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Excess androgen production in subcutaneous adipose tissue of women with polycystic ovarian syndrome is not related to insulin or LH

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Abstract

The purpose of this study was to investigate androgen production and the role of insulin and LH in its regulation in subcutaneous adipose tissue (SAT) of women with polycