypothesized that these TGF-beta ligands would further augment the impairment in exercise-induced signalling.

5.2 Methods

5.2.1 Participants

Myotube cultures were established from six women with PCOS who were overweight, and six healthy women were used for this study (total n =12) (For more details, see Methodology section 3.1).

5.2.2 Assessment of exercise capacity

See Methodology section 3.2

5.2.3 Muscle Biopsy

See Methodology section 3.3

5.2.4 Human Primary culture of myotubes

See Methodology section 3.7

5.2.5 TGFB and AMH myotubes experiment

To determine if the circulating factors TGF-beta 1 and AMH differently influence primary myotube responses to *in vitro* EPS, the following four conditions were applied to all the cultures: no treatment control, EPS, EPS+TGF-beta 1 (5ng/ml) and EPS+AMH (30 ng/ml). On day 5 of differentiation, media was changed from all cells to a serum-free medium, with or without the respective treatment (TGF-beta 1 or AMH). EPS protocol commenced on day 6, for more details, see methodology section 3.9.

5.2.6 Electrical pulse stimulation *in vitro* contraction

A chronic low-frequency stimulation protocol of 11.5 V, 2 ms, 1Hz was applied for 6 hours in serum-free low glucose media (alpha MEM). For more details, see Methodology section 3.10.

5.2.7 Western blot analysis

Targets quantified in this study were Phospho-p38 MAPK, PGC1α, phospho CREB, NUR77, NOR1, Phospho-AMPK, Phospho-ACC, GLUT4, Phospho and total AKT, Phospho and total MTOR (for more details, see Methodology section 3.12)

5.2.8 Statistical analysis

Clinical characteristics were compared with two-tailed unpaired student's t-test. A linear mixed model analysis was used to examine the differences between treatments, groups, and within donor variation. Changes in protein expression and phosphorylation were assessed using a mixed-effects model (2 groups and 4-time points). Significant effects of interaction (group x time), Time (Basal control vs EPS with and without treatment) and group (Lean Con vs OW PCOS). The distribution of the data was tested using the Shapiro-Wilk test. Post-hoc analysis of significant interaction and main effects was carried out using the Sidak multiple comparisons test. Data in text for basal expression and phosphorylation of proteins is presented as relative difference percentage ± SD 95% CI [lower, upper], P-value. (For additional details See Methodology section 3.13)

5.3 Results

5.3.1 Clinical characteristics

Clinical characteristics of participants involved in this study, overweight women with PCOS and healthy lean controls, are summarised in **Table 5**.

Table 5 Clinical characteristics of participants

	PCOS	Healthy controls	p-value
number of participants	6	6	
Age	28 ± 3	24 ± 3	0.02
Body weight (kg)	104.6 ± 14	66.4 ± 14.4	< 0.001
BMI	38.4 ± 5.5	22.3 ± 3.0	< 0.001
Lean mass (%)	48 ± 5	68.6 ± 5.0	< 0.001
Fasting glucose (mM)	4.8 ± 0.2	4.4 ± 0.3	0.03
Fasting insulin (uIU/m)	16.6 ± 5.4	9.9 ± 2.9	0.02
$VO2_{peak}$ *(ml/kg/min)*	24.3 ± 6.5	45.0 ± 6.5	< 0.001
GIR (mg/lbmkg/min)	6.0 ± 2.3	16.7 ± 5.5	0.003
*Insulin sensitivity index ((GIR/LBM)/SS INS) *100*	6.6 ± 2.8	16.07 ± 4.0	0.001

5.3.2 Stress kinase

5.3.2.1 phospho-p38 MAPK

There was an increase in p38 MAPK phosphorylation following EPS with and without treatment. (Group effect, P = 0.09; treatment effect, P = 0.003; Interaction effect, P = 0.16) (percentage of relative change ± SD, 95% CI, P value). Post-hoc analysis revealed a significant increase in healthy myotubes following EPS alone (221 ± 346%, 95% CI, [-56, 498], P = 0.04), and EPS with TGF beta 1 (5ng/ml) (397 ± 748%, 95% CI, [-201, 996], P = 0.001) **(Figure 14)**.

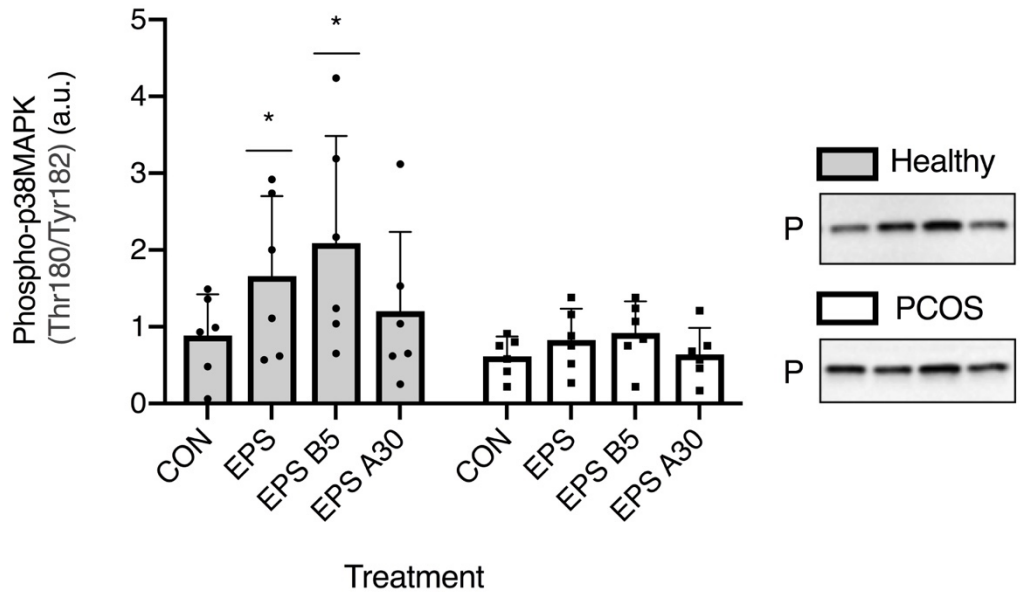

Figure 14. Phosphorylation of p38 MAPK

Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *Significantly different from basal control. Representative images shown for Phosphorylation (P) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

5.3.3 Transcription factors

5.3.3.1 phospho-CREB

There was an increase in CREB phosphorylation following EPS with and without treatment. (Group effect, P = 0.12; Treatment effect, P = 0.04; Interaction effect, P = 0.06) (percentage of relative change ± SD, 95% CI, P value). A statistically significant increase occurred in healthy myotubes following EPS alone (137 ± 267%, 95% CI, [-77, 351], P = 0.01). EPS with TGF-beta 1 (5ng/ml) (112 ± 204%, 95% CI, [-52, 276], P = 0.01), and EPS with AMH (30ng/ml) (115 ± 227%, 95% CI, [-67, 297], P = 0.02) **(Figure 15A).**

5.3.3.2 PGC1α

There were no differences in the expression of PGC1α following EPS with and without treatments and no differences between groups (Group effect, P = 0.86; Treatment effect, P = 0.90; Interaction effect, P = 0.23) **(Figure 15B).**

5.3.3.3 NOR1

There were differences in expression of NOR1 between groups. Overall, myotubes from women with PCOS had greater expression of NOR1 with no effect of treatment (Group effect, P = 0.001; treatment effect, P = 0.41; Interaction effect, P = 0.57) (Percentage difference ± SD, 95% CI, P value). Differences were observed in the following conditions: EPS alone (103 ± 59%, 95% CI, [56, 150], P = 0.001). EPS with TGF beta (5ng/ml) (89 ± 83 %, 95% CI, [22, 155], P = 0.004), and EPS with AMH (30ng/ml) (100 ± 110 %, 95% CI, [12, 188], P = 0.002) **(Figure 16A)**.

5.3.3.4 NUR77

There were no differences in the expression of NUR77 following EPS with and without treatments and no differences between groups (Group effect, P = 0.92; Treatment effect, P = 0.46; Interaction effect, P = 0.87) **(Figure 16B)**.

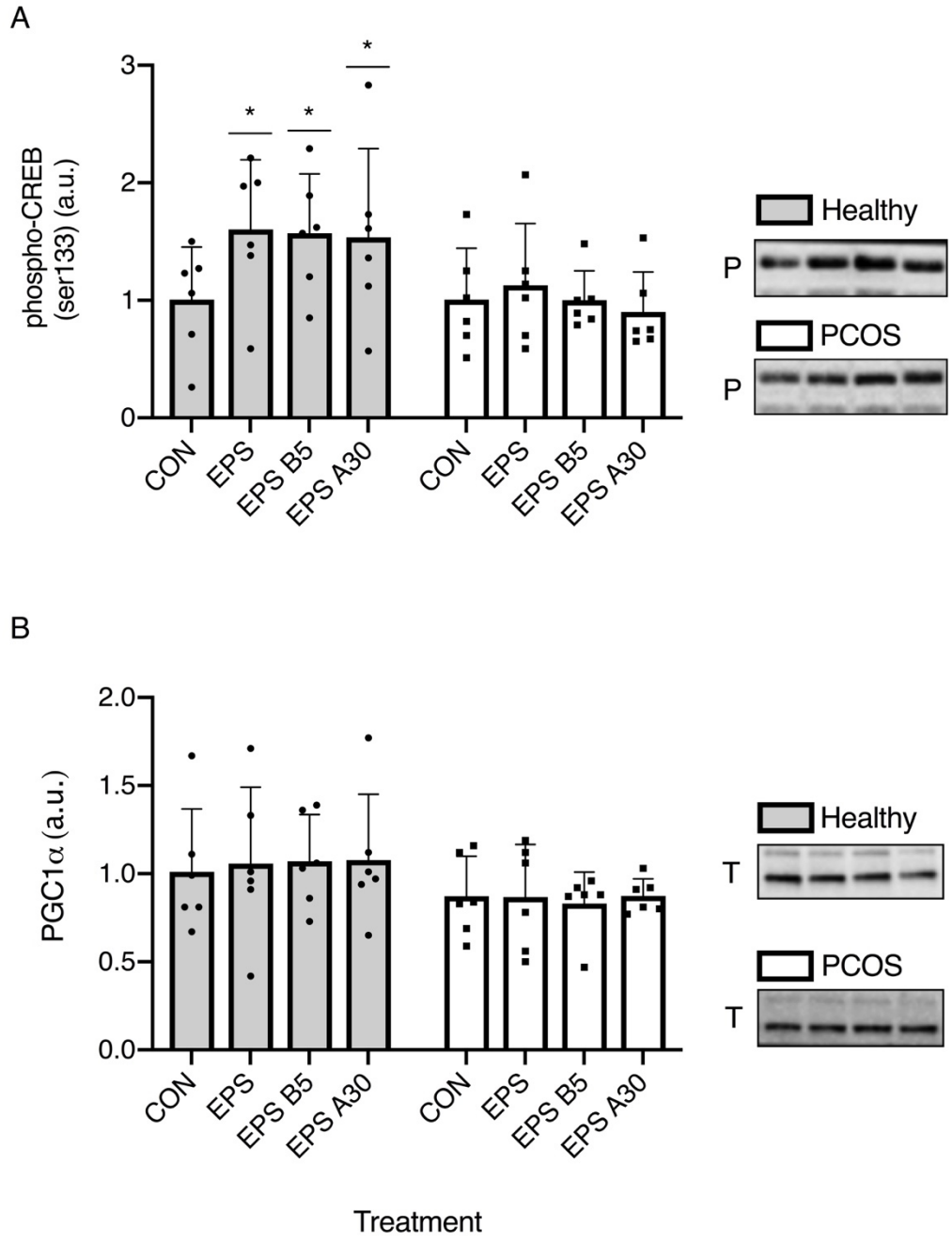

Figure 15. Transcription Factors: CREB, PGC1α

A) Phosphorylation of $CREB_{ser133}$. B) Total Expression of PGC1α. Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *Significantly different from basal control. Representative images shown for Phosphorylation (P) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

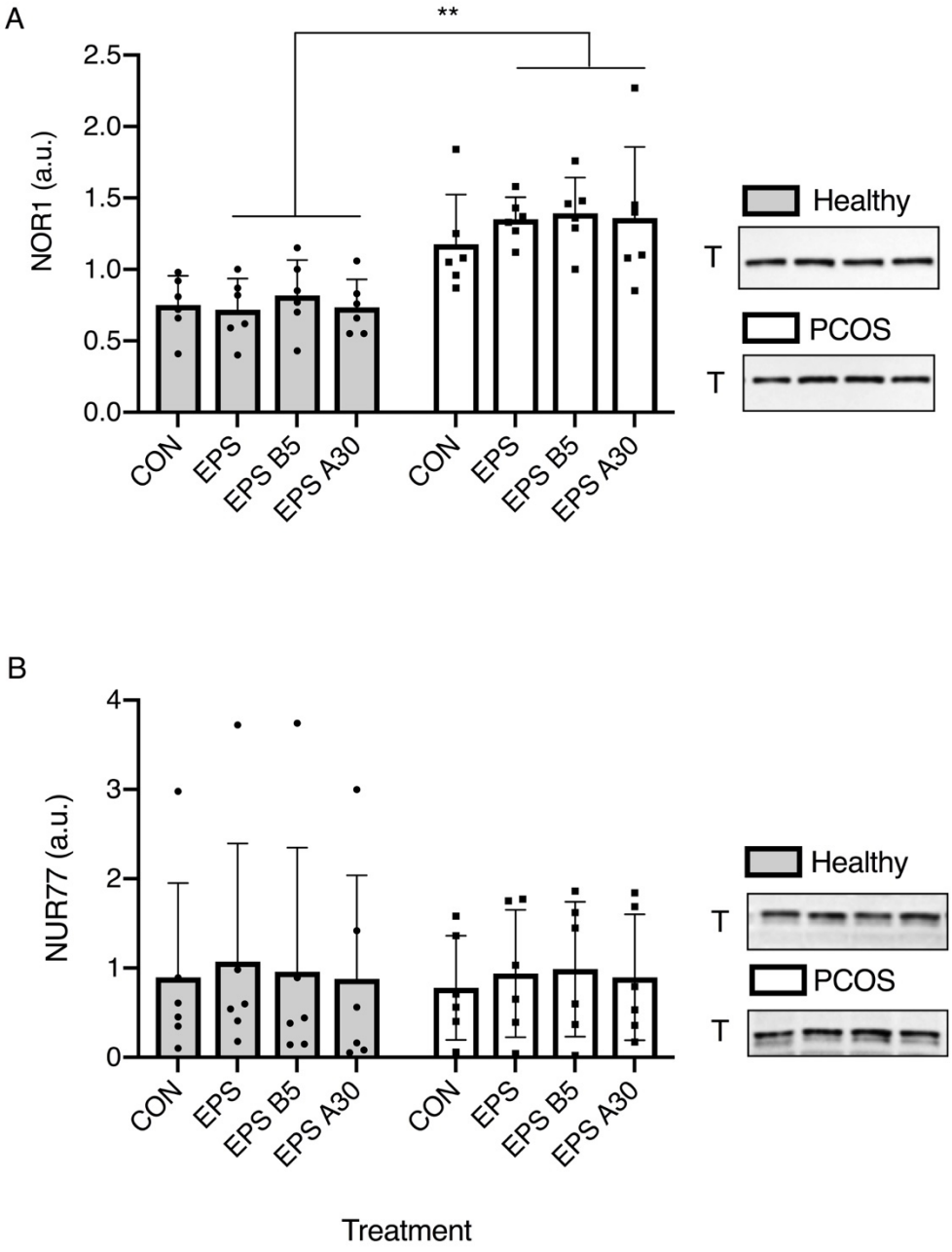

Figure 16. Transcription Factors: NOR1, NUR77

A) Total Expression of NOR1 B) Total Expression of NUR77. Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *significantly different from basal control. **Significant difference between groups Representative images shown for Total (T) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

5.3.4 Glucose homeostasis

5.3.4.1 AMPK

There were differences in the phosphorylation of AMPK between groups, with myotubes from healthy controls showing greater expression of AMPK across all conditions (Group effect, P = 0.04; Treatment effect, P = 0.69; Interaction effect, P = 0.95). Post-hoc analysis identified no differences (P > 0.06) **(Figure 17A)**.

5.3.4.2 ACC

There was appeared to be an increase in the phosphorylation of ACC following EPS and EPS with AMH (Group effect P = 0.35; Treatment effect, P = 0.04; Interaction effect, P = 0.92). Post-hoc analysis identified no differences (P > 0.15) **(Figure 17B)**.

5.3.4.3 GLUT4

There were no differences in the expression of GLUT4 following EPS with and without treatments and no differences between groups (Group effect, P = 0.28; treatment effect, P = 0.61; Interaction effect, P = 0.78) **(Figure 18A)**.

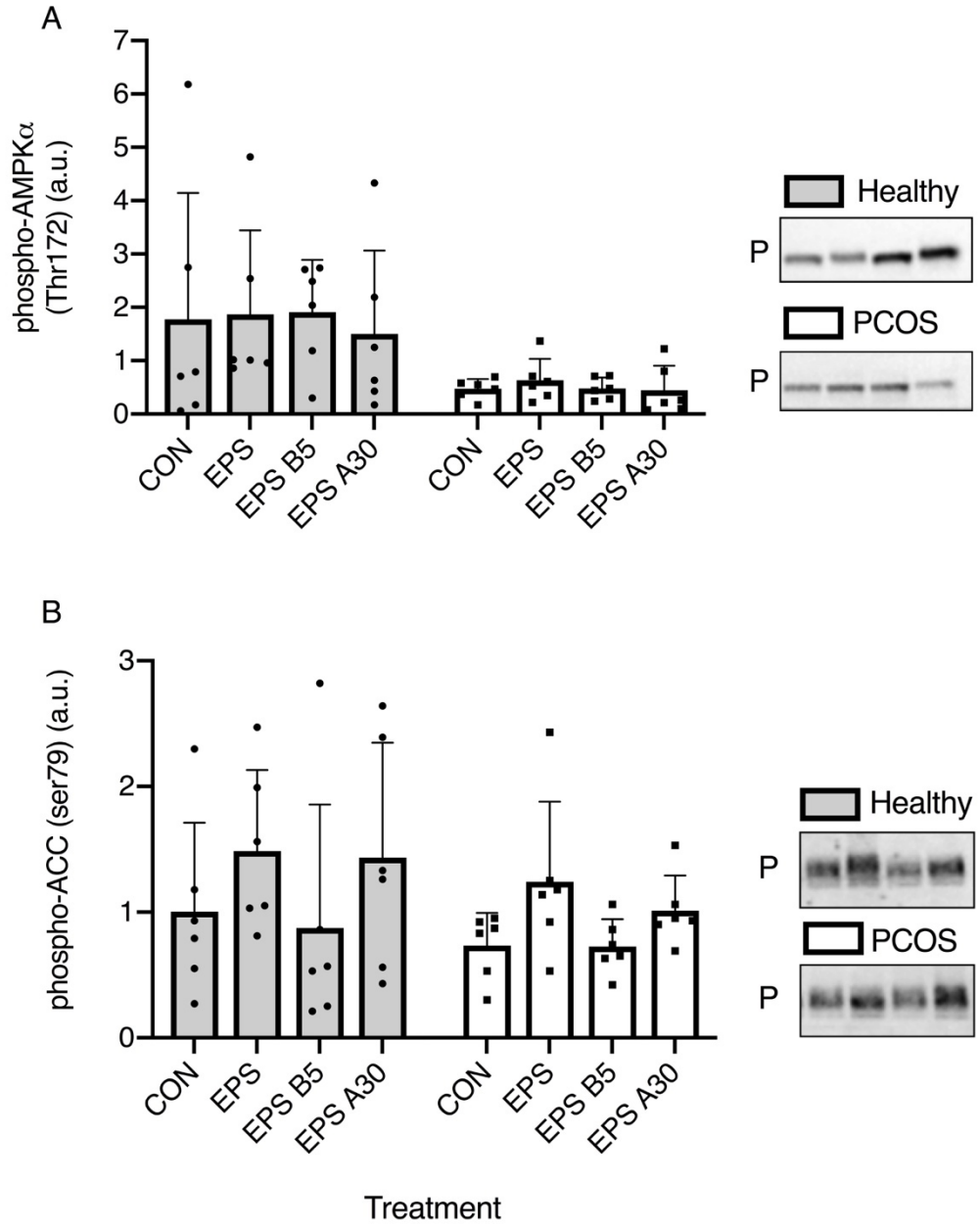

Figure 17. Glucose Homeostasis: p-ACC, p-AMPK

A) Phosphorylation of $AMPK_{Thr172}$ B) Phosphorylation of ACC_{ser79}. Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *Significantly different from basal control. **significant difference between groups Representative images shown for Phosphorylation (P) and Total (T) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

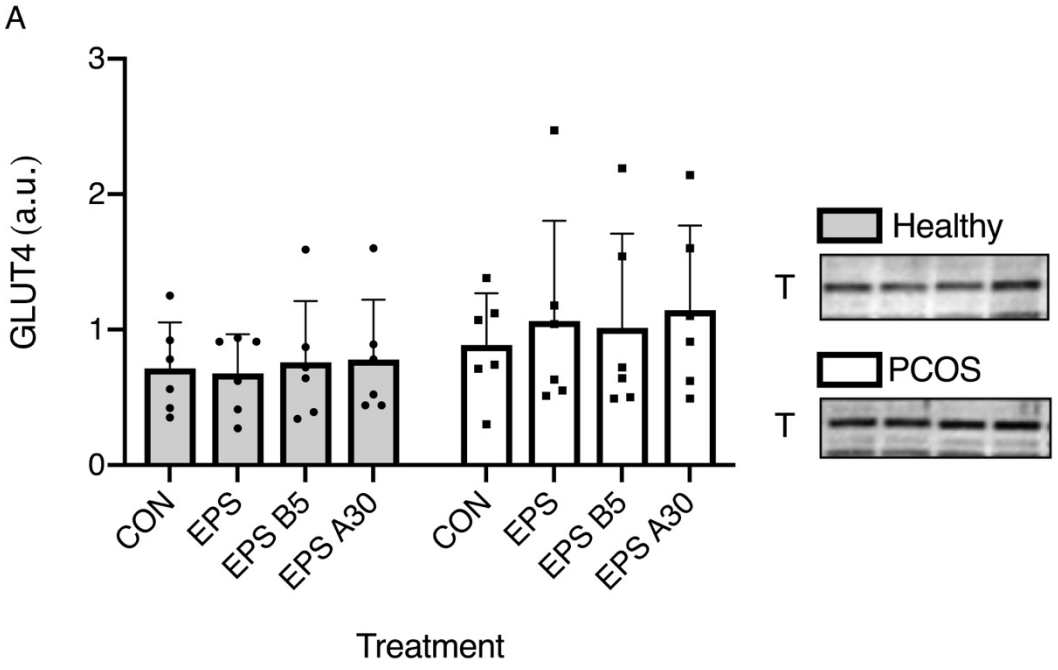

Figure 18. Glucose Homeostasis: GLUT4 expression

A) Total Expression of GLUT4. Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *Significantly different from basal control. **Significant difference between groups Representative images shown for Phosphorylation (P) and Total (T) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

5.3.5 Hypertrophy

5.3.5.1 phospho/total AKT

There were no differences in the phosphorylation of Akt following EPS with and without treatments and no differences between groups (Group effect, P = 0.48; treatment effect, P = 0.39; Interaction effect, P = 0.36) **(Figure 19A)**.

5.3.5.2 phospho/total MTOR

There were no differences in the phosphorylation of mTOR following EPS with and without treatments and no differences between groups (Group effect, P = 0.61; treatment effect, P = 0.16; Interaction effect, P = 0.98) **(Figure 19B)**.

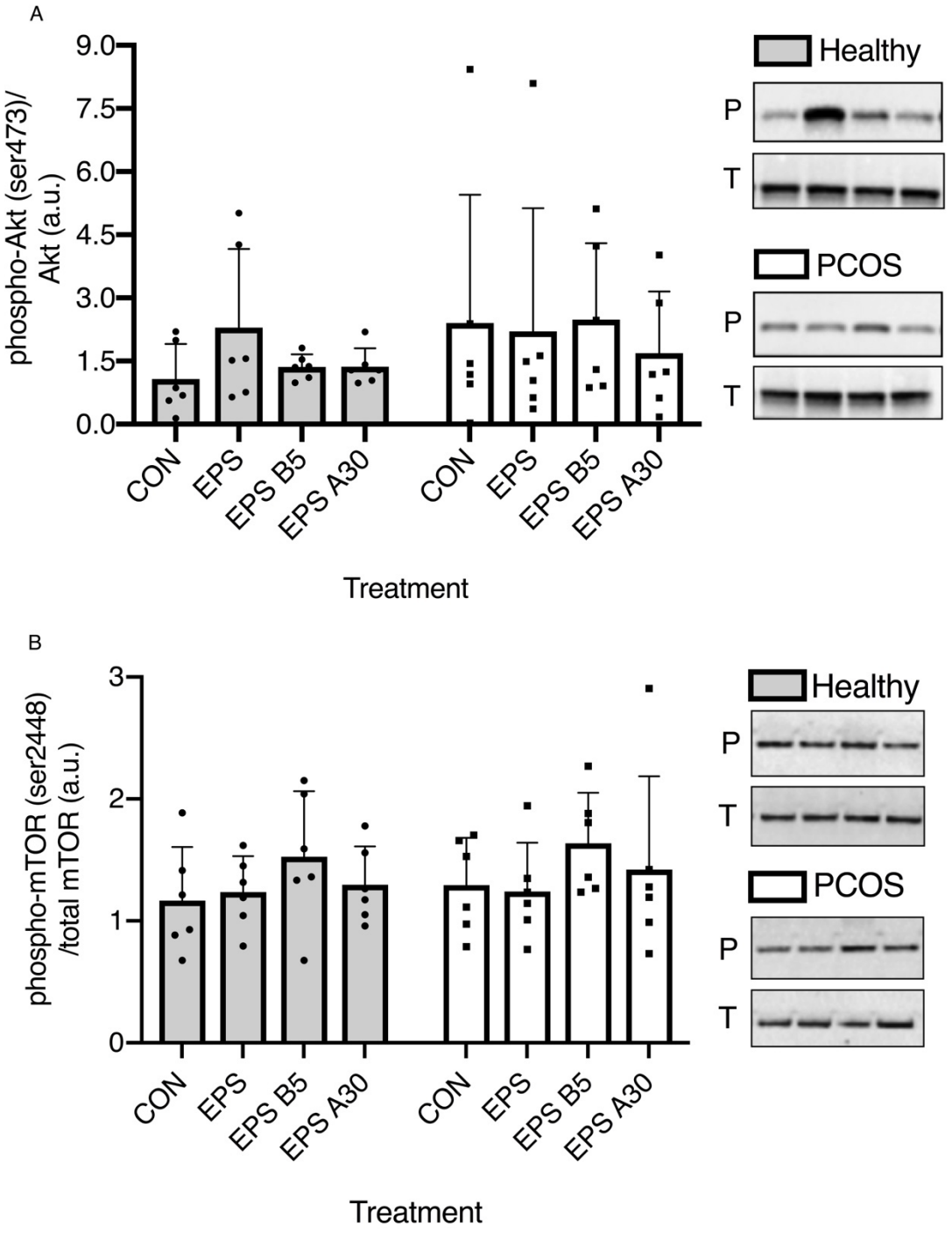

Figure 19. Hypertrophy

A) mTOR phosphorylation relative to total mTOR expression. B) AKT phosphorylation relative to total AKT expression. Control (C) as no treatment and following 6 hours of EPS (EPS) with TGF-beta 1 (5 ng/ml) (EPS B5) and AMH (30 ng/ml) (EPS A30. Data reported as Mean ± SD. *Significantly different from basal control. **Significant difference between groups Representative images shown for Phosphorylation (P) and Total (T) protein expression shown for (Healthy: n = 6, PCOS: n = 6).

5.4 Discussion

5.4.1 Do myotubes from women with PCOS display impaired exercise-induced signal transduction in response to EPS?

For this study, we used EPS as a model of *in vitro* exercise combined with exposure to the TGF-beta ligands, TGF-beta 1 and AMH, to understand if myotubes from women with PCOS display a similar pattern in exercise signal transduction to myotubes from healthy women. Furthermore, we wished to determine if TGF-beta ligands influenced these responses, which have been associated with the pathology of PCOS and linked to a reduction in the metabolic benefits of exercise. Our data provide further preliminary evidence of differences in skeletal muscle adaptive responses to exercise; however, whether these findings translate *in vivo* remains to be determined.

5.4.1.1 Stress kinase

. Exercise has been shown to cause an acute increase in the activation of the mitogen-activated protein kinases (MAPK) pathway in skeletal muscle (Bartlett et al., 2012; Coffey et al., 2006; Petersen et al., 2012). Interestingly, phosphorylation of this pathway via JNK, p38MAPK and NF-κB has been associated with greater post-exercise insulin sensitivity (Parker et al., 2016). Activation of the MAPK pathway has been linked to IL6 expression and GLUT4 translocation (Nedachi et al., 2008; Whitham et al., 2012). Indeed, the inhibition of p38 MAPK leads to a decrease in insulin-stimulated glucose uptake (Sweeney et al., 1999). In our study, we observed an increased phosphorylation of p38MAPK following EPS in myotubes from control women, but this effect appeared to be absent in the myotubes from women with PCOS. Although we have not assessed glucose uptake in these cells after EPS, nor expression of IL6 and GLUT4 translocation, it could be suggested that the lack of phosphorylation of P38MAPK in myotubes from women with PCOS may result in attenuation of the metabolic benefits of an exercise stimulus. However, others have shown that activation of p38MAPK during *in vitro* contraction of rat soleus muscle was not required to improve post-contraction insulin sensitivity (Geiger et al., 2005). The role of p38 MAPK in regulating post-exercise glucose uptake and insulin sensitivity is still under debate. While it would appear to play a part, further investigations are needed to elucidate its role and that of other signalling pathways that may be involved.

5.4.1.2 Transcription factors

We observed an increase in the activation of CREB following EPS in the control myotubes, while this effect appeared to be absent in the PCOS myotubes. CREB has been shown to act as a regulator of myogenic and mitochondrial gene expression, influencing structural and metabolic adaptions to exercise (Bruno et al., 2014; Catoire et al., 2012). Similarly, the phosphorylation of CREB has been observed following *in vivo* high-intensity exercise (Egan et al., 2010; Widegren et al., 1998) and in response to contractile activity (Bruno et al., 2014), supporting our *in vitro* data in myotubes from healthy individuals. CREB is capable of enhancing the transcription of PGC1α (Baldelli et al., 2014; Wu et al., 2006) with enhanced transcription likely leading to an increase in mitochondrial biogenesis following exercise (Baar et al., 2002). However, we observed no differences in the expression of PGC1α following EPS in both groups. This may be due to the sample time point, with cells being lysed immediately post EPS. In line with that, it has been shown that PGC1α mRNA and protein expression is at its highest at 3 hours after exercise (Granata et al., 2017; Little et al., 2011), which would explain our results. To date, only a few studies with EPS have demonstrated increases in PGC1α gene expression in C2C12 (Burch et al., 2010) and human primary myotubes (Hjorth et al., 2015; Lambernd et al., 2012), and only one study has shown protein changes in C2C12 myotubes (Burch et al., 2010).

While there was a difference in expression of NOR1 between groups, EPS with or without the addition of TGF-beta ligands had no effect on the expression of NOR1 and NUR77 in both groups. Nuclear orphan receptors NOR1/NR4A3 and NUR77/NR4A1 have been identified as important regulators of exercise-induced skeletal muscle adaptations (Pearen and Muscat, 2018). However, in human studies, exercise increases the gene but not the protein expression of NOR1 and NUR77. Additionally, the expression of skeletal muscle NOR1 and NUR77 have been shown to been insulin-responsive in healthy individuals but not in those with type 2 diabetes, with exercise training improving this response (Mey et al., 2019).

5.4.1.3 Glucose homeostasis

Muscle contractions require ATP and phosphocreatine (PCr), leading to a reduction in glycogen. This leads to the subsequent activation of AMPK. Previously, EPS has been shown to activate AMPK and acetyl-CoA carboxylase (ACC) in myotubes from healthy individuals and with metabolic diseases (Al-bayati et al., 2019; Audrey E Brown et al., 2015; Lambernd et al., 2012; Park et al., 2019). This response often occurs in conjunction with an increase in p38MAPK and ACC post-exercise *in vivo* (Bartlett et al., 2012; Combes et al., 2015; Gibala et al., 2009). In our study, despite seeing increases in p38 MAPK, there was no increase in $AMPK_{thr172}$ following EPS in both groups. It should be noted that myotubes from women with PCOS had a marked reduction in basal AMPK expression when compared with healthy control myotubes. This matches findings of Hansen et al. 2019, who showed lean women with PCOS have a 50% basal reduction in phosphorylation of $AMPK_{thr172}$ (Hansen et al., 2019). Another component of AMPK signalling is the phosphorylation of ACC, leading to an increase in fatty acid oxidation following acute exercise (Chen et al., 2000; Hoffman et al., 2015). Following EPS in our study, both groups displayed an increase in ACC phosphorylation observed by previous studies. Post-exercise GLUT4 expression has been shown to increase following acute exercise in some, but not all studies (Kraniou et al., 2006; Richter and Hargreaves, 2013) and in the case of EPS no published studies to date have observed increases in GLTU4 protein expression in human primary or C2C12 myotubes (Nikolić et al., 2017). We showed no changes in GLUT4 expression and no differences between groups following EPS. However, EPS has been shown to increase GLUT4 translocation independently of changes in GLUT4 protein expression (Park et al., 2019). Thus, future studies should assess GLUT4 translocation in EPS experiments with myotubes from women with PCOS.

5.4.1.4 Hypertrophy

Following EPS there were no changes in either group for the phosphorylation of Akt_{ser473} and $mTOR_{ser2448}$. Beyond its role in insulin signalling, the Akt/mTOR pathway is activated during hypertrophy, inhibiting this pathway leading to atrophy (Bodine et al., 2001). However, we did not assess downstream targets of mTOR pathway (p70s6K, 4EBP1, and eIF2α). The lack of hypertrophic signalling may be related to the EPS protocol used, we observed an increase in MYH7 and MYH2 gene expression (Unpublished data Moreno-Asso et al.) representative of

slow-twitch fibre activation/recruitment suggesting stimulus applied (11.5 V, 2 ms, 1Hz for 6 hours) is more akin to aerobic exercise. However, similar EPS protocol (12 V, 2 ms, 1Hz for 8 hours) is sufficient stimulus to trigger phosphorylation of mTOR and 4E-BP1, with a subsequent increase in myotube diameter 8 hours post EPS (Tarum et al., 2017). These differences may be equated to sex-specific responses, with the majority of their participants being male in comparison to our study, where all participants were females. Sex-specific differences in myotubes have been observed in DNA methylation and differentiation (Davegårdh et al., 2019) and in response to a hypertrophic stimulus such as testosterone (Salehzadeh et al., 2011). Therefore, the lack of hypertrophic signalling in our myotubes may be due to sex-specific response.

5.4.1.5 Do TGF-beta ligands influence the response to *in vitro* exercise in myotubes?

The effects of TGF-beta ligands AMH and TGF-beta 1 on EPS-induced contraction appears to be limited. In previous studies, it has been observed that low dose of TGF-beta 1 (2 ng/ml) can improve contractile function in 3D muscle constructs following electrical stimulation, via altered structure and contractile activity (Weist et al., 2013). However, we were not able to detect other effects of TGF-beta ligands on myotubes during EPS, indicating that TGF-beta ligands did not influence *in vitro* exercise-induced skeletal muscle signalling. This suggests that the effects of TGF-beta ligands observed *in vivo* may stem from different cell types such as fibroblasts or via interactions with environmental factors. Another potential explanation is that the effects of TGF-beta 1 may be more prominent post-exercise during the recovery phase rather than directly influencing contractile responses.

5.4.2 Limitations and Future studies

5.4.2.1 Other *in vitro* models to mimic exercise: 3D models and optogenetics

Since starting this study, there have been several advances in the *in vitro* models used to mimic *in vivo* exercise. Many are implementing the use of 3D tissue-engineered skeletal muscle from myotubes to create more physiologically representative *in vivo* skeletal muscle allowing for electrical and mechanical stimulation (Turner et al., 2019). This approach would help to

overcome some of the issues we faced in our study; when greater a voltage was applied and cells would detach, and also get a more homogenous stimulation of all myotubes. However, these models can be costly and require large numbers of cells, given that studies utilising biopsies have multiple analysis to conduct, and this may not be a feasible option. Another option utilising similar principles is human skeletal micro-muscles combined with optogenetics, which can be carried out using 96-well plates with elastomeric poles. This model demonstrated capabilities of force generation, hypertrophy and metabolic flux (Afshar et al., 2020; Mills et al., 2019). Future studies should consider utilising these new approaches.

5.4.2.2 Contractile properties of human primary myotubes in comparison with C2C12 myotubes

In addition, there has been an increase in the number of studies that have utilised EPS. One key finding is that C2C12 display greater increase in EPS-induced glucose uptake with a greater abundance of key contractile proteins than human primary myotubes (Abdelmoez et al., 2019). Similarly, when comparing human primary myotubes to intact human muscle fibres, it is noted that myotubes have a lack of expression in calcium handling proteins (RyR1, DHPR, SERCA1, SERCA2), which may account for the lack of visible contraction seen during EPS (Nikolić et al., 2017). This may be effective for manipulating the physiological environment to understand how TGF-beta ligands or other drug-targetable pathways influence exercise responses; however, it does not allow to study specific characteristics of the donor. Others have proposed the use of a co-culture model of human primary and C2C12 myotubes to improve contraction inducible responses, e.g. calcium channels activation and myokine secretion (Chen et al., 2019), however, this approach is not appropriated when studying human disorder-associated differences (condition-specific) in a human population.

5.4.2.3 Time course and physiological endpoints

A key limitation of our study is that cells were only harvested at one single time point, immediately post EPS. Others who have employed a time course have shown differential expression of proteins and genes from 60-180mins post EPS (Park et al., 2019; Pattamaprapanont et al., 2016). Having a time course may have revealed additional changes that could occur during the post-exercise recovery phase. This study was part of a series of

experiments, and due to constraints in resources, we were unable to assess metabolic responses such as glucose uptake, glycogen synthesis and fatty acid oxidation, which would have informed better about physiological changes. Future studies may wish to assess these outcomes to see if the differences observed in exercise-induced signalling resulted in differences in metabolic parameters and consider a sample collection within a time course to capture all EPS-induced changes.

5.4.2.4 The effects of obesity on contractile function

A potential confounder in our results is the lack of a BMI-matched control group, therefore, our findings should be interpreted with caution. The BMI differences between our groups (PCOS: 38.4 ± 5.5 vs Healthy: 22.3 ± 3.0 kg/m^2) may in part explain the differences observed. It is known that obesity negatively affects skeletal muscle contractile function *in vivo,* due to inflammation, lipid accumulation, altered fibre type distribution, structural remodelling and reduced expression of contractile proteins (Straight et al., 2020; Tallis et al., 2018). It has been demonstrated that myotubes co-cultured with obese visceral adipose tissue display a reduction in the expression of myogenic and contractile proteins, leading to atrophic effects (Pellegrinelli et al., 2015). However, in the absence of the visceral adipose tissue, the obese myotubes did not display this response. In the context of EPS, myotubes from obese donors show blunted contraction-induced responses in insulin signalling and glucose metabolism in comparison to healthy myotubes (Al-bayati et al., 2019; Feng et al., 2015; Park et al., 2019). Future studies investigating the effects of PCOS on contractile responses should include BMI-matched control women and lean women with PCOS. This approach would be more comprehensive and would allow investigators to account for the effects of obesity.

5.4.3 Conclusions

Collectively, we observed limited differences in response to EPS between myotubes from women with PCOS and healthy controls. The differences we did observe may point towards reduced responsiveness to EPS. At the same time, the effect of TGF-beta ligands on contractile responses was minimal. Follow-up work should focus on replicating these findings with additional target pathways, including an assessment of metabolic function via glucose uptake,

glycogen synthesis and fatty acid oxidation, and assessing temporal responses and utilising new advances in technology such as 3D cultures. These approaches would allow for a better understanding of the physiological implications of altered exercise-induced signalling transduction.

5.4.3.1 Key findings

- Myotubes from women with PCOS and healthy controls display minimal differences in exercise-induced signalling transduction.
- TGF-beta ligands, AMH and TGF-beta 1, do not appear to influence the signalling responses in myotubes to the *in vitro* contraction by EPS.
- Myotubes from healthy women had an increase in the EPS-induced phosphorylation of p38MAPK and CREB.

6 **STUDY 3:** Cross-sectional Analysis of Insulin Sensitivity, Insulin Signalling and TGF-beta Signalling in Women with and Without PCOS

6.1 Introduction

It has long been known that women with PCOS, both lean and overweight, have significant peripheral insulin resistance compared to matched controls (Dunaif et al., 1989). A possible explanation was first identified with excessive serine phosphorylation of the insulin receptor in skeletal muscle of women with PCOS (Dunaif et al., 1995). Follow-up work demonstrated a reduction in IRS-1-associated PI3K activity during the initial response to insulin stimulation (Dunaif et al., 2001), although this defect was only apparent in the initial 30 minutes of the euglycemic-hyperinsulinemic clamp and was absent at 90 minutes. Dunaif and colleagues' seminal works have served as the catalyst for further investigations, utilising euglycemic-hyperinsulinemic clamps combined with muscle biopsies. Defects in skeletal muscle insulin signalling observed in insulin-resistant women with PCOS have included; IRS-1, ERK1/2 (Corbould et al., 2006; Rajkhowa et al., 2009), Akt and AS160 (Hojlund et al., 2008), atypical protein kinase C (Beeson et al., 2004), glycogen synthase kinase (Glintborg et al., 2008), and mTOR (N. Stepto et al., 2020), all of which could potentially influence GLUT4 translocation and glucose uptake. However, other studies have not been able to identify defects in skeletal muscle insulin signalling during euglycemic-hyperinsulinemic clamps despite the given population of overweight and lean women with PCOS having severe insulin resistance (Ciaraldi et al., 2009; Hansen et al., 2019). Although there are many subsequent studies exploring the molecular mechanisms of insulin resistance in women with PCOS, there is currently a lack of consensus regarding specific defects in insulin signalling and the underlying mechanisms responsible for peripheral insulin resistance in women with PCOS.

A potential mechanism contributing to the development of peripheral insulin resistance may be ECM remodelling and aberrant TGF-beta signalling. It has been observed that individuals who are insulin resistant have increased skeletal muscle collagen I and III, accompanied by a reduction in PI3K-p85 expression and IRS-1 tyrosine phosphorylation (Berria et al., 2006). Additional human studies show that rapid weight gain or lipid infusion is capable of inducing

upregulation of ECM related genes (Richardson et al., 2005; Tam et al., 2014). This excess collagen and stroma deposition may act as a physical barrier for glucose transport and insulin signalling.

Active TGF-beta 1 binds to TGF-beta co-receptors I and II then phosphorylates SMAD 2 and SMAD 3 and forms a complex with SMAD4, which is translocated to the nucleus and upregulates ECM related genes (Derynck and Budi, 2019; Hu et al., 2018; K. K. Kim et al., 2018). This promotes the production of collagen and fibronectin, which are important for tissue repair, although excessive activation of TGF-beta signalling leads to fibrosis (Walton et al., 2017). ECM remodelling in women with PCOS has been shown to occur in the ovaries via TGF-beta signalling and is attributed to reproductive dysfunction (Hatzirodos et al., 2011; Raja-Khan et al., 2014). Similarly, women with PCOS are predisposed to hepatic steatosis and non-alcoholic fatty liver disease (Petta et al., 2017; Rocha et al., 2017). Preliminary evidence from our group shows an increase in tissue density and profibrotic gene expression profile in the skeletal muscle of women with PCOS (Hutchison et al., 2012; N. Stepto et al., 2020). This presents the possibility that PCOS is a profibrotic condition, and that effects observed in ovaries may also extend to other peripheral tissues, such as skeletal muscle and adipose tissue, and impact metabolic function. In line with that, circulating TGF-beta1 levels have been related to adiposity in humans and have been shown to regulate glucose homeostasis in animal models (Alessi et al., 2000; Yadav et al., 2011). Also, TGF-beta ligands and activation of SMAD signalling have been suggested to interact with Akt and mTOR signalling influencing muscle mass and metabolism (Conery et al., 2004; Remy et al., 2004; Trendelenburg et al., 2009; Winbanks et al., 2012), indicating a link between TGF-beta signalling and skeletal muscle metabolic function. However, the role of TGF-beta signalling regulating insulin resistance through the extracellular matrix remodelling in skeletal muscle in PCOS and its contribution to the development of metabolic dysfunction has yet to be established.

6.1.1 Aims and hypothesis

We aimed to assess the peripheral insulin sensitivity and identify insulin signalling defects that have previously been observed in the skeletal muscle of women with PCOS. It was hypothesized that women with PCOS would display a reduction in insulin sensitivity in comparison to healthy women who were lean or overweight. Furthermore, we also

hypothesized that the reduction in insulin sensitivity would be accompanied by an increase in the phosphorylation of IRS-1_{ser312}, a negative regulator of insulin signalling and a decrease in the phosphorylation of AKT and AS160, resulting in impaired glucose transport. Additionally, we aimed to determine if TGF-beta signalling is dysregulated and contributes to the development of insulin resistance in women with PCOS. It was hypothesized that the skeletal muscle of women with PCOS would have a higher expression of collagens, which may create a physical barrier to insulin, and/or overexpression of TGF-beta 1 that may act as an inhibitor of insulin signalling.

This study used a cross-sectional study design to compare peripheral insulin sensitivity, skeletal muscle signalling, and other clinical parameters between overweight women with and without PCOS and lean healthy women.

6.2 Methods

6.2.1 Participants

A total of 45 women took part in this study: 29 overweight women with PCOS (OWPCOS), 6 overweight healthy women (OW con) and 10 lean, healthy women (Lean Con) (See Methodology section 3.1).

6.2.2 Metabolic and body composition assessment

See Methodology section 3.2

6.2.3 Muscle Biopsy

Muscle biopsies were obtained before the euglycemic-hyperinsulinemic clamps and during the steady-state phase of the clamp, which can be defined as the final 30 minutes (See Methodology section 3.3)

6.2.4 Bloods and pathology analysis

See Methodology section 3.6

6.2.5 Western blot analysis

Targets assessed in this study were: TGF-beta 1, TGF-beta 2, TGF-beta receptor 1, Collagen IV, I and III, phospho SMAD3, phospho AKT_{ser473}, AKT 2, phospho $mTOR_{ser2448}$, PI3K P85, PI3K P110, $AS160_{Thr642}$, GLUT4, Phospho ACC_{ser79}, Phospho $AMPK\alpha_{Thr172}$, Phospho $ERK1/2_{Thr202/Tyr204}$, phospho $P38MAPK_{Thr180/Tyr182}$, Phospho $FOXO1_{ser256}$ (See Methodology section 3.12)

6.2.6 Statistical analysis

Clinical characteristics and basal expression of TGF-beta related proteins were analysed using a one-way ANOVA. In cases where clinical characteristics were only available for two groups, unpaired samples t-test was used to detect differences. Pearson correlation coefficient was used to examine the relationship between TGF-beta signalling and glucose-infusion rate. Basal to insulin-stimulated changes in protein expression and phosphorylation were assessed using a mixed-effects model (3 groups and 2-time points). Significant effects of interaction (group x time), Time (Basal vs insulin-stimulated) and group (Lean Con vs OW Con vs OW PCOS). When significant effects were observed, Sidak posthoc test was used to identify differences. The distribution of the data was tested using the Shapiro-Wilk test. Skewed data were log-transformed and using log2. Data in text for insulin sensitivity and cardiorespiratory fitness were reported as follows: mean difference ± SD, 95% CI [lower, upper], P-value. Data in text for basal expression of TGF-beta related proteins is presented as mean difference percentage ± SD 95% CI [lower, upper], P value. Data in text for insulin signalling proteins are presented as percentage of fold-change from basal ± SD, 95% CI [lower, upper], P-value. (Additional details in Methodology section 3.13).

6.3 Results

6.3.1 Clinical Characteristics

Overall clinical profiles between groups outlined in **Table 6.**

Table 6. Clinical characteristics of women with PCOS, overweight controls and lean healthy controls.

	Lean Controls	Overweight Controls	Overweight PCOS	P-value OW con VS Lean con	P-value OW PCOS VS Lean con	P-value OW PCOS VS OWcon
General						
No. of participants	10	6	29			
Age (years)	26 ± 6	30 ± 7	32 ± 6			
Height (cm)	170 ± 1	160 ± 1	164 ± 1			
Phenotype						
A	-	-	9			
B	-	-	4			
C	-	-	4			
D	-	-	8			
Body composition						
Weight (kg)	66.4 ± 12.3	79.7 ± 13.3	99.1 ± 23.4	0.49	<0.001	0.11
BMI (kg m-2)	22.9 ± 3.0	31.3 ± 3.2	36.8 ± 8.2	0.02	<0.001	0.23
Lean mass (%)	67 ± 4	52 ± 3	49 ± 6	<0.001	<0.001	0.58
Fat mass (%)	30 ± 5	46 ± 3	47 ± 6	<0.001	<0.001	0.98
Glucose Homeostasis						
Fasting glucose (mM)	4.4 ± 0.4	4.4 ± 0.4	4.9 ± 0.5	0.99	0.01	0.04
Fasting insulin (uIU/mL)	9.2 ± 2.5	-	17.8 ± 6.1	n/a	0.006	n/a
HOMA-IR	1.8 ± 0.6	-	3.8 ± 1.7	n/a	0.001	n/a
HbA1c (%)	5.1 ± 0.2	5.1 ± 0.2	5.2 ± 0.2	ns	ns	ns
Insulin Sensitivity						
GIR (mg/lbmkg/min)	15.5 ± 4.7	4.8 ± 1.0	7.1 ± 3.1	<0.001	<0.001	0.37
Hormonal status						
Testosterone total (nmol/L)	1.0 ± 0.4	1.15 ± 0.6	1.6 ± 0.6	0.98	0.05	0.26
Testosterone free (pool/L)	13.3 ± 5.3	19.05 ± 11.9	34.6 ± 15.6	0.79	<0.001	0.04
SHBG (nmol/L)	65.5 ± 30.5	50.81 ± 26.6	32.2 ± 18.0	0.52	0.001	0.22
Dihydrotestosterone (nmol/L)	0.4 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.25	0.10	0.001
Androstenedione (nmol/L)	3.5 ± 1.0	3.3 ± 1.5	4.8 ± 1.4	0.97	0.04	0.04
Estradiol (pmol/L)	216.5 ± 208.5	277.5 ± 258.9	261.5 ± 225.6	ns	ns	ns
AMH (pmol/L)	88.1 ± 40.5	-	57.1 ± 25.3	n/a	0.01	n/a
Lipids						
Cholesterol (mmol/L)	4.1 ± 0.7	4.4 ± 1.0	4.8 ± 0.8	0.85	0.04	0.50
Triglycerides (mmol/L)	0.7 ± 0.1	1.2 ± 0.4	1.3 ± 0.6	0.15	0.007	0.96
HDL (mmol/L)	1.6 ± 0.3	1.3 ± 0.2	1.4 ± 0.3	ns	ns	ns
LDL (mmol/L)	2.1 ± 0.6	2.5 ± 0.9	2.9 ± 0.8	0.64	0.02	0.66
LDL:HDL ratio	1.4 ± 0.5	1.9 ± 0.6	2.2 ± 0.7	0.26	0.006	0.81
Liver function						
AST (IU/L)	20.8 ± 5.3	19.0 ± 5.2	24.8 ± 11.5	ns	ns	ns
ALT (IU/L)	18.9 ± 10.6	18.3 ± 7.8	35.0 ± 26.1	ns	ns	ns

Data reported as mean and SD. AST: aspartate aminotransferase ALT: alanine aminotransferase. HOMA-IR: fasting insulin (mU/L)*fasting plasma glucose (mmol/L)/22.5.Between group analysis performed using one-way ANOVA. ns: not significant as determined by one-way ANOVA. n/a; not measured. ns; not significant.

6.3.1.1 Body Composition

Overweight women with PCOS display greater body weight, BMI, fat mass and a lower lean mass compared to lean controls. The body composition profile was similar between overweight women with PCOS and overweight controls **(Table 6)**.

6.3.1.2 Glucose homeostasis

Overweight women with PCOS have higher levels of fasting blood glucose, insulin, and HOMA-IR than lean and overweight controls. There were no differences in HBA1c between groups **(Table 6)**.

6.3.1.3 Hormonal status

Overweight women with PCOS have higher levels of free and total testosterone, androstenedione, and lower levels of serum AMH and SHBG, compared to lean women without PCOS. Compared to overweight controls, women with PCOS have higher levels of free testosterone and androstenedione, with lower levels of dihydrotestosterone. There were no differences in estradiol between groups **(Table 6)**.

6.3.1.4 Lipids

Overweight women with PCOS have higher levels of cholesterol, triglycerides, LDL and LDL: HDL ratio compared to lean controls. The lipid profile was similar between overweight women with PCOS and overweight controls **(Table 6)**.

6.3.1.5 Liver Function

No apparent differences in liver function as measured by aspartate aminotransferase and alanine aminotransferase were observed between groups **(Table 6)**.

6.3.2 Insulin sensitivity

Significant differences between groups in insulin sensitivity as determined by the euglycemic–hyperinsulinemic clamp, calculated as per Glucose infusion rate (GIR) ($P < 0.001$). Lean controls were more insulin sensitive than both overweight controls (mean difference GIR ± SD: 10.66 ± 3.60, 95% CI [6.29,15.02] $P < 0.001$) and overweight women with PCOS (mean difference GIR ± SD: 8.36 ± 1.57, 95% CI [5.20,11.52], $P < 0.001$). There were no significant differences in insulin resistance between overweight controls and overweight women with PCOS ($P = 0.37$). Women with PCOS were 94% less insulin sensitive than lean, healthy control women **(Table 6)**.

6.3.3 TGF-Beta signalling

6.3.3.1 TGF-beta 1 and TGF-beta 2

There were no detectable differences between groups for the expression of TGF-beta 1 ($P = 0.10$) **(Figure 20A).** Overall, there were significant differences between groups for the expression of TGF-beta 2 ($P = 0.02$). Lean controls had greater expression of TGF-beta 2 than overweight women with PCOS (mean difference percentage ± SD: 28 ± 27, 95% CI [2,51], $P < 0.0001$) **(Figure 20B)**.

6.3.3.2 TGF-beta Receptor 1 and TGF-beta Receptor 2

Overall, there were significant differences between groups for the expression of TGF-beta receptor 1 ($P < 0.001$). Overweight controls had greater expression of TGF-beta receptor 1 than lean controls (mean difference percentage ± SD: 47 ± 22, 95% CI [4,56], $P = 0.02$) and overweight women with PCOS (mean difference percentage ± SD: 53 ± 24, 95% CI [42,125], $P < 0.001$) **(Figure 20C).** The expression of TGF-beta receptor 2 could not be detected.

6.3.3.3 Collagen IV, I and III

There were no detectable differences between groups for the expression of Collagen IV, I or III ($P > 0.50$) **(Figure 20D,20E,20F).**

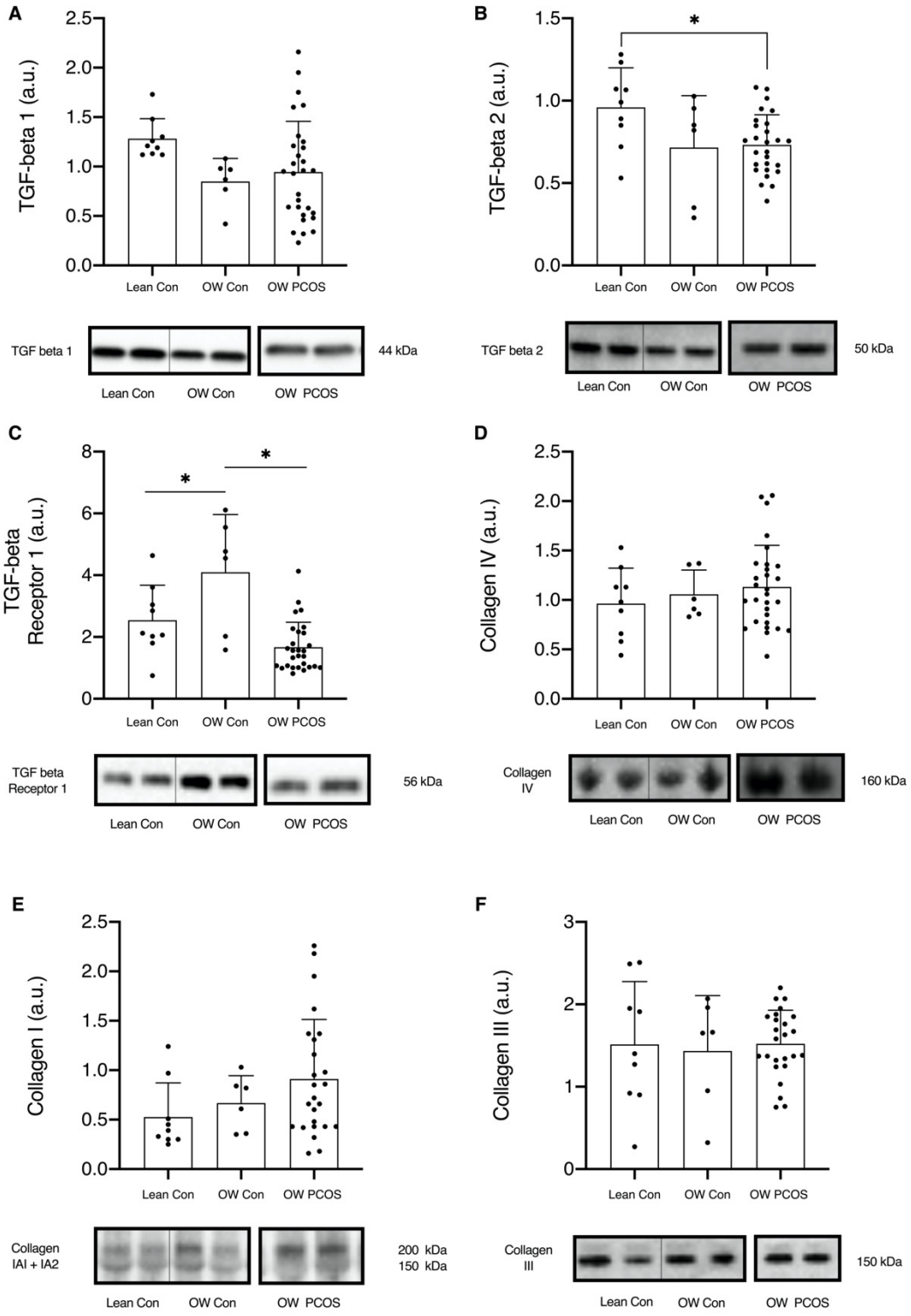

Figure 20. Basal expression of TGF-beta ligands, receptors and collagen IV, I and III.

TGF-beta 1 expression B) TGF-beta 2 expression C) TGF-beta receptor 1 D) Collagen IV E) Collagen I F) Collagen III. Representative blots for protein expression of TGF-beta signalling and collagen IV from muscle samples obtained under resting conditions for 2 participants per group. *signifies statistically significant difference between groups. Data presented as mean ± SD. (Lean con: n = 9, OW con: n = 6, OW PCOS: n = 25). Data analysed using one-way ANOVA and sidak posthoc.

6.3.3.4 SMAD3

The phosphorylation of SMAD 3 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.07; Time effect, P = 0.85; Interaction effect, P = 0.62). **(Figure 21)**.

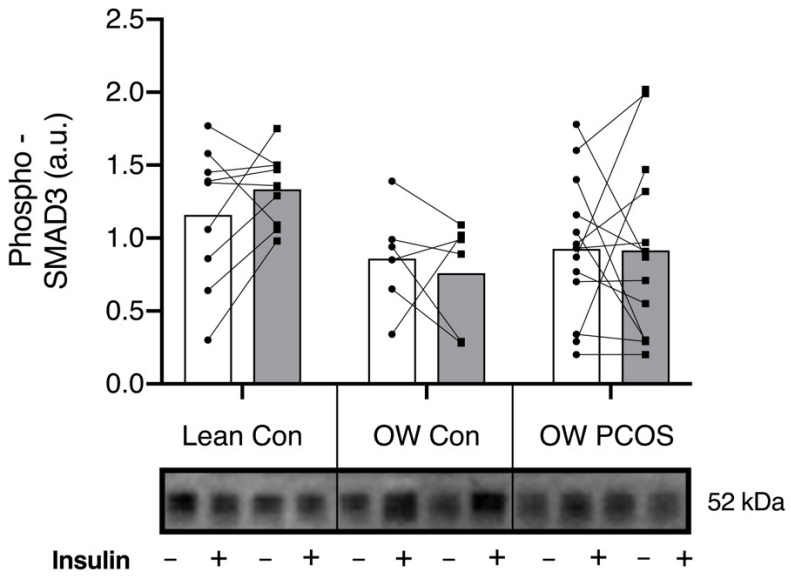

Figure 21. SMAD3 phosphorylation at baseline and during insulin stimulation

Data presented mean ± SD. (Lean con: n = 9, OW con: n = 6, OW PCOS: n = 13). Data analysed using mixed-effects modelling and sidak post-hoc.

6.3.3.5 Relationship between TGF-beta signalling, clinical measures and insulin sensitivity

There was no relationship between skeletal muscle TGF-beta signalling and insulin sensitivity (Figure 26.). Serum free testosterone had a significant and negative association with insulin sensitivity (P = 0.001). Skeletal muscle TGBR2 expression had a significant negative association with serum total testosterone (P = 0.01), free testosterone (P = 0.01), and androsterone (P = 0.004). All other associations observed were related to normal/typical physiological responses.

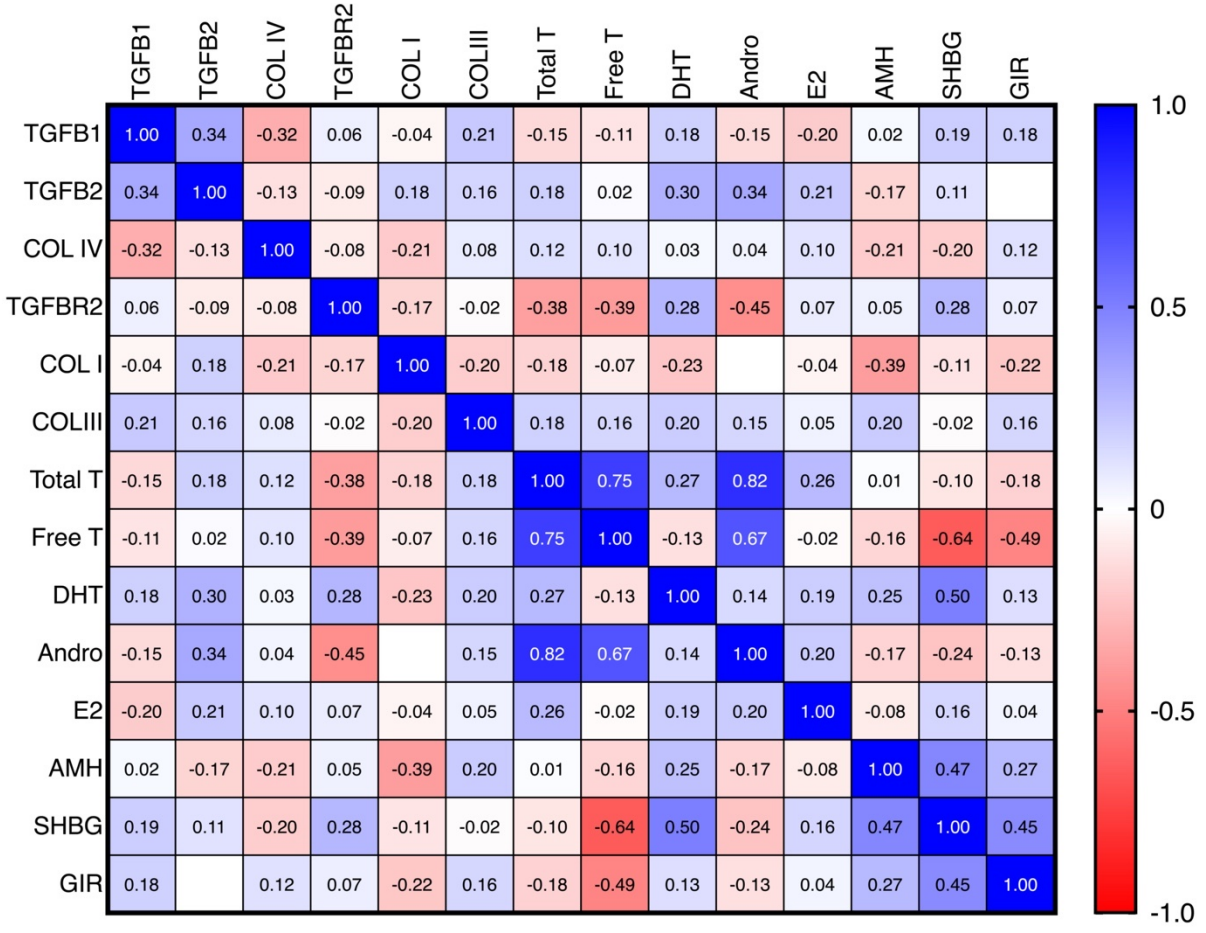

Figure 22. Correlation matrix for skeletal muscle TGF-beta Ligands, clinical measures and insulin sensitivity.

Pearson correlation coefficient was used to determine the relationship between variables (n = 40, all groups combined). Correlational matrix showing Pearson correlation coefficient r values. The correlation matrix heat maps demonstrate significant positive (dark blue) and negative (dark red) relationships between variables.

6.3.4 Insulin Signalling

6.3.4.1 Insulin receptor substrate 1

The phosphorylation of IRS-1_{ser312} was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.54; Time effect, P = 0.24; Interaction effect, P = 0.71) **(Figure 23A)**.

6.3.4.2 AKT

The phosphorylation of Akt_{ser473} was different between groups at baseline and during insulin stimulation (Group effect, P = 0.69; time effect, P < 0.001; Interaction effect, P = 0.24). There was a significant increase in phosphorylation of Akt_{ser473} following insulin stimulation in the lean controls (relative change± SD: 543 ± 346%, 95% CI[317,769], P = 0.003) and OW PCOS(relative change ± SD: 389 ± 382%, 95% CI[181,596], P < 0.001), but not in the OW Con group (relative change ± SD: 129 ± 186%, 95% CI[-20,278], P = 0.70) **(Figure 23B)**. The expression of AKT2 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.19; Time effect, P = 0.12; Interaction effect, P = 0.39) **(Figure 23C)**.

6.3.4.3 MTOR

The phosphorylation of mTOR was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.51; Time effect, P = 0.26; Interaction effect, P = 0.60) **(Figure 23D.)**.

6.3.4.4 PI3K

The expression of PI3K p85 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.76; Time effect, P = 0.80; Interaction effect, P = 0.04). There was a significant interaction effect but post-hoc analysis did not identify any differences (P > 0.20) **(Figure 23E)** The expression of PI3K p110 was different between basal and insulin stimulation (Group effect, P = 0.80; Time effect, P = 0.006; Interaction effect, P = 0.11) There

was a significant decrease in the expression of PI3K p110 following insulin stimulation in the lean control group only (relative change ± SD: -27 ± 58%, 95% CI[-65,11],P = 0.01) **(Figure 23F)**.

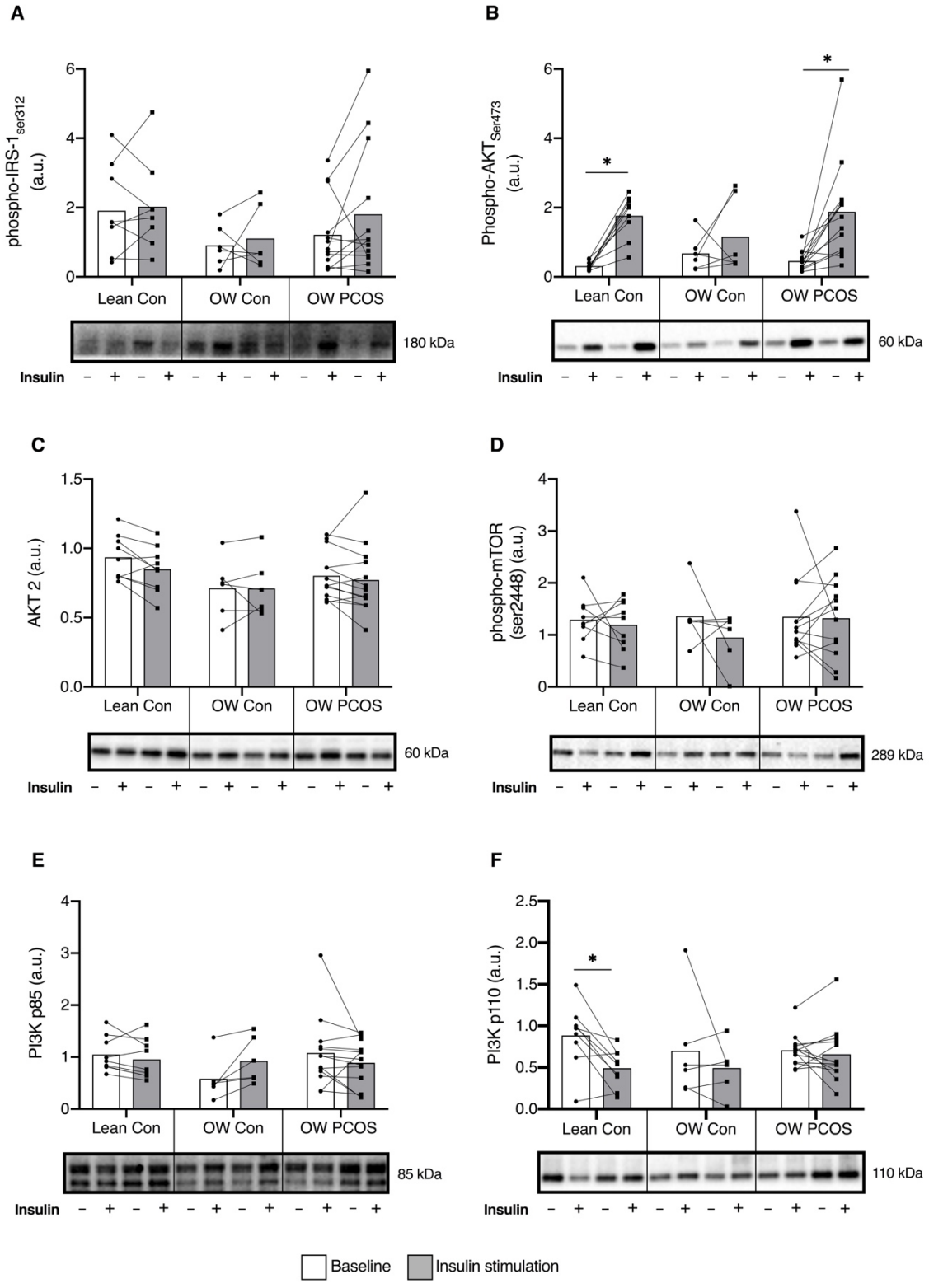

Figure 23. Insulin signalling proteins at baseline and during insulin-stimulation.

A) phosphorylation of IRS-1ser312 B) phosphorylation of AKTser473 C) Expression of AKT2 D) phosphorylation of mTORser2448 E) Expression of PI3K p85 F) Expression of PI3K p110. Data presented mean ± SD. *represents significant differences between basal and insulin stimulation. (Lean con: n = 9, OW con: n = 6, OW PCOS: n = 13). Data analysed using mixed-effects modelling and sidak posthoc test.

6.3.4.5 AS160

The phosphorylation of AS160/TBC1D4 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.09; Time effect, P = 0.06; Interaction effect, P = 0.99) **(Figure 24A)**.

6.3.4.6 GLUT4

The expression of GLUT4 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.99; Time effect, P = 0.53; Interaction effect, P = 0.58) **(Figure 24B)**.

6.3.4.7 AMPK signalling

The phosphorylation of ACC_{ser79} was decreased follow insulin stimulation across all groups (Group effect, P = 0.20; Time effect, P = 0.01; Interaction effect, P = 0.81). Post-hoc analysis identified no differences (P > 0.26) **(Figure 24C)**. The phosphorylation of $AMPKalpha_{Thr172}$ was different between groups at baseline and during insulin stimulation (Group effect, P = 0.01; Time effect, P = 0.58; Interaction effect, P = 0.13). Overweight controls had a higher basal expression compared to lean controls (mean difference ± SD (a.u.): 1.35 ± 0.44, 95%CI [1.09,1.61], P = 0.004) and overweight women with PCOS (mean difference ± SD (a.u.): 1.32 ± 0.38, 95%CI [1.08,1.57], P = 0.004) **(Figure 24D)**.

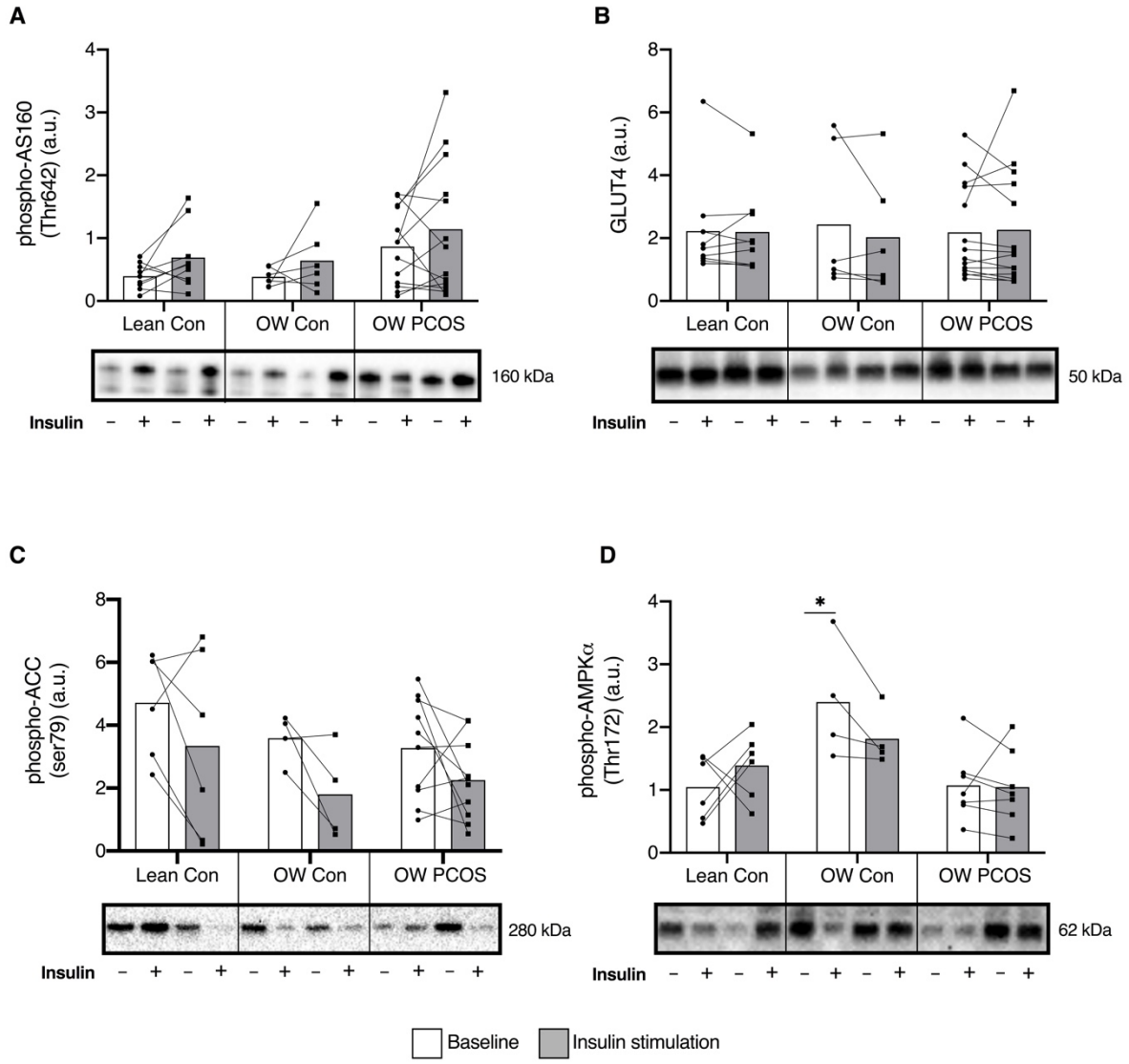

Figure 24. GLUT4 and AMPK Signalling at baseline and during insulin stimulation.

A) Phosphorylation of AS160/TBC1D4 Thr642 B) GLUT4 Expression C) Phosphorylation of ACC ser79 D) Phosphorylation of AMPKalpha Thr172. Data presented mean ± SD. *represents significant differences between groups under basal conditions. For AS160, Acc, and GLUT4: (Lean con: n = 9, OW con: n = 6, OW PCOS: n = 13) For AMPK (Lean con: n = 6, OW con: n = 4, OW PCOS: n = 7). Data analysed using mixed effects modelling and sidak post-hoc.

6.3.4.8 Stress kinases

The phosphorylation of ERK1/2 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.41; Time effect, P = 0.10; Interaction effect, P = 0.25) (Figure 22A.) The phosphorylation of p38 MAPK was different between groups at baseline and during insulin stimulation (Group effect, P = 0.01; Time effect, P = 0.59; Interaction effect, P = 0.13) **(Figure 25A)**. The basal and insulin-stimulated phosphorylation of p38 MAPK was significantly higher in overweight women with PCOS compared to Lean controls (Basal: mean difference ± SD (a.u.): 11.92 ± 8.40, 95%CI [9.16,14.77], P < 0.001, insulin-stimulated: mean difference ± SD (a.u.): 9.63 ± 4.24, 95%CI [6.79,12.47], P = 0.006) Following insulin stimulation phosphorylation of p38 MAPK was significantly higher in women with PCOS compared to overweight controls (insulin-stimulated: mean difference ± SD (a.u.): 9.93 ± 4.51, 95%CI [6.99,12.88], P = 0.01) **(Figure 25B.)**.

6.3.4.9 FOXO1

The phosphorylation of FOXO1 was similar between groups at baseline and during insulin stimulation (Group effect, P = 0.71; Time effect, P = 0.55; Interaction effect, P = 0.003), a significant interaction was detected, post-hoc analysis identified no differences (P > 0.17) **(Figure 25C.)**.

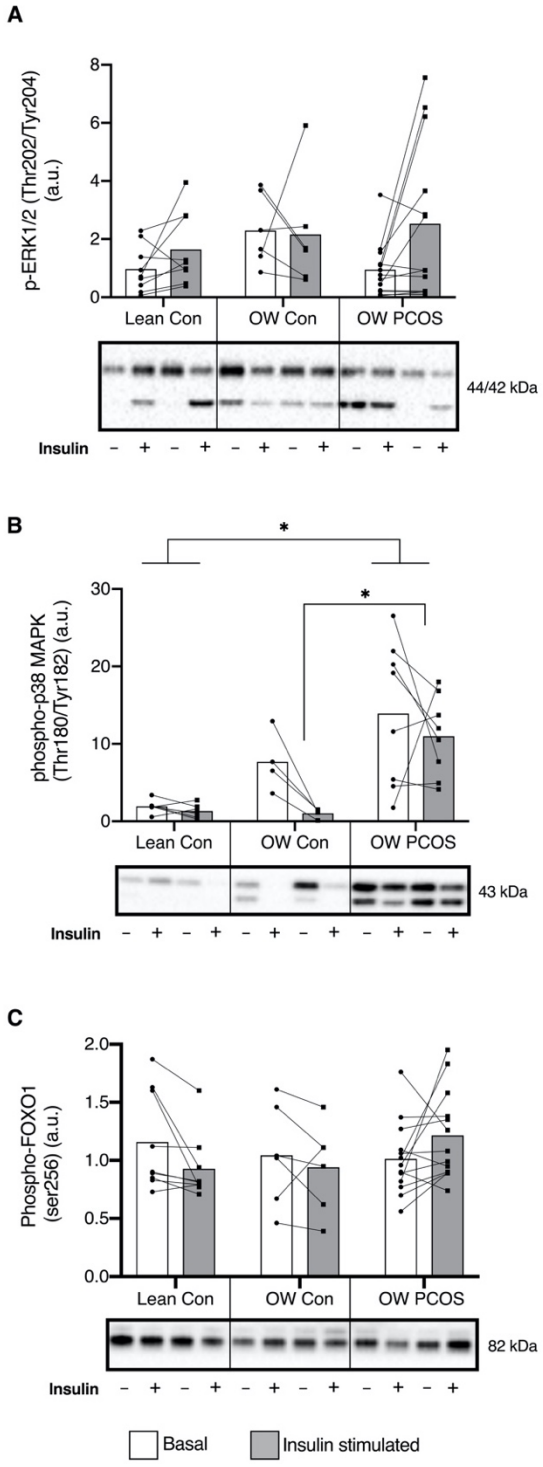

Figure 25. Stress Kinase and FOXO1

A) Phosphorlyation of ERK1/2 Thr202/Tyr 204) B) Phosphorlyation of p38 MAPK Thr180/Tyr182 C) Phosphorlyation of FOXO1 ser256. Data presented mean ± SD. *represents significant differences between groups. For ERK1/2 and FOXO1: (Lean con: n = 9, OW con: n = 6, OW PCOS: n = 13). For P38MAPK: (Lean con: n = 6, OW con: n = 4, OW PCOS: n = 8). Data analysed using mixed effects modelling and sidak post-hoc test.

6.4 Discussion

6.4.1 Clinical characteristics and insulin sensitivity in women with PCOS and without PCOS

The clinical profile of women with PCOS and controls outlined in **Table 6** may help to explain the underlying insulin resistance observed. In our study, overweight women with PCOS had elevated fasting insulin, glucose and HOMA-IR compared to lean controls, with no differences in HBA1c. Coupled with metabolic impairments in women with PCOS, there was also a marked reduction in lean mass and an increase in fat mass, which was not observed when compared to overweight healthy controls. It has long been known that body composition influences glucose metabolism with a positive relationship between insulin sensitivity and percentage of skeletal muscle mass and a negative relationship with percentage of fat mass (Yki Jarvinen and Koivisto, 1983). In addition, skeletal muscle is the primary site of postprandial and insulin-stimulated glucose uptake (DeFronzo and Tripathy, 2009). This at least partly accounts for the reduction in insulin sensitivity in overweight women with and without PCOS.

Insulin resistance is known to be highly prevalent in women with PCOS and can occur independently of obesity (Stepto et al., 2013; Tosi et al., 2017). Our data show that overweight controls and overweight women with PCOS have a comparable reduction in insulin sensitivity when compared to lean controls. Caution is required in the interpretation of these results, given that we had only six overweight controls. Obesity presents as a large confounding factor in understanding insulin resistance in women with PCOS, and it has been demonstrated that women with PCOS have a 27% reduction in insulin sensitivity as measured by the euglycemic–hyperinsulinemic clamp, compared to matched controls (Cassar et al., 2016b). Obesity itself worsens the reduction in insulin sensitivity by ~15% and to a greater extent in women with PCOS (Cassar et al., 2016b). A limitation of our work is the lack of inclusion of a group with lean women with PCOS, which would help separate the effects of obesity and PCOS. Although the inclusion of that group was initially planned, this population has been challenging to recruit given the lower prevalence.

A distinguishing feature of the overweight women with PCOS was an increase in free testosterone (bioactive form) when compared to both healthy and overweight controls. The link between excess androgen production and insulin resistance in women with PCOS is poorly understood. Insulin resistance and hyperinsulinemia can increase androgen production via decreased SHBG in the liver, causing an increase in bioactive free testosterone (Wallace et al., 2013; Wang et al., 2019). Similarly, in our study women with PCOS had a significant reduction in SHBG compared to lean controls with no difference with overweight controls. This aligns with the lack of differences observed in insulin resistance between both overweight groups. Although SHBG levels were similar, only women with PCOS had elevated free testosterone. This highlights that the elevation in free testosterone may result from other factors beyond SHBG, such as the presence of elevated levels of luteinizing hormone (LH), which stimulates androgen production from theca cells in PCOS ovaries (Rosenfield et al., 2016). The effects of androgens on skeletal muscle have not been directly assessed in women with PCOS. However, it is known that hyperandrogenism leads to preferential storage of intra-abdominal fat and increases the number of subcutaneous adipocytes (Dumesic et al., 2016). This may promote ectopic fat accumulation leading to metabolic dysfunction and insulin resistance (Dumesic et al., 2019). This provides a mechanism by which hyperandrogenism may indirectly influence skeletal muscle insulin resistance, which supports the hypothesis previously proposed by Corbould (Corbould, 2008). However, the role of hyperandrogenism may account for some but not all cases of insulin resistance in women with PCOS, as not all women with PCOS have hyperandrogenism.

Although AMH levels have been shown to be positively correlated with HOMA-IR in all phenotypes of PCOS (Wiweko 2018), our findings indicate that there is no link between serum AMH and insulin resistance. Instead, there is a more close relationship between testosterone and reproductive dysfunction (Cassar et al., 2014). Serum AMH has been previously associated with irregular menstrual cycles and the severity of PCOS (Abbara et al., 2019). In our study, cohort overweight women with PCOS displayed lower levels of serum AMH compared to lean women (PCOS: 57.1 ± 25.3, Lean control: 88.1 ± 40.5 (pmol/L)). This unexpected finding may be a result of biological variability, considerably change over the lifespan and assay selection (Bungum et al., 2018; Tremellen and Zander-Fox, 2015). This presents a challenge in a clinical context where often a single measurement is obtained and questions the validity of AMH in the diagnosis of PCOS. It is clear that AMH plays a role in the reproductive function of PCOS,

and more research is required to validate the assays in a variety of populations before being incorporated into clinical testing for PCOS (Teede et al., 2019).

We reported that women with PCOS had elevated blood lipids, cholesterol, triglycerides and LDL compared to lean healthy controls but were similar to overweight controls. This highlights the contribution of obesity rather than PCOS. It has been reported that women with PCOS have higher rates of hepatic steatosis and non-acholic fatty liver disease (Petta et al., 2017; Rocha et al., 2017). However, in the case of our study, there were no differences in circulating biomarkers of liver function; AST and ALT. This goes against our hypothesis of PCOS being a profibrotic condition.

6.4.2 Does TGF-beta signalling contribute to insulin resistance in women with PCOS?

We aimed to determine if TGF-beta signalling could be a potential mechanism for the development of insulin resistance in women with PCOS. Contrary to our initial hypothesis based upon the findings from previous studies (Hutchison et al., 2012; N. Stepto et al., 2020), women with PCOS did not appear to present with aberrant TGF-beta signalling or increased accumulation of collagen I, III, IV in the skeletal muscle **(Figure 20)**. The only identifiable difference was a reduction in TGF-beta 2 expression in skeletal muscle from women with PCOS compared to lean control women **(Figure 20B.)** Aberrant TGF-beta signalling can occur via an increase in the activation of SMAD3. We observed no differences in SMAD3 phosphorylation at baseline and during insulin stimulation. Furthermore, we have observed no differences in gene expression of SMADs, collagens or ECM enzymes between women with PCOS and lean controls (unpublished data Moreno-Asso et al.). There is a good agreement between increases in gene expression and protein expression of collagens and other ECM proteins (Makhnovskii et al., 2020). Together these findings suggest that skeletal muscle TGF-beta signalling is not dysregulated and was not related to skeletal muscle insulin resistance in overweight women with PCOS.

In our study, women with PCOS had a significant reduction in skeletal muscle TGF-beta 2 expression compared to healthy lean controls **(Figure 20B)**. TGF-beta 2 has been shown to act as an adipokine and increase glucose uptake in C2C12 myotubes (Takahashi et al., 2019). This

may point to the role of TGF-beta ligands in regulating insulin sensitivity. Pre-clinical models have demonstrated that other TGF-beta ligands such as follistatin and myostatin are capable of regulating metabolic homeostasis (Eilers et al., 2020; Han et al., 2019). The regulation of TGF-beta ligands and signalling appears to be complex, given their role in multiple processes such as cell proliferation, differentiation, tissue remodelling, and immune function (Xu et al., 2018). Their function is dependent on the physiological environment, and for instance, under conditions of high glucose or ROS, TGF-beta1 exerts profibrotic effects (Abrigo et al., 2016; Li et al., 2003). Preliminary evidence from this study and **Study 1** shows that TGF-beta ligands are capable of influencing metabolism; however, these effects may occur independently of the development of tissue fibrosis.

6.4.3 Skeletal muscle insulin signalling and insulin resistance in women with PCOS

We observed an increase in phosphorylation of p38MAPK at baseline in women with PCOS compared to lean healthy controls **(Figure 25B)**. An increase in the basal phosphorylation of p38MAPK has been shown in the skeletal muscle of individuals with type 2 diabetes, which may be linked to hyperglycemia and hyperinsulinemia (Koistinen et al., 2003). Also increased p38MAPK activity has been related to oxidative stress and reactive oxygen species (ROS) and an increase in pro-inflammatory cytokines (Audrey E. Brown et al., 2015; Diamond-Stanic et al., 2011). Measures specific to reactive oxygen species and inflammation were beyond the scope of the current study but may present an opportunity worthy of further investigation. In the context of PCOS, ROS has been linked to insulin resistance (González et al., 2006a, 2006b).

Moreover, skeletal muscle MAPK signalling has been found to be dysregulated in PCOS with the phosphorylation of ERK1/2 being elevated under basal (Ciaraldi et al., 2013) and insulin stimulation conditions (Corbould et al., 2006; Rajkhowa et al., 2009). These defects in mitogenic signalling have been attributed to an increase in phosphorylation of inhibitory IRS-1serine. However, in our study we observed no differences in basal or insulin-stimulated phosphorylation of IRS-1_{ser312} and ERK1/2 **(Figure 23A and 25A)**. In women with PCOS with an inflammatory profile in plasma cytokines and in skeletal muscle signalling, an acute bout of exercise has been shown to reduce these effects (Dantas et al., 2019), suggesting that this is an acquired pathology rather than an intrinsic defect. Despite elevated p38MAPK phosphorylation

in our study, we have seen no differences in insulin signalling. Future studies should explore other targets that might be affected by excessive p38MAPK phosphorylation.

Our study aimed to replicate previous findings demonstrating whether or not insulin resistance could be attributed to defects in the expression or phosphorylation of insulin signalling cascade (Diamanti-Kandarakis and Dunaif, 2012; Stepto et al., 2019a). We did not detect any defects in skeletal muscle insulin signalling in biopsies obtained during the steady-state phase of by the euglycemic–hyperinsulinemic clamp compared to lean and overweight women. In particular, the insulin-stimulated phosphorlyation of Akt_{ser473} during the clamp was similar between overweight women with PCOS and lean controls **(Figure 23B)**, despite significantly lower GIR in the women with PCOS. In line with our results, other studies have observed no specific defects in skeletal muscle insulin signalling in overweight and lean women with PCOS during insulin stimulation (Ciaraldi et al., 2009; Hansen et al., 2019). In these studies, the underlying insulin resistance either could not be explained by the data collected (Ciaraldi et al., 2009) or was attributed to downregulation of AMPK and ACC phosphorylation, with increased skeletal muscle lipids (Hansen et al., 2019). Again, these findings are contrary to ours, where we see no differences in the phosphorylation AMPK and ACC **(Figure 24C and 24D)**. Comparably, a reduction of insulin sensitivity via lipid infusion occurs without any changes in insulin signalling (Dubé et al., 2014; Høeg et al., 2011). This may indicate that changes in mitochondrial function and fatty acid oxidation may be responsible for metabolic dysfunction observed in our study. Together, the data from our study and previous studies suggest that insulin resistance in women with PCOS may result from proinflammatory factors and excessive ROS. This suggests that insulin resistance in women with PCOS is not a direct result of defects in insulin signalling. It is important to take into account that there are multiple mechanisms for the development of insulin resistance. In particular, due to the heterogeneity of PCOS, there may not be a single factor or specific defect that is responsible.

6.4.4 Limitations and future directions

This study is not without limitation. Firstly, the sample size was not the same across groups, with the number of lean and overweight control women being lower than the numbers of women in the PCOS group. Furthermore, given the heterogeneity of PCOS, greater numbers across all phenotypes are needed to better characterize factors contributing to peripheral insulin

resistance in each metabolic profile and identify phenotype-specific signalling defects. As the data presented here is part of an ongoing trial, a greater number of participants will complete the study. It may have a better representation of matched controls and different PCOS phenotypes. Secondly, a more comprehensive analysis of TGF-beta signalling is required to determine if it plays a role in skeletal muscle, assessing additional targets, structural remodelling via scans and tissue histology.

Due to the differences in BMI between the overweight women with PCOS and the overweight women in the control group (PCOS: 36.8 vs OW con: 31.3 kg/m^2), it is not possible to fully distinguish whether the observations made in this study were due to obesity or PCOS. Although the differences in BMI were not statistically significant, they may be of clinical relevance. Our study, increased phosphorylation of p38 MAPK in skeletal muscle at rest and during the euglycemic-hyperinsulinemic clamp was the only defect observed. In the context of the effects of obesity, chronic activation of p38 MAPK by ROS has been shown to induce insulin resistance in obese individuals and those with type 2 diabetes (Bengal et al., 2020; Audrey E. Brown et al., 2015). This would indicate the elevation in p38 MAPK may result from obesity rather than PCOS, highlighting the need for appropriate BMI-matched controls in PCOS research.

Future studies may wish to utilise a systems biology approach using *omics* platforms to assess transcriptional and translational defects in many metabolic pathways rather than relying solely on a targeted approach. Also, the relationship between adipose tissue and skeletal muscle in insulin resistance in PCOS may offer a new avenue for further research. The search for a unifying cause of insulin resistance in women with PCOS may be futile given the heterogeneity of the condition and confounding factors such as obesity. Therefore, future studies should focus on investigating phenotype-specific defects.

6.4.5 Conclusions

In conclusion, peripheral insulin resistance in overweight women with PCOS was not related to the previously observed defects in insulin signalling and could not be accounted for by TGF-beta signalling and collagen deposition.

6.4.5.1 Key findings

- Main clinical features that separated women with and without PCOS were elevations in free testosterone and androstenedione, a sign of hyperandrogenism. Surprisingly, women with PCOS had lower levels of AMH compared to lean controls.
- Overweight women with PCOS display lower protein expression of skeletal muscle TGF-beta 2 but no differences in SMAD phosphorylation nor collagen I, III, IV were observed.
- Overweight women with PCOS displayed intact skeletal muscle metabolic insulin signalling.
- Women with PCOS have higher basal phosphorylation of p38 MAPK, suggesting that inflammatory factors or reactive oxygen species may be contributing to insulin resistance.

7 **STUDY 4:** Effect of High-intensity Interval Training and Moderate-intensity Training on Insulin Sensitivity and TGF-beta Signalling in Overweight Women with PCOS

7.1 Introduction

The ovaries of women with PCOS display a marked increase in collagen deposition and fibrotic tissue due to TGF-beta signalling dysregulation (Hatzirodos et al., 2011; Raja-Khan et al., 2014). Given the systemic nature of TGF-beta signalling and activity, it is conceivable that it may play a major role in the aetiology of PCOS affecting other tissues. This may provide the missing link between the metabolic and ovarian abnormalities present in women with PCOS. Previous data from our lab showed that women with PCOS who are overweight appear to have lesser improvements in insulin sensitivity compared to BMI-matched controls (Harrison et al., 2012). This has also been supported by others showing similar responses in lean women with PCOS (Hansen et al., 2020). However, the underlying mechanism of this impairment in the insulin-sensitizing effect of exercise training in women with PCOS is poorly understood.

Preliminary evidence suggests that TGF-beta signalling may be partly responsible for this unfavourable response. Stepto et al., 2020, showed that women with PCOS who are overweight display a gene expression pattern in skeletal muscle that is favourable for the development of fibrosis and ECM remodelling (N. Stepto et al., 2020), with greater gene expression of COL1A2, COL3A1, DCN, TGFBR2 and LOX, following exercise training intervention these genes remain elevated with COL1A2, COL3A1, and LOX expression is increased. This elevation in profibrotic genes (N. Stepto et al., 2020), along with elevated circulating levels of AMH and TGF-beta 1 (Cassar et al., 2014; Tal et al., 2013), and an increase in skeletal muscle tissue density (Hutchison et al., 2012) in women with PCOS, suggests that TGF-beta ligands and ECM remodelling may play a role in modulating insulin sensitivity in response to exercise training.

The interaction between insulin resistance and the ECM is becoming more apparent, although there is still scope for further investigation. Dysregulation of ECM remodelling via elevated TGF-beta ligands and inflammation results in excess collagen deposition, creating a physical barrier that could impair diffusion of glucose and insulin, contributing to insulin resistance (Berria et al., 2006; Richardson et al., 2005; Watts et al., 2013). A recent study identified a link between the ECM and insulin sensitivity following bariatric surgery with and without nine months of exercise training in women with severe obesity (Dantas et al., 2020). They noted that improvements in insulin sensitivity coincided with a reduction in collagen I and III, basal phosphorylation of SMAD2/3 and in the thickness of the basement membrane (Dantas et al., 2020). Moreover, individuals with type 2 diabetes who have an increase in skeletal muscle TGF-beta 1 activity show a lack of improvement in insulin sensitivity following exercise training, linked to a marked reduction of genes involved in mitochondrial biogenesis; PGC1α, and TFAM (Böhm et al., 2016). Therefore, the role of aberrant TGF-beta signalling in women with PCOS may be responsible for peripheral insulin resistance and the suppression of the insulin sensitizing effects of exercise training.

7.1.1 Aims and hypothesis

We aimed to determine if 12 weeks of high-intensity or moderate-intensity exercise training can improve VO_{2peak} and insulin sensitivity in overweight women with PCOS. It was hypothesized that greater exercise intensities would be required to see improvements in VO_{2peak} and insulin sensitivity, to overcome the impaired insulin-sensitizing response to exercise observed in women with PCOS. We also aimed to establish if TGF-beta signalling is altered in response to 12 weeks of high-intensity or moderate-intensity exercise training in women with PCOS. It was hypothesized that exercise training would reduce the profibrotic signalling associated to TGF-beta 1 and SMAD3 and increase the expression of the adipokine TGF-beta 2.

7.2 Methods

7.2.1 Participants

A total of 25 women with PCOS who were overweight were randomized into the following groups MICT (n = 12/enrolled, n=10/completed) and HIIT (n = 13/enrolled, n=11/completed). Randomisation was completed by an independent statistician using computerised sequence generation at an allocation ratio of 1:1. The randomisation was stratified based upon BMI, with ranges of <35 kg/m^2 or >35 kg/m^2. The sample size was estimated a priori using G*power 3.1.9.2 software (Faul et al., 2007). Calculations were carried based upon data from previous PCOS training studies. Changes of +32.25% in insulin sensitivity (GIR (mg/lbmkg/min) (Hutchison et al., 2012, 2011; Konopka et al., 2015; N. K. Stepto et al., 2020) and of +24% VO_{2peak} (ml/kg/min) (Patten et al., 2020) were used as the expected effect size. To detect within-group differences, a total sample size of 26 (13 per group) will be required with a Power of 80% and $\alpha = 0.05$. We aimed to recruit an additional 8 participants to account for a 30% drop-out rate. Previous exercise training studies in women with PCOS have used groups sizes of 10-20 (Patten et al., 2020; Stepto et al., 2019b). Prior to baseline testing, physical activity levels were monitored for seven days using Actigraph™ accelerometer to determine if participants were meeting the weekly recommended physical activity levels. In the case of our participant's, none were meeting the weekly recommended physical activity levels (data not shown). Only participants that completed testing for the main outcome variables of insulin sensitivity and exercise capacity pre-and post-training intervention were included in the final analysis of this study (See Methodology section 3.1).

7.2.2 Metabolic and body composition assessment

See Methodology section 3.2

7.2.3 Muscle Biopsy

Muscle biopsies were obtained pre-and post-exercise intervention. However, only a subset agreed to pre-and post-exercise training biopsies in each group MICT (n=4) and HIIT (n=9). (See Methodology section 3.3)

7.2.4 Assessment of exercise capacity

See Methodology section 3.4

7.2.5 Exercise training intervention

See Methodology section 3.5

7.2.6 Bloods and pathology analysis

See Methodology section 3.6

7.2.7 Western blot analysis

Targets assessed in this chapter were TGF-beta 1, TGF-beta 2, TGF-Beta Receptor 1, Phospho-SMAD 3, Collagen IV, PGC1α, and GLUT4 (for more details, see Methodology section 3.12).

7.2.8 Statistical analysis

A linear mixed model analysis was used to compare the effectiveness of the exercise interventions for all pre-post measures. Pre-training and post-training changes in protein expression and phosphorylation were assessed using a mixed-effects model (2 groups and 2-time points). Significant effects of interaction (group x time), Time (pre-training vs post-training) and group (MICT vs HIIT). When significant effects were observed, Sidak posthoc test was used to identify differences. The distribution of the data was tested using the Shapiro-Wilk test. Data in text for insulin sensitivity and cardiorespiratory fitness were reported as

follows: relative change (%) ± SD, 95% CI [lower, upper], P-value. Data in text for TGF-beta and metabolic-related proteins are presented as relative change (%) ± SD, 95% CI [lower, upper], P-value. (For additional details see methodology section 3.13).

7.3 Results

7.3.1 Clinical characteristics

The clinical profiles following the 12-week exercise intervention are outlined in **Table 7.**

Table 7. Clinical characteristics of women with PCOS before and after the 12-week exercise intervention.

	Moderate-intensity continuous training (MICT)		High-intensity interval training (HIIT)	
General				
Number of participants	10		11	
Age (years)	29 ± 5		35 ± 5	
Height (cm)	162 ± 0.1		165 ± 0.1	
Phenotype				
A	4		4	
B	0		2	
C	2		1	
D	4		4	
	Pre	**Post**	**Pre**	**Post**
Body composition				
Weight (kg)	99.0 ± 28.3	99.9 ± 28.0	96.4 ± 20.3	96.0 ± 19.6
Body mass index (kg m-2)	37 ± 10	38 ± 10	35 ± 7	35 ± 7
Lean mass (%)	48 ± 5	48 ± 5	50 ± 6	51 ± 6
Fat mass (%)	49 ± 5	51 ± 5[a]	45 ± 7	46 ± 7
Glucose homeostasis				
Fasting glucose (mmol/l)	4.9 ± 0.5	4.7 ± 0.3	4.9 ± 0.4	4.6 ± 0.3
Fasting insulin (uIU/mL)	17.8 ± 6.1	18.5 ± 5.8	17.8 ± 10.4	16.7 ± 12.8
HOMA-IR	3.7 ± 1.4	3.8 ± 1.3	3.8 ± 2.4	3.7 ± 3.1
HbA1c (%)	5.3 ± 0.2	5.4 ± 0.2	5.1 ± 0.2	5.2 ± 0.2
Insulin sensitivity				
GIR (mg/lbmkg/min)	7.9 ± 3.0	8.3 ± 3.3	7.7 ± 3.0	10.2 ± 3.3[a]
Insulin sensitivity index (ISI)	7.4 ± 3.3	7.6 ± 3.9	8.3 ± 4.2	11.20 ± 5.0[a]
Hormonal status				
$Testosterone^{total}$ (nmol/L)	1.7 ± 0.6	1.3 ± 0.6	1.6 ± 0.6	1.6 ± 1.0
$Testosterone^{free}$ (pmol/L)	34.3 ± 17.1	28.3 ± 17.7	33.4 ± 14.7	28.4 ± 15.8
SHBG (nmol/L)	27.9 ± 14.2	26. 4 ± 11.6	35.9 ± 22.0	38.0 ± 22.7
Dihydrotestosterone (nmol/L)	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
Androstenedione (nmol/L)	4.6 ± 1.9	3.8 ± 1.2	5.0 ± 1.1	4.5 ± 1.9
Estradiol (pmol/L)	327.1 ± 265.1	282.8 ± 285.3	235.9 ± 207.6	235.0 ± 150.0
Anti-müllerian hormone (pmol/L)	49.2 ± 20.6	42.2 ± 19.4	63.4 ± 25.9	67.4 ± 46.0
Lipids				
Cholesterol (mmol/L)	5.0 ± 0.8	5.0 ± 0.7	4.8 ± 0.8	4.8 ± 1.0
Triglycerides (mmol/L)	1.4 ± 0.6	1.7 ± 0.6[b]	1.0 ± 0.4	1.0 ± 0.4
high-density lipoprotein (mmol/L)	1.4 ± 0.3	1.3 ± 0.3	1.5 ± 0.3	1.5 ± 0.4
Low-density lipoprotein (mmol/L)	3.0 ± 0.8	3.0 ± 0.7	3.1 ± 0.8	3.0 ± 0.9
LDL:HDL ratio	2.2 ± 0.7	2.4 ± 0.7[a]	2.2 ± 0.6	2.1 ± 0.7
Liver function				
AST (IU/L)	20.6 ± 11.6	20.2 ± 13.5	24.0 ± 13.7	20.3 ± 7.8
ALT (IU/L)	33.0 ± 16.3	28.7 ± 19.3	33.0 ± 29.0	23.3 ± 12.1

Data are mean and SD. AST: aspartate aminotransferase ALT: alanine aminotransferase Insulin sensitivity index was calculated using the following formula:(Glucose infusion rate/lean body mass)/Steady state insulin)*100. HOMA-IR: fasting insulin (mU/L)* fasting plasma glucose (mmol/L)/22.5. [a]denotes significant difference from pre-training. [b]denotes significant differences between groups. Following HIIT: significant improvements in ISI and GIR (P = 0.01, P = 0.02, respectively). Following MICT significant increase in fat mass (P = 0.001), LDL:HDL ratio (P = 0.008) and higher levels of triglycerides following training compared to HIIT group (P = 0.01).

7.3.1.1 Body composition

Following 12 weeks of MICT or HIIT, there were no changes in body weight, BMI or lean mass. Fat mass was slightly increased following MICT (mean change: 2.30 ± 1.71% 95% CI [0.97,3.62], P = 0.001) **(Table 7)**.

7.3.1.2 Glucose homeostasis

Following 12 weeks of MICT or HIIT, there was no changes in common clinical markers of glucose homeostasis: fasting glucose, fasting insulin, HOMA-IR or HbA1c. Insulin sensitivity index derived from the clamp showed no changes following MICT and increased following HIIT **(Table 7)**.

7.3.1.3 Lipids

Following MICT, triglycerides were elevated compared to the HIIT group (mean difference: 0.67 ± 0.70 mmol/L, 95% CI [0.12,1.22], P = 0.01). MICT also resulted in increase in the LDL:HDL ratio (mean change: 0.23 ± 0.21%, 95% CI [0.06,0.17], P = 0.008). While following HIIT there was no changes in lipids **(Table 7)**.

7.3.1.4 Liver function

Following 12 weeks of MICT or HIIT, there were no changes in liver function as measured by aspartate aminotransferase (AST) and alanine aminotransferase (ALT) **(Table 7)**.

7.3.2 Cardiorespiratory fitness

There were no differences between groups at baseline or following training for VO_{2peak}. The 12 weeks of exercise training resulted in an increase in VO_{2peak} independently of the type of intervention completed. (Group effect P = 0.11; training effect, P < 0.001; Interaction effect, P = 0.10). Both MICT and HIIT resulted in similar improvement in VO_{2peak}; MICT (relative change ± SD: 17 ± 8%, 95% CI [12,22], P < 0.001), HIIT (relative change ± SD: 22 ± 11%, 95% CI [16,29], P < 0.001). **(Figure 26A.)**.

7.3.3 Insulin sensitivity

There were no differences between groups at baseline or following training for the glucose infusion rate. Following 12 weeks of exercise training there was a main effect of training on glucose infusion rate (Group effect P = 0.51; training effect, P = 0.03; Interaction effect, P = 0.11). Post-hoc analysis indicated that HIIT resulted in a significant increase in insulin sensitivity; HIIT (relative change ± SD: 47 ± 59%, 95% CI [13,82], P = 0.02), MICT (relative change ± SD: 8 ± 24%, CI [-7,22], P = 0.87) **(Figure 26B.)**.

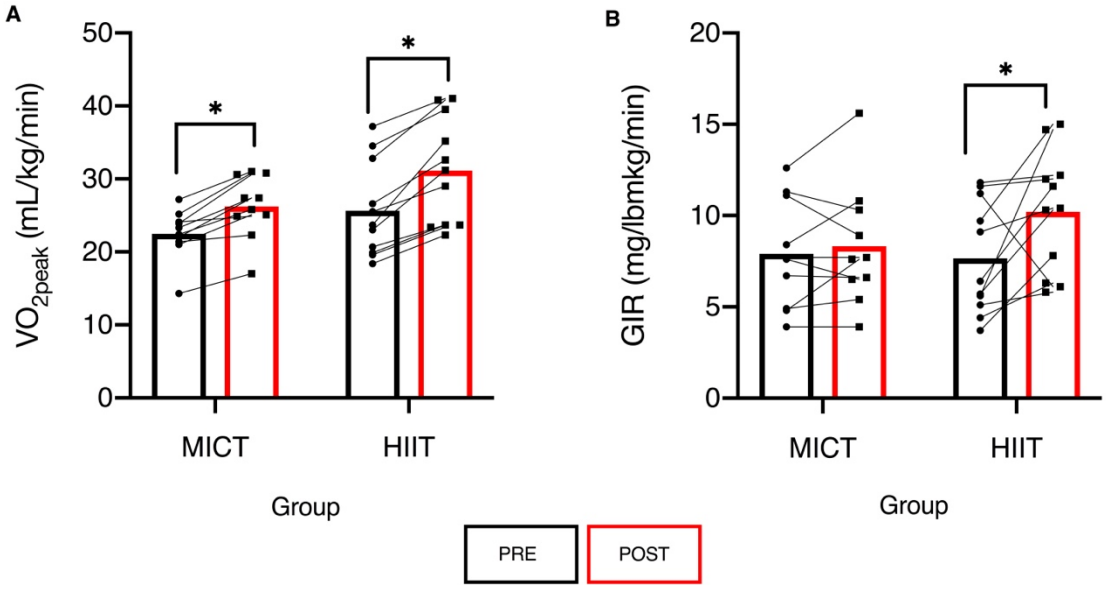

Figure 26. pre- and post-intervention data for VO_{2peak} and Glucose infusion rate.
A) VO_{2peak} obtained during maximal graded exercise test pre- and post-12-week exercise intervention.
B) Glucose infusion rate (GIR) a measure of insulin sensitivity obtained during euglycemic–hyperinsulinemic clamp. GIR was taken from the steady-state period during the final 30mins of the clamp.
*** signifies statistically significant difference from pre-post intervention. Data presented mean ± SD. (MICT: n = 10, HIIT: n = 11).**

7.3.4 TGF-Beta signalling

7.3.4.1 TGF-beta 1 and TGF-beta 2

The expression of TGF-beta ligands, TGF-beta 1 and 2 were not different between groups and did not change following the 12-week exercise interventions. TGF-beta 1 (Group effect P = 0.58; training effect, P = 0.40; Interaction effect, P = 0.85) **(Figure 27A.)**. TGF-beta 2 (Group effect P = 0.53; training effect, P = 0.47; Interaction effect, P = 0.23) **(Figure 27B.)**.

7.3.4.2 TGF-beta receptor 1 and TGF-beta receptor 2

The expression of TGF-beta receptor 1 was not different between groups at baseline and post training, there was a main effect of training for the expression of TGF-beta receptor 1 (Group effect P = 0.42; training effect, P = 0.01; Interaction effect, P = 0.07). Post hoc analysis identified significant increase following the MICT intervention (relative change ± SD: 82 ± 103%, 95% CI [-19,183], P = 0.02) HIIT (relative change ± SD: 24 ± 29%, 95% CI [5,42], P = 0.62) **(Figure 27C.)**. The expression of TGF-beta receptor 2 could not be detected.

7.3.4.3 SMAD 3

There were no differences between groups at baseline or following training for phosphorylation of SMAD 3. (Group effect P = 0.66; training effect, P = 0.04; Interaction effect, P = 0.96). There was a main effect of training, but Post-hoc analysis identified no differences (P > 0.13) **(Figure 27D.)**.

7.3.4.4 Collagen IV

The expression of collagen IV was not different between groups and was not altered following the 12-week exercise intervention, (Group effect P = 0.96; training effect, P = 0.18; Interaction effect, P = 0.16). **(Figure 27E.)**.

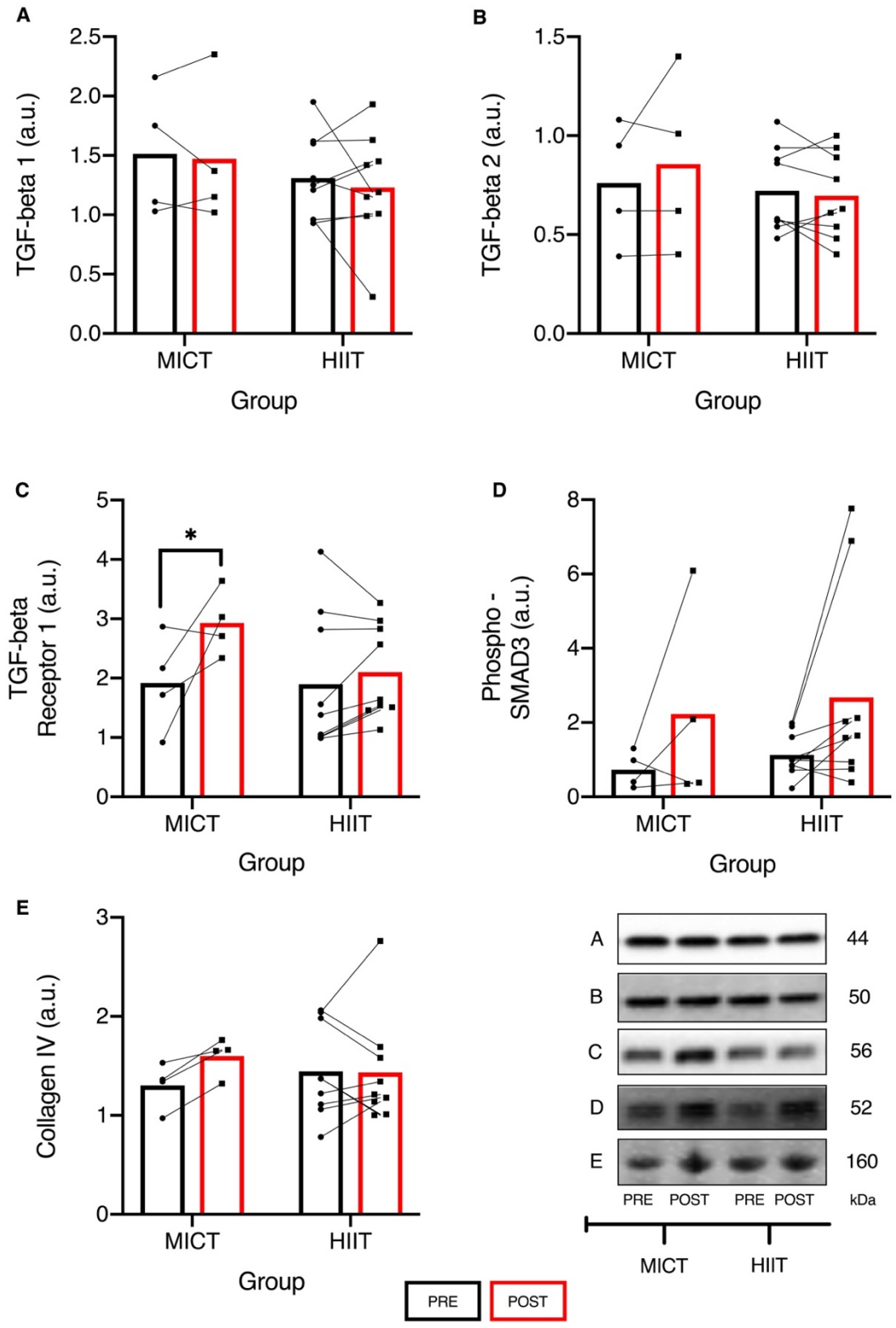

Figure 27.Pre-post intervention TGF-beta signalling protein expression.

Total TGF-beta 1 B) Total TGF-beta 2 C) Total TGF-beta receptor 1 D) SMAD3 Phosphorylation E) Total Collagen IV. * signifies statistically significant difference from pre-post intervention. Data presented mean ± SD. (MICT: n = 4, HIIT: n = 9).

7.3.5 Metabolic protein expression

7.3.5.1 PGC1α

No differences were detected between groups at baseline or post training for PGC1α expression. There was a main effect for training with an increase in PGC1α expression in the HIIT group and a decrease in the MICT group. (Group effect P = 0.90; training effect, P = 0.32; Interaction effect, P = 0.02). MICT: (relative change ± SD: -22 ± 36%, 95% CI [-57,13], P = 0.61) HIIT: (relative change ± SD: 36 ± 18%, 95% CI [25, 48], P = 0.01) **(Figure 28A)**.

7.3.5.2 GLUT4

There were no differences detected between groups at baseline or following 12 weeks of exercise training. There was a main effect of training with an increase in GLUT4 expression. (Group effect P = 0.16; training effect, P < 0.001; Interaction effect, P = 0.19). Both MICT and HIIT resulted in similar increases in GLUT4; MICT (relative change ± SD: 27 ± 16%, 95% CI [11,43], P = 0.001), HIIT (relative change ± SD: 20 ± 11%, 95% CI [12,27], P = 0.001) **(Figure 28B)**.

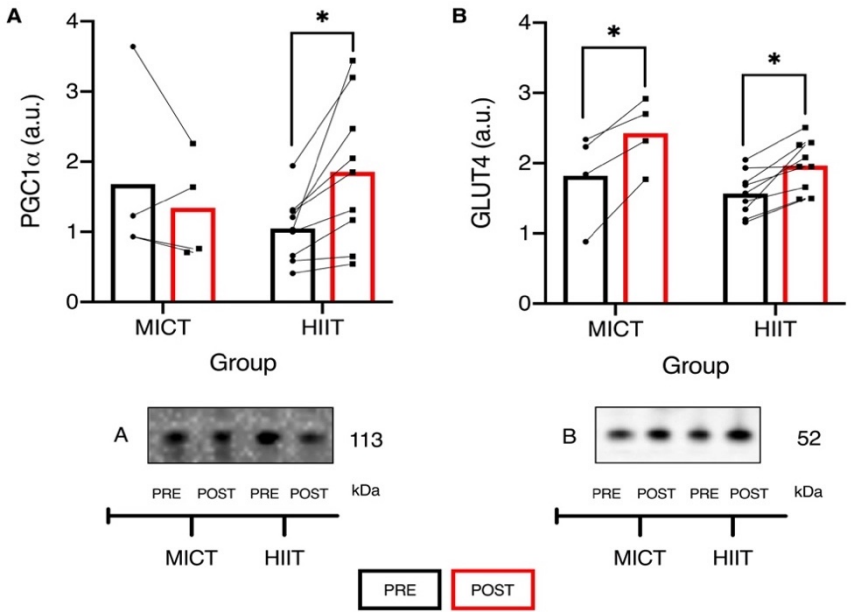

Figure 28. Metabolic protein expression pre-post 12-week training intervention.
A) Total PGC1α B) Total GLUT4 * signifies statistically significant difference from pre-post intervention. Data presented mean ± SD. (MICT: n = 4, HIIT: n = 9).

7.4 Discussion

7.4.1 Are women with PCOS resistant to the insulin sensitizing effect of exercise training?

No changes were observed in common clinical markers of glucose homeostasis: HOMA-IR, fasting glucose and insulin, and HbA1c. HIIT of 75 min per week resulted in improvements in insulin sensitivity by 47% and exercise capacity by 22%, and MICT of 150 min per week resulted in improvements in insulin sensitivity by 8% and exercise capacity by 17%. While both interventions improved cardiometabolic health, this effect seemed to be more pronounced following HIIT. Collective evidence shows similar improvements in insulin sensitivity, 16-45% increase, in women with PCOS following aerobic exercise training interventions (Harrison et al., 2012; Hutchison et al., 2012, 2011; Konopka et al., 2015). Similar findings have been reported in T2DM individuals, who had an improvement in insulin sensitivity of 25-50% following exercise interventions utilising HIIT or MICT (Cassidy et al., 2017; Conn et al., 2014). Our findings build upon previous studies demonstrating that regular aerobic exercise is beneficial for improving insulin sensitivity in women with PCOS, assessed by the gold-standard euglycemic-hyperinsulinemic clamp. However, we did not see improvements in commonly used clinical measures such as HbA1c, fasting insulin and glucose, and HOMA-IR. This may indicate that the beneficial effects of exercise on metabolic health may be underestimated or even go undetected when relying on common clinical measures. Our findings are in agreement with a recent meta-analysis from our group (Patten et al., 2020), highlighting that both moderate and vigorous exercise are capable of improving cardiometabolic health outcomes. The results from the meta-analysis also indicated that vigorous exercise might be more beneficial than moderate exercise for improving HOMA-IR, change in HOMA-IR following exercise training of vigorous-intensity: -36% or moderate-intensity: -16%).

It has been proposed that women with PCOS have partial resistance to the insulin sensitizing effects of exercise. In line with that, overweight women without PCOS following HIIT showed greater improvements in insulin sensitivity than women with PCOS (23 vs 16%, respectively) (Harrison et al., 2012). Similarly, in lean hyperandrogenic women with PCOS, a 14-week exercise intervention consisting of HIIT and resistance exercise failed to improve insulin-

stimulated glucose uptake, insulin signalling and blood flow (Hansen et al, 2020). This may suggest that the PCOS phenotype, in particular hyperandrogenism, may be influencing the metabolic adaptations to exercise. However, our intervention study only included women with PCOS; therefore, we cannot entirely rule out a partial resistance to insulin sensitizing effects, but this seems unlikely given the substantial improvements that we observed.

Improvements in peripheral insulin sensitivity following HIIT are probably a result of greater depletion skeletal muscle glycogen and recruitment of type II fibres than MICT (Egan et al., 2010; Kristensen et al., 2015; Lee-Young and Canny, 2009). This subsequently leads to greater metabolic adaptations with increases in GLUT4 expression and mitochondrial biogenesis (increase in PGC1α) (Baar et al., 2002; Chinsomboon et al., 2009), as observed in our study. Previously, it has been established that increases in PGC1α expression are related to training intensity, with HIIT being a potent stimulus for increasing PGC1α and mitochondrial biogenesis (Bishop et al., 2019). This could result in altered substrate utilization and influence whole-body metabolism. While PGC1α is decreased in individuals with metabolic diseases and can be improved with exercise, a direct link between PGC1α and improvements in insulin sensitivity has yet to be established *in vivo.* The improvements in insulin sensitivity following HIIT occurred in conjunction with an increase in PGC1α, suggesting an increased exercise-induced mitochondrial function. However, both groups had an increase in GLUT4 abundance, suggesting that this not a limiting factor in insulin sensitivity in women with PCOS. These findings are in line with a previous study indicating that GLUT4 translocation in overweight women with PCOS is comparable to that on overweight controls following an acute moderate treadmill exercise, despite being more insulin resistant (Dantas et al., 2015). Previously, both HIIT and MICT have been shown to increase GLUT4 expression in type I and II fibres, as well as GLUT4 translocation to the plasma membrane, allowing GLUT4 to be more readily available for fusion with GLUT4 vesicles upon insulin stimulation (Bradley et al., 2015; Richter and Hargreaves, 2013). In the case of our study, the localization of GLUT4 was not assessed, and we can only confirm an increase in GLUT4 expression.

There were large variances in the improvement of insulin sensitivity following HIIT and MICT in our study, with improvements ranging from 3%-163%. Individuals with higher baseline insulin sensitivity displayed lesser improvements following our exercise interventions. This

may be related to the volume of exercise training performed only meeting the minimum recommended guidelines and suggests that individualised/personalized exercise programming are necessary due to the heterogeneity of the condition. It is important not to discount the role of moderate-intensity exercise for women with PCOS. Konopka and colleagues have shown that following 12 weeks of MICT 60 min at 65% of VO_{2peak} resulted in a 47% improvement in GIR (insulin sensitivity) (Konopka et al., 2015). When comparing these results to our study, there are notable difference: 5 vs 3 days of training per week, session time 60 vs 50 minutes, and intensity 65% vs 50-60% VO_{2peak}. Thus, despite minimal improvements in insulin sensitivity following MICT in our study, there is still a role of MICT in exercise prescription for women with PCOS with greater exercise volume being required. Taken together, women with PCOS do not appear to be resistant to the insulin sensitizing effect of exercise training when the exercise prescribed is of higher intensities or higher volumes.

7.4.2 Does exercise training alter skeletal muscle TGF-beta signalling in women with PCOS?

We aimed to investigate the effects of exercise training on skeletal muscle TGF-beta signalling in overweight women with PCOS. This study followed up the findings from Stepto et al., which attributed insulin resistance and attenuated responses to exercise training to aberrant TGF beta signalling (N. Stepto et al., 2020). Based upon our findings, increased insulin sensitivity occurred independently of changes in TGF-beta and ECM related proteins. Following 12 weeks of MICT or HIIT, there were no changes in TGF-beta 1, TGF-beta 2 and collagen IV, with both groups seeing a non-significant increase in the phosphorylation of SMAD3. Following MICT only there was an increase in the expression of TGF-beta receptor 1. The effect of exercise intensity on ECM remodelling has only been explored in a few acute studies few studies, Holm et al. found similar rates of total collagen protein fractional synthesis following low-load and high load unilateral knee extensions (Holm et al., 2010). Together with the findings from our study, these results may indicate that higher exercise intensities are not required for ECM remodelling via collagens deposition and synthesis. It has recently been reported that when bariatric surgery is combined with nine months of exercise training in severely obese women, improvements in insulin sensitivity are linked to reductions in skeletal muscle collagen deposition, capillary basement membrane thickness, and TGF-β1/SMAD3 signalling (Dantas et al., 2020). In the case of our study, the intervention may not have been

long enough (12 weeks compared to 9 months) to induce structural changes in the ECM. This study also points to the beneficial effect of combined aerobic and resistance exercise intervention rather than a single exercise modality.

It has previously been demonstrated that increases in SMAD3 phosphorylation results in suppression of PGC1α (Tiano et al., 2015), decrease in skeletal muscle glucose uptake (Yadav et al., 2011), and promotes muscle atrophy (Goodman et al., 2013) in animal models and C2C12 mouse myoblasts. In the context of our study, both MICT and HIIT resulted in an increase in SMAD3 phosphorylation with no negative effects observed in muscle mass or metabolism. The lack of SMAD3 signalling has been shown to impair muscle regeneration and decrease TFAM following acute muscle damage in mice (Ge et al., 2012) and to play a role in muscle hypertrophy (Mendias et al., 2016), which may suggest that increased SMAD3 signalling could also have beneficial effects. The role of SMAD3 in muscle physiology is still not fully understood, and any potential negative effects in other conditions/settings may have been mitigated by beneficial effects of exercise such as increases in PGC1α expression observed following HIIT. The effects and function of TGF-beta signalling may be modulated by other factors such as inflammation and ROS. In our study, MICT increased TGF-beta receptor 1 protein expression but not HIIT, and the reasons for this are not clear. A possible explanation can be ascertained from research focusing on renal fibrosis, whereby increases in PGC1α expression reduced activation of SMAD signalling by reducing TGF-beta receptor 1 expression (Choi et al., 2019). In the MICT group, lack of increase in expression of PGC1α coincided with an increase in TGF-beta receptor 1 expression. However, these findings should be taken with caution due to the small sample size of MICT biopsies (n = 4). In addition, further investigation is needed, given that greater expression of TGF-beta receptor 1 corresponds with increases in TGF-beta activity.

Although the ECM and TGF-beta signalling does not appear to be a limiting factor for insulin sensitizing effects of exercise training in women with PCOS, we cannot fully rule out the role of the ECM in regulating insulin sensitivity in women with PCOS given the complexity of this structure and lack of understanding regarding biological communication with skeletal muscle cells (Gillies and Lieber, 2011). In a recent meta-analysis reviewing the composition of ECM in connective tissue, it was identified by proteomics that the skeletal muscle ECM proteins are predominantly made up of collagen I and III, with other collagens, versican, elastin and

fibronectin accounting for the rest of ECM protein expression (McKee et al., 2019). A similar systemic approach comparing the skeletal muscle of women with PCOS and matched controls may overcome the limitation of assessing individual protein expression. This would allow for a clear assessment of the ECM structure as a whole, and the relative expression of the different components, creating a more accurate method of analysis.

7.4.3 Exercise prescription for women with PCOS

Based on the findings from our study, the current exercise guidelines may not be sufficient for women with PCOS to see improvements in clinical parameters beyond insulin sensitivity and exercise capacity. Our study aligns with a previous observational study, showing that time spent doing vigorous physical activity reduced the odds of metabolic syndrome in women with PCOS by 22% per hour of activity (Greenwood et al., 2016). This effect occurred independently of total activity time and was absent for moderate activity.

Despite the positive effects of both of our exercise interventions, HIIT and MICT, on the peak oxygen consumption and a significant improvement with HIIT on the underlying insulin resistance, there was no beneficial effect of neither of the exercise interventions on body composition, hormonal status or lipids. A recent meta-analysis of exercise intervention studies in women with PCOS showed beneficial effects of exercise on fasting insulin, HOMA-IR, cholesterol and VO_{2peak} (Kite et al., 2019). Similar to our study, there was little to no beneficial effects of exercise on androgens and other hormones (Kite et al., 2019). Another meta-analysis in healthy women showed beneficial effects of exercise to decrease circulating sex hormones, in particular when the exercise interventions included high-intensity exercise (Ennour-Idrissi et al., 2015). In particular, resistance exercise in women with PCOS has shown favourable effects on reduction of Free androgen index, testosterone, and AMH (Almenning et al., 2015; Kogure et al., 2018), suggesting a potential for exercise to improve hyperandrogenism and hormonal status of women with PCOS. Based upon our findings showing beneficial effects of aerobic training on cardiometabolic health, together with other strength training studies showing changes on the hormonal profile, a combined intervention may be optimal. Women with PCOS are genetically predisposed to obesity and increased visceral adiposity (Day et al., 2015; Durmus et al., 2017), which may be detrimental for both metabolic and reproductive health. Therefore, exercise can maintain weight loss or mitigate weight gain in women with

PCOS (Barber et al., 2019). In line with our findings, HIIT and MICT resulted in improvements in insulin sensitivity without changing body weight, lean mass or fat mass. This highlights that exercise alone is not effective means to achieve weight loss; however, it can maintain weight and has other health benefits. One of the major barriers to implement exercise interventions in women with PCOS is a lack of time and low motivation and compliance (Blackshaw et al., 2019). One of the strengths of our study was the fact that all exercise training sessions were supervised, which resulted in superior outcomes. Consequently, adherence was greater than 90%. Thus, more studies are required to optimize exercise prescription to achieve these outcomes and assure motivation and compliance.

7.4.3.1 Implementing recommendations from the evidence based-guidelines for women with PCOS

A recent opinion piece outlining the recommendations from evidence-based guidelines for exercise and physical activity in women with PCOS identified key research areas requiring further investigation, which are relevant to the findings of our study (Moran et al., 2020; Stepto et al., 2019b). These included optimizing exercise training regimes and the effect of long-term exercise interventions ≥ 12months. We have demonstrated that a 12-week high-intensity interval exercise training may have a greater beneficial effect on metabolic health compared to 12-week moderate-intensity continuous exercise training, but further studies should replicate these findings and assess sustainability. In addition, research should focus on optimizing regimes to improve different clinical aspects of PCOS (metabolic, hormonal and reproductive health) and phenotype specific-interventions. Given the short duration of our exercise interventions (3months), it may be postulated that longer-term intervention would have resulted in greater improvements in cardiometabolic health and altered hormonal status.

7.4.4 Limitations and future studies

Overall, there are several limitations with our study, in particular, the small sample size (n = 4) for biopsies in the MICT group compared with the HIIT group (n = 9). Comparing groups due to the unequal sample size resulted in a reduction in statistical power and increased the chance of type I errors occurring. In our study, post-intervention biopsies were optional as we did not want this to be a barrier for women completing the exercise intervention and post-testing. Also,

the limited overall sample size may not have been sufficient to detect differences between groups.

In relation to the assessment of ECM and TGF-beta signalling proteins, some targets still have to be analysed such as collagen I and III, key components of intramuscular connective tissue in skeletal muscle (McKee et al., 2019). Following this analysis, we will be able to see if our intervention altered skeletal muscle ECM composition. In addition, we attempted to quantify CTGF (profibrotic regulator) and perlecan (multi-functional heparan sulfate proteoglycan) (Yamashita et al., 2018) via western blot, but no protein was detected.

Given that PCOS is a life-long condition and exercise is recommended as first-line therapy for management, long-term trials are needed to assess sustainability and feasibility of the incorporation of regular exercise as part of everyday life. This would allow for the determination of long-term benefits beyond improving insulin sensitivity, such as reproductive and mental health outcomes. Our study and others across the literature are of a short-term nature with exercise interventions lasting 3 to 4 months. Future studies should aim to run long-term interventions, although it is appreciated this is not without challenges given the personnel and resources required to achieve this. Another consideration for the interpretation of our findings is that our study was lab-based, and all exercise sessions were supervised. It would be useful to see if similar results could be achieved in a community-based study.

7.4.5 Conclusions

We were able to demonstrate that exercise training, both HIIT and MICT, improved insulin sensitivity and VO_{2peak}, when interventions were matched for MET.min/week, but only HIIT resulted in significant improvements in insulin sensitivity. Improvements in insulin sensitivity occur independently of skeletal muscle TGF-beta signalling/expression and changes in body composition. Neither interventions altered hormonal status, lipids or markers of liver function. Collectively, exercise training, irrespective of the intensity, is an effective lifestyle intervention for improving cardiometabolic health in women with PCOS.

7.4.5.1 Key findings

- Both moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT) similarly improved VO_{2peak}, but improvements in insulin sensitivity were greater following high-intensity interval training (HIIT).
- Improvements in insulin sensitivity occurred independently of changes in skeletal muscle TGF-beta signalling, collagen deposition or body composition.
- Hormonal status, lipids and liver function were unaffected by 12 weeks of exercise training regardless of exercise intensity.

8 General Discussion

8.1 Overall aim

To date, a variety of studies have attempted to understand the mechanisms of insulin resistance in women with PCOS by quantifying insulin signalling in the peripheral tissues and relating it to clinical measures of insulin sensitivity. However, few have attempted to understand if aspects of the pathophysiology of PCOS are contributing factors to these defects and the subsequent insulin resistance. Lifestyle modification is considered first-line therapy for the management of PCOS and is regarded as an effective means of improving metabolic health. Currently, there is a lack of understanding regarding the effectiveness of these approaches on clinical outcomes in women with PCOS and a need for specifically designed interventions for women with PCOS taking into account their PCOS phenotype and associated clinical features. The overall aim of this thesis was to determine if TGF-beta signalling is dysregulated in the skeletal muscle of overweight women with PCOS and subsequently if it was related to insulin resistance. In addition to attempting to understand if TGF-beta signalling was altered by exercise training and related to improvements in insulin sensitivity. This aim was achieved through a combination of *in vivo* and *in vitro* investigations. The results of this thesis provide novel insights into the role of TGF-beta signalling and insulin resistance in overweight women with PCOS.

8.2 Conclusions

The main conclusions of each of the studies are as follows:

Study 1: The metabolic phenotype was not retained in cultured in myotubes from women with PCOS suggesting that external environmental factors, combined with intrinsic defects, play an essential role in developing peripheral insulin resistance. We identified that two TGF-beta ligands known to play a role in the pathophysiology of PCOS, TGF-beta 1 and AMH, had distinct effects on insulin signalling and glucose uptake in myotubes from women with and without PCOS. TGF-beta 1 may trigger metabolic conditions conducive to the development of tissue fibrosis, inducing aerobic glycolysis. Conversely, AMH caused a reduction in glucose

uptake and insulin signalling changes with a decrease in PI3Kp110 expression and an increase in the phosphorylation of IRS-1_{ser312}.

Study 2: Myotubes from women with PCOS displayed minimal differences in responsiveness to an *in vitro* model of contraction (EPS) compared to myotubes from healthy controls, which show increased phosphorylation of p38 MAPK and CREB. When combined with *in vitro* contraction, TGF-beta 1 and AMH had minimal effects on exercise-induced signalling transduction.

Study 3: Women with PCOS did not have any identifiable defects in skeletal muscle insulin signalling despite having severe insulin resistance. Likewise, TGF-beta signalling was not dysregulated, with the expression of TGF-beta ligands, SMADs and collagens being comparable between women with and without PCOS. Skeletal muscle TGF-beta signalling was not related to insulin resistance.

Study 4: Exercise training, regardless of the intensity, improved the cardiometabolic health of women with PCOS. Both HIIT and MICT resulted in a significant increased $VO_{2peak,}$ but only HIIT resulted in significant improvements in insulin sensitivity independently of changes in body composition or other clinical parameters. Collectively, exercise training, irrespective of the intensity, is an effective lifestyle intervention for improving cardiometabolic health in women with PCOS. Exercise training did not cause any changes in TGF-beta signalling in overweight women with PCOS.

8.2.1 Discussion of key findings

8.2.1.1 The role of TGF-beta ligands and associated signalling in metabolism

Multiple studies have hinted that TGF-beta signalling and ECM remodelling may play a role in the development of insulin resistance in women with PCOS. Our *in vitro* data from **Study 1** investigated a possible mechanism by which TGF-beta ligands can influence glucose homeostasis. Previous results from C2C12 myotubes suggest that TGF-beta 1 treatment increases phosphorylation of SMAD3, which can suppress insulin-stimulated phosphorylation of AKT and AS160 (Dantas et al., 2020; Guo et al., 2019). In contrast to these results, our study

showed TGF-beta 1 resulted in increases in glucose uptake without changes in proximal insulin signalling. The contrasting response with our results and studies from C2C12 myotubes may be explained by differences in structure and function, metabolic behaviours and donor variability (Abdelmoez et al., 2019). On the other hand, AMH had not been previously investigated in the context of skeletal muscle metabolism. We found that AMH was capable of reducing glucose uptake via an increase in the phosphorylation IRS-1$_{ser312}$, a defect that has been previously associated with insulin resistance in PCOS.

Although we demonstrated that TGF-beta 1 and AMH could alter glucose metabolism and insulin signalling in primary cultured myotubes, it appears that these findings do not translate *in vivo*. In **Study 3,** we observed insulin resistance in the absence of any signalling defects in skeletal muscle tissue, a lack of aberrant TGF-beta signalling, and higher levels of serum AMH in controls compared to overweight women with PCOS. The response or contribution of TGF-beta signalling in peripheral insulin resistance could be occurring via other tissues or organs. A recent review focusing on proteomics of insulin resistance in metabolically active organs (adipose tissue, skeletal muscle and liver) found ECM and fibrosis-related proteins in adipose tissue to be responsible for the pathology of insulin resistance, while in skeletal muscle that was associated to mitochondrial related proteins (Li et al., 2020). In line with this, TGF-beta 1 has been linked with subcutaneous adipose tissue insulin resistance (Dumesic et al., 2019). It has recently been observed in obese women that ECM contributes to insulin resistance via adipose accumulation of lumican, which enhances collagen deposition and impairs insulin signalling (Guzmán-Ruiz et al., 2020). This suggests that TGF-beta signalling may play a role in adipose tissue influencing metabolic function rather than in skeletal muscle. Altogether, this indicates that the dysregulation of TGF-beta signalling is a consequence of insulin resistance rather than a cause per se, and it may serve as a biomarker of metabolic dysfunction in women with PCOS.

There is evidence to suggest changes in the extracellular matrix occur in conjunction with changes in insulin sensitivity, but how the two are directly linked is still poorly understood. Typically, insulin resistance has been associated with increased collagen deposition; however, we have seen no changes in the main component of basement membrane collagen IV, collagen I (epimysium) or collagen III (perimysium). It should be noted that the pathology of fibrosis is also determined by collagen organization and distribution, which we did not assess. Previously,

it had been postulated that TGF-beta ligands are capable of impairing metabolic responsiveness to exercise training in T2D patients (Böhm et al., 2016) and may be related to insulin resistance (Williams et al., 2015). In **Study 3,** we found that insulin sensitivity was not related to TGF-beta signalling. In **Study 4,** we found that improvements in insulin sensitivity occurred independently of changes in TGF-beta signalling. This suggests that TGF-beta signalling is not a limiting factor for improvements in insulin sensitivity following exercise training in women with PCOS.

It is important to note that this thesis only focused on the metabolic role of two out of thirty-three TGF-beta ligands and their main associated signalling pathway (Moses et al., 2016). These ligands are known to interact and share receptors, modulating a plethora of biological processes. We assessed these ligands for their potential negative effects; however, their responses are dependent on the cellular environment. Under normal physiological conditions, they play an important role in tissue regeneration, regulating stem cell proliferation, differentiation and migration (Xu et al., 2018). The activation of these TGF-beta ligands is regulated by a number of factors such as pH, ROS and matrix metalloproteinases (Annes et al., 2003). In conditions of inflammation and oxidative stress, excessive activation of latent TGF-beta ligands could result in detrimental effects. Other TGF-beta ligands such as follistatin and myostatin can influence insulin action in skeletal muscle in PCOS-like, suggesting an association of these ligands with insulin resistance in women with PCOS (Eilers et al., 2020; Han et al., 2019). Testosterone can directly influence TGF-beta signalling in mouse satellite cells, resulting in the inhibition of phosphorylation of SMAD2/3 and an increase in follistatin (Braga et al., 2012). This highlights the complex regulation of TGF-beta signalling, with more research being required to understand these complex multifactorial cytokines and their role in the pathology of PCOS and other disease states.

8.2.1.2 Comparison of *in vivo* and *in vitro* data: whole muscle *vs* primary cultured myotubes

In **Study 3,** we found no observable differences in metabolic insulin signalling in the skeletal muscle samples obtained from women with PCOS at rest and during insulin-stimulated conditions, whereas in **Study 1,** myotubes from women with PCOS displayed impaired insulin-stimulated phosphorylation of AKT when compared to lean, healthy women. This defect has

not been previously observed *in vitr*o but has been identified *in vivo* (Hojlund et al., 2008). The reason for these disparities in our study is not clear and remains to be determined. However, it is unlikely that this difference fully accounts for insulin resistance, given that only a small percentage of AKT phosphorylation is required for GLUT4 translocation and glucose uptake (Hoehn et al., 2008; Whitehead et al., 2001). We identified in **Study 3** that basal phosphorylation of p38MAPK was elevated in skeletal muscle of women with PCOS. However, these findings were not observed when the cells were in culture **(study 2).** In one of the few studies assessing skeletal muscle insulin signalling *in vivo* and *in vitro,* Ciaraldi and colleagues showed that skeletal muscle and myotubes from women with PCOS both display a reduction of ~50% in insulin-stimulated glucose uptake in the absence of any insulin signalling defects (Ciaraldi et al., 2009). This highlights that insulin resistance can occur independently of defects in the insulin signalling cascade in women with PCOS.

Taken together, combined results from **Study 1, Study 2** and **Study 3** suggest that expression of signalling components is intact and that assessment of activity of these proteins may be where defects are occurring. This consideration aligns with previous findings showing a normal abundance of PI3K p85 but a reduction in insulin-stimulated PI3K activity in skeletal muscle and myotubes (Corbould et al., 2005; Dunaif et al., 2001). In the case of our studies, we only assessed the expression of proteins and not the activity. The lack of agreement between studies in skeletal muscle tissue and cultured myotubes may be determined by the severity of PCOS and the corresponding *in vivo* physiological environment, where the level of hyperinsulinemia, hyperandrogenism and other factors may cause metabolic reprogramming. The metabolic differences seen *in vivo* and *in vitro* from the same donor may indicate that environmental factors are driving insulin resistance. This presents an opportunity to manipulate the extracellular milieu in cell culture to mechanistically explore environmental factors contributing to insulin resistance in PCOS.

The measure of glucose uptake in culture is influenced by the expression of glucose transporters, which seems to differ from what is observed *in vivo*. In myotubes, the expression of GLUT1 is higher while GLUT4 is lower than *in vivo* skeletal muscle (Al-Khalili et al., 2003; Henry et al., 1995; Sarabia et al., 1992). In myotubes, GLUT1 is responsible for basal glucose uptake whilst GLUT4 facilitates insulin-stimulated glucose uptake mimicking *in vivo* responses (Al-Khalili et al., 2003; Henry et al., 1995; Sarabia et al., 1992). Therefore, the

elevated expression of GLUT1 and subsequent increase in basal glucose uptake may contribute to the lack of insulin-stimulated glucose uptake observed in both myotubes from lean, healthy controls and women with PCOS. This may subsequently influence the interpretation of the retention of the *in vivo* metabolic phenotype. Indeed, in **Study 1**, the relationship between insulin sensitivity measured by a euglycemic-hyperinsulinemic clamp and *in vitro* glucose uptake in myotubes was poor.

8.2.1.3 Mechanisms of insulin resistance

The findings from our *in vivo* and *in vitro* studies, together with previous studies, pose the question of what develops first, insulin resistance or defects in insulin signalling in women with PCOS. Due to the challenges of the diagnosis and women being diagnosed with PCOS at various different stages of life, it makes it difficult to determine when insulin resistance first develops. It has been observed that obese adolescent girls with PCOS display a decrease in peripheral insulin sensitivity, decreased adiponectin and metabolic inflexibility compared to matched girls without PCOS (J. Y. Kim et al., 2018). The mechanisms responsible for the development of insulin resistance remain a mystery not only in PCOS but also in other metabolic conditions (Fazakerley et al., 2019).

One factor to consider when comparing our results with others is the timing of the biopsies. It has previously been suggested that defects occur in the early phase, 15-30 minutes, of insulin stimulation with a decrease in IRS-1-associated PI3K activity, which was no longer present after 90minutes (Dunaif et al., 2001). Previous data from our lab showing skeletal muscle insulin signalling during the early stage of the clamp, after 30 minutes (N. Stepto et al., 2020), and our data in **Study 3** obtained during steady-state both found insulin signalling to be intact despite women with PCOS presenting with insulin resistance. Based on our data, it would appear that insulin resistance is developed by environmental factors and low physical activity levels. A recently proposed mechanism for the development of insulin resistance is chronic hyperinsulinemia, which is common in women with PCOS. It has been demonstrated that prolonged insulin exposure in human primary myotubes and C2C12 results in a reduction in insulin-stimulated glucose uptake (Turner et al., 2020, 2018). This is accompanied by a reduction in phosphorylation of IRS-1 tyrosine and AKT (Turner et al., 2020). Another study in C2C12 myotubes reported that chronic hyperinsulinemia resulted in the downregulation of

insulin receptor mRNA (Cen et al., 2019). Furthermore, animal models have been used to demonstrate the role of circulating insulin in the regulation of adiposity and weight gain (Page et al., 2018). This occurs as insulin is capable of suppressing lipolysis, leading to increased lipid uptake and storage. This mechanism of insulin resistance goes against traditional thinking that hyperinsulinemia is a consequence rather than a cause of insulin resistance, and it requires more research before it becomes an accepted mechanism. Regarding our studies, this idea may make sense as obese women with PCOS have elevated fasting insulin **(Study 3),** and improvements in insulin sensitivity were accompanied by a non-significant decrease in fasting insulin following HIIT and MICT **(Study 4)**.

In **Study 3,** we observed an elevation of phosphorylation of p38MAPK in skeletal muscle of women with PCOS. This may indicate that mitochondrial dysfunction and excess ROS production as factors responsible for the development of insulin resistance in these women. This may occur via either fatty acid metabolites and intracellular diglycerides or a decrease in substrate oxidation influencing the activity of the electron transport chain (Montgomery and Turner, 2015; Shukla and Mukherjee, 2020). Similar to other defects observed, studies assessing mitochondrial function in women with PCOS are not conclusive. Hutchinson et al. show no observable defects in mitochondrial respiration and function (Hutchison et al., 2012), while Konopka et al. observed increased mitochondrial ROS production and downregulation of ADP to oxygen ratio (Konopka et al., 2015). Although the mechanism of insulin resistance remains to be determined, all of the defects observed to date are able to be improved via lifestyle intervention. It should be acknowledged that the odds of a single factor or defect being the cause of insulin resistance in women with PCOS are highly unlikely. It may be more reasonable to consider multiple possibilities with distinct pathways and patterns within each phenotype.

8.2.1.4 Exercise training and insulin sensitivity

It has been proposed that individuals with metabolic diseases have an impaired response to exercise, where improvements in insulin sensitivity and glucose homeostasis do not occur or occur to a lesser extent than healthy populations. Indeed, this hypothesis has been presented in exercise training studies of women with PCOS. The findings of **Study 4** support other studies indicating that women with PCOS are able to benefit from exercise training with substantial

improvements in insulin sensitivity. The determining factor of this response appears to be more related to intensity, volume and frequency, with an exercise training of a high-intensity of 3 sessions per week **(Study 4)** or moderate-intensity exercise of 5 sessions per week (Konopka et al., 2015), resulting in substantial improvements in insulin sensitivity. In our study (**Study 4**), 10/11 participants following HIIT had an improvement in insulin sensitivity, independently of changes in body composition. Collectively, this data suggests that women with PCOS are not resistant to the beneficial effects of exercise, but rather previous studies have used insufficient exercise dose as determined by frequency, intensity, and time. In our study, we observed improvements in insulin sensitivity independent of weight loss or changes in body composition, demonstrating a direct effect of exercise training (HIIT and MICT) on insulin sensitivity. Indeed, weight loss in exercise intervention studies is a confounding factor making it difficult to separate the effects of exercise and weight loss on insulin sensitivity. This would suggest that improvements in insulin sensitivity were reflective of enhanced skeletal muscle glucose uptake. It has been previously demonstrated that improvements in insulin sensitivity can occur following exercise training with and without changes in body composition (Poehlman et al., 2000).

It is important to note that the beneficial effects of exercise on insulin sensitivity are transient and related to the acute effects of the last session performed. As previously mentioned, following a period of inactivity, high levels of insulin sensitivity are rapidly diminished (Heath et al., 1983). A recent study showed that post-training improvements were present 24 hours after the last exercise bout but were no longer apparent after four days of training cessation, regardless of exercise intensity of the training in obese men and women (Ryan et al., 2020). This highlights the need for regular physical activity and exercise to maintain normal insulin sensitivity. Also, when interpreting or comparing results between studies, the time between the last exercise session and measurement of insulin sensitivity should be considered.

8.2.1.5 GLUT4 translocation in women with PCOS

Exercise training is an effective stimulus for increasing GLUT4 abundance (Richter and Hargreaves, 2013). In **Study 4,** we were able to show an increase in skeletal muscle GLUT4 expression; however, this does not resolve whether glucose transport is compromised at baseline or improved by exercise training. In women with PCOS, post-exercise GLUT4

translocation has been assessed via indirect measures assessing total and membrane protein expression of GLUT4 (Dantas et al., 2015). They found that women with PCOS had similar GLUT4 translocation following acute exercise to BMI matched controls. Furthermore, GLUT4 translocation in the postprandial state has not been assessed in women with PCOS and may have important implications in the development of insulin resistance. Recent advances in this area allow quantification of GLUT4 translocation to subcellular locations in skeletal muscle using transmission electron microscopy and fluorescence microscopy (Knudsen et al., 2020). An *in vitro* approach with a cell culture model has been developed utilising human serum to stimulate GLUT4 translocation in cultured myotubes with serum being collected from participants in a fasted and fed state (Cogan et al., 2019). This approach using conditioned media could be used to assess the effects of post-exercise serum from women with and without PCOS to better understand the insulin sensitising effects of exercise. This approach would be particularly interesting in women with PCOS due to investigating the role of circulating hormones and other environmental factors in skeletal muscle insulin resistance.

8.3 Limitations and Considerations

A strength of our studies is that we used the euglycemic-hyperinsulinemic clamp technique, often considered the gold-standard to determine insulin sensitivity (DeFronzo et al., 1979). A principle of this technique is that the constant infusion of insulin induces a hyperinsulinemic state, which increases glucose disposal from skeletal muscle and adipose tissue and suppresses hepatic glucose production (Muniyappa et al., 2008). Although we did not use stable isotope tracer [6,6-^{2}H] of glucose to account for hepatic glucose production, future studies should utilize tracers to improve the accuracy of the measurement of insulin sensitivity. Another challenge of the euglycemic-hyperinsulinemic clamp is the interpretation of data obtained and its clinical relevance, given that there are no reference ranges for GIR (Tam et al., 2012). Also, comparing results between studies is challenging given differences in insulin doses used (range: 30-120 mU/min/m^2). The results can be expressed relative to body weight, body surface area or fat-free mass. We used the same insulin dose of insulin for all groups in order to allow group comparisons. Several different methods have been used to set cut off points for insulin resistance determined by a clamp, which can influence the percentage of participants defined as insulin resistant. An example is the one established by Stern and colleagues, who used mixed

bimodal modelling on data from 2,321 participants who had insulin sensitivity measured by clamp using an insulin infusion rate of 40 mU/min/m^2 (the same as our studies) to define insulin resistance cut-off point of 5 mg/lbmkg/min (Stern et al., 2005). In the case of our studies, if we applied this cut-off, 36% of the women with PCOS would be classified as insulin resistant. In contrast, previous work from our lab defined insulin resistance as GIR of less than the 25th percentile of lean controls (Stepto et al., 2013). If we apply the latter to our studies, 100% of the women with PCOS would be classified as insulin resistant. Another study in women with PCOS defined reference limit of 11.76 mg/lbm/min (Tosi et al., 2017). If this is applied to our study, 84% of women with PCOS would be classified as insulin resistant. This calls for the need for standardized definitions of insulin resistance based upon clamp-derived data to ensure that the prevalence of insulin resistance can be accurately reported.

A confounding factor in the assessment of phenotypic retention and identification of signalling defects from whole muscle to myotubes in our work is the reliance on a single technique for signalling and metabolism, western blotting and 2-deoxy glucose uptake respectively **(Study 1 and 2)**. This limited approach may have meant that we had overlooked characteristics or defects present. A more comprehensive approach to assessing myotubes' metabolic function could include glycogen synthesis, glucose oxidation, lipid uptake, lipid oxidation, and mitochondrial ATP synthesis, all of which are well-established assays. However, supporting our findings (**Study 1**), a more comprehensive approach by Eriksen and colleagues assessing metabolic function observed that insulin resistance and mitochondrial dysfunction *in vivo* was no longer present *in vitro* (Eriksen et al., 2010, 2011). Another limitation is the use of western blotting. We utilized several aspects to minimize error and improve reproducibility by optimizing conditions for each target and utilising the stain-free method. However, there are factors out of our control that may influence our findings, such as the quality of antibodies (Gilda et al., 2015). The use of additional techniques such as immunohistochemistry would be useful to support our findings and allow for observation of localization and changes in tissue morphology. Other techniques such as proteomics present as an attractive alternative to western blotting, which provides with the ability to quantify vast numbers of targets and identify new pathways. Nevertheless, this technology is not as accessible as western blotting.

A significant limitation for studies 1, 2 and 3 is the lack of a BMI-matched control group, which could be a significant confounder for the findings of this thesis. In an ideal scenario, a study

with the following groups: (1) lean control group, (2) lean women with PCOS, (3) obese control group, and (4) obese women with PCOS, would be carried out to take into account the interaction of PCOS and obesity. Additional aspects, such as physical activity levels and PCOS phenotype, should also be matched to minimize confounders. Indeed, obese women with and without PCOS have many similar traits, including excess central adiposity, insulin resistance, and a greater risk of developing type 2 diabetes. Furthermore, approximately 60% of women with PCOS are either overweight or obese, and this is associated with the severity of metabolic features (Lim et al., 2013). However, it has been stated that insulin resistance can occur in the absence of obesity in women with PCOS (Stepto et al., 2013), suggesting that factors independent of obesity contribute to insulin resistance in PCOS. The interaction between obesity and PCOS provides a challenge for many researchers. In the case of our studies, the majority of the major outcomes and physiological responses assessed in this thesis can be affected by obesity, including insulin signalling, insulin sensitivity, contractile function, extracellular matrix composition, contractile function and oxidative stress. Therefore, we cannot fully account for the effects of obesity to understand the real impact of PCOS.

Like many of the studies conducted to date focusing on skeletal muscle insulin resistance in women with PCOS, our sample size was limited. This may be partly attributed to the time-demand of our study and the use of invasive techniques such as biopsies acting as barriers to recruitment. A potential strategy to reduce the number of biopsies would be to utilize an *ex vivo* approach incubating a proportion of the muscle biopsy in insulin. In addition, we had a strict inclusion criterion to ensure that other factors did not influence the primary outcome of insulin sensitivity. The limited sample sizes observed across the board in mechanistic and intervention based PCOS research calls for a more collaborative approach between groups. Our studies lack a broad representation across all PCOS phenotypes, making it challenging to translate the effectiveness of our findings for all women with this syndrome. A major limitation of our studies is the lack of a group of lean women with PCOS to account for the contribution of the condition versus obesity. To this accord in our study, insulin sensitivity was comparable between overweight women with and without PCOS, indicating an important contribution of obesity.

8.4 Future Research

8.4.1 Tissue crosstalk

Similar to the observations in the skeletal muscle of women with PCOS, insulin resistance and signalling have been explored in the subcutaneous adipose tissue. Women with PCOS display adipose tissue insulin resistance *in vivo*, but it has been shown that cultured preadipocytes displayed normal insulin responsiveness and no insulin signalling defects (Corbould and Dunaif, 2007; Dumesic et al., 2019). Moreover, contrary to myotubes, adipocytes have decreased GLUT4 expression (Rosenbaum et al., 1993). More recently, pathways related to PI3K-AKT, MAPK, TGF-beta and androgen signalling were found to be differently regulated in the adipose tissue (Kokosar et al., 2016). Interestingly, TGF-beta 2 is secreted from the adipose tissue in response to increases in lactate during exercise (Takahashi et al., 2019). They also showed that TGF-beta 2 stimulates glucose uptake and fatty acid oxidation in skeletal muscle suggesting inter-organ communication. Therefore, the crosstalk between adipose and skeletal muscle tissues may be a future avenue of research within PCOS. Samples obtained from women with PCOS could be examined by using a co-culture model in conjunction with hormone treatments. The assessment of myokines and adipokines released under different conditions may provide insights into how these tissues act under stress-induced conditions such as exercise or hyperinsulinemia. In addition, the characterization of these myokines and adipokines is relatively unexplored, presenting opportunities for the discovery of new therapeutics.

8.4.2 Retention of donor characteristics *in vitro*

The use of human primary myotube cultures allows for the identification of intrinsic defects and environmental in the development of metabolic diseases. A number of studies have indicated that myotubes from women with PCOS do not retain the donor characteristics (Eriksen et al., 2010, 2011). Accordingly, in **Study 1,** we showed a lack of retention of the metabolic phenotype in myotubes from women with PCOS. However, to date, the analysis employed have been limited to targeted approaches such as western blots, qPCR and metabolic measures, and a more comprehensive study has not been done. Given that the specific aetiology

of this underlying insulin resistance is unknown, targeted approaches may miss defects, which may be responsible for metabolic abnormalities. This could also be the case when assessing donor characteristic retention in cultured cells. Myotubes from individuals with type 2 diabetes have been studied via proteomics and RNA sequencing (Al-Khalili et al., 2014; Frederiksen et al., 2008; Thingholm et al., 2011), and were unable to detect any intrinsic defects at basal conditions that may explain altered metabolism in the myotubes although these have been demonstrated to retain metabolic features of their type 2 diabetic donor (Ciaraldi et al., 1995; Gaster et al., 2012, 2002; Henry et al., 1995). This highlights the complexity of insulin resistance and how the mechanism involved might be different between conditions. Furthermore, it has been shown that *in vivo* characteristics such as fibre type and mitochondrial content are retained *in vitro* in early passages (4-5) but not in subsequent passages (Covington et al., 2015). Myotubes cultured beyond passage 5, show downregulation of metabolic function and an increase in senescent cells (Nehlin et al., 2011). Therefore, experiments should be carried out in early passages to minimize the loss of *in vivo* characteristics in myotubes, as done in our studies.

Future studies should create an in-depth profile of early-passaged cultured cells from women with PCOS in basal and insulin-stimulated states in order to understand how metabolic defects are occurring and whether metabolic characteristics are retained in cultured myotubes. Additionally, in order to ascertain if environmental factors contribute to metabolic dysfunction and related signalling, future studies may wish to consider culturing cells in serum from the same donor. This may allow for the identification of circulating factors that are influencing insulin sensitivity. Remarkably, benefits of exercise training *in vivo* on glucose and lipid metabolism are retained *in vitro* (Bourlier et al., 2013; Lund et al., 2017). This suggests that metabolic reprogramming of satellite cells by exercise *in vivo* is evident in cultured myotubes and present opportunities for understanding how different populations respond to exercise in this *in vitro* model. The approach of culturing myotubes from muscle biopsies before and after exercise training interventions may be useful to assess whether the impact of exercise training is similar in distinct populations. In cases where underlying defects are observed, this approach would be able to determine whether exercise can normalize these defects and shift cells towards a healthy phenotype.

8.4.3 Exercise and lifestyle in women with PCOS

Based on our findings in **Study 4,** future interventions should ensure that exercise training is of a sufficient volume or intensity to improve insulin sensitivity and cardiorespiratory fitness. Whilst we showed beneficial effects of a short-term (12 weeks) exercise intervention, long-term trials (up to 12 months) are required to determine if long-term interventions would result in more significant improvements in metabolic health and if the effects extend beyond metabolic health to other clinical parameters. Other factors to explore that may further optimize the benefits of exercise for women with PCOS are the timing of exercise around meals: fasted and fed state and modulating the frequency of training to encompass shorter bouts throughout the day. An important but overlooked factor is the effect of exercise on appetite regulation considering that women with PCOS display dysregulation of ghrelin (Kasim-Karakas et al., 2007; Moran et al., 2004). Furthermore, the acute responses to different types of exercise in women with PCOS are poorly characterized. Only skeletal muscle GLUT4 and insulin signalling gene expression profiles have been explored in response to an acute bout of exercise. A simple study assessing glucose uptake and insulin sensitivity via a euglycemic-hyperinsulinemic clamp post-exercise may be sufficient to assess the acute insulin sensitizing effects of exercise without the need for invasive biopsy procedures. Therefore, future clinical studies should consider all these factors and investigate the acute effect of exercise on insulin sensitivity.

It is important to note that exercise is only one component of lifestyle modification for women with PCOS, and other lifestyle factors, in particular diet and nutrition, may have an additional beneficial impact. A recent review has shown that lifestyle interventions comprising of diet and exercise showed combined intervention is more beneficial than exercise alone, in particular for reducing BMI and adiposity (Naderpoor et al., 2015). Given that we were unable to see changes in body composition with exercise alone, regardless of a significant improvement in peripheral insulin sensitivity, it may be suggested that combined dietary and exercise intervention would be more effective than on its own.

8.4.4 Further assessment of TGF-beta signalling in women with PCOS

Several experimental approaches could be applied to explore the role of the TGF-beta ligands and their signalling pathways in PCOS pathologies (insulin resistance, glucose metabolism and hyperandrogenism). In particular, animal models could initially be utilized. Indeed, PCOS-specific animal models utilising prenatal androgen or AMH exposure have already been used to re-create the PCOS-phenotype (Risal et al., 2019; Tata et al., 2018), and whereby offspring develop a PCOS-like phenotype displaying reproductive dysfunction and metabolic perturbations. Other TGF-beta ligands could be used in this prenatal exposure model to explore whether they develop similar PCOS-like phenotypes. This could provide critical insights into the role of TGF-beta ligands on early metabolic reprogramming. This approach could be applied to various animal models, from non-human primates to rodent models, depending on outcomes of interest and resources available (Stener-Victorin et al., 2020). The use of targeted treatments on PCOS-like animal models may provide an *in vivo* approach to investigate the direct effect of TGF-beta pathways in PCOS, by using neutralizing antibodies, siRNA or small peptide-base strategies. These type of treatments have been designed to target different parts of the pathway, including ligand activation, receptor blocking, receptor kinase inhibition, mRNA disruption and transcription disruption (Akhurst, 2017).

Currently, several neutralizing antibodies are designed to target only the active isoforms of TGF-beta ligands producing anti-fibrotic and tumour reducing effects *in vitro* and *in vivo* (Bedinger et al., 2016; Liu et al., 2021). Specifically for skeletal muscle, others have utilized anti-Activin receptor IIA and IIB antibodies to inhibit the TGF-beta ligands myostatin, activin A, and GDF11, and promote muscle growth or reduce muscle wasting in mice (Morvan et al., 2017). Another study treating mouse models of obesity and T2D with anti-TGF-β1 neutralization antibody (1D11) has shown that these animals are protected from the development of metabolic disease (Yadav et al., 2011). This protection was conferred by reducing SMAD3 signalling in the adipose tissue and reducing inflammatory cells and cytokines. Following these studies, the role of TGF-beta signalling in PCOS could be explored by selective inhibition of different TGF-beta receptors or ligands to improve peripheral insulin resistance or ovarian fibrosis.

Human trials with targeted TGF-beta signalling treatments have already been conducted, particularly in the context of fibrotic conditions and cancers. However, the benefits displayed in preclinical models have failed to translate into human trials due to a systemic inhibition promoting inflammation, tumorigenesis and drug-related toxicity (Lodyga and Hinz, 2020). This is not surprising given the systemic nature of TGF-beta signalling and its roles in homeostasis in particular immune function. These adverse effects may be overcome with the drug delivery field advancing in cell and tissue-specific approaches such as extracellular vesicles as delivery vehicles (Zhao et al., 2020), as well as the development of selective TGF-beta inhibitors (Martin et al., 2020) and drugs such as nintedanib and pirfenidone, which target TGF-beta induced transcription of the profibrotic genes COL1A1, COL3A1 and fibronectin 1 (Knuppel et al., 2017).

8.5 Clinical implications

We sought to determine if aberrant TGF-beta signalling extended beyond reproductive tissue to the peripheral tissues, influencing insulin signalling and sensitivity. From our findings, it would appear that fibrosis or ECM remodelling is tissue-specific and does not necessarily extend systemically. More research is required to rule out the role of TGF-beta signalling and maladaptive ECM remodelling in other tissues and organs. From a drug development perspective, the primary source of aberrant TGF-beta signalling is reproductive tissues. Therefore, target treatments should focus on resolving the defects in the ovaries rather than whole-body approach. Many clinical trials of TGF-beta inhibitors targeting receptors or specific ligands have focused on cancers and pulmonary fibrosis (Akhurst, 2017; Lodyga and Hinz, 2020; Mahalanobish et al., 2020). This is challenging given the diverse role of TGF-beta ligands and their role in many different cell types. We demonstrate *in vitro* that the TGF-beta ligands AMH and TGF-beta 1 can influence metabolism and insulin signalling in skeletal muscle myotubes; therefore, the measurement of these ligands in circulation may be warranted.

The lack of clarity regarding the mechanism governing insulin resistance makes it difficult for target therapies to be developed. The likelihood of a central defect in insulin signalling in women with PCOS being the cause of insulin resistance does not seem viable, given the lack of consistency in findings to date. Also, if there was a genetic defect, it would be present in multiple tissues, but this has not yet been observed in women with PCOS, who present isolated

defects in a single tissue or cell type. In the case of our study, the higher expression of MAPK at baseline in the skeletal muscle would suggest elevations in ROS and inflammatory factors. Future investigations should seek to determine how common medications prescribed to women with PCOS, such as insulin sensitizers and oral contraceptives, influence TGF-beta ligands and ROS production.

Although the precise mechanisms of peripheral insulin resistance in women with PCOS are still not clear, lifestyle intervention appears to have positive effects. We have shown that a time-efficient weekly exercise training regime performed under supervision effectively improves metabolic health as measured by a euglycemic-hyperinsulinemic clamp. It should be noted that using standard clinical markers such as fasting glucose, fasting insulin, and HbA1c was not sufficient to detect improvements in metabolic health. Also, improvements in insulin sensitivity occurred independently of changes in body weight and composition. It is not feasible to run euglycemic-hyperinsulinemic clamps regularly in a clinical setting, given the labour, cost, and limited clinical benefit of this level of information. This highlights the need for more sensitive surrogates/biomarkers when assessing the benefits of lifestyle interventions. Notably, we had a strict inclusion criteria for our participants, excluding women regularly taking prescribed medications such as metformin and oral contraceptives (McCartney and Marshall, 2016) as these could influence the primary outcomes of our study. However, it is important to include these women in future trials and investigate how medications influence the response to exercise interventions.

Therefore, understanding the molecular mechanisms involved in insulin resistance will be vital for developing future PCOS-specific therapies, which may include combined lifestyle interventions, to ultimately improve the metabolic health and well-being of women with PCOS.

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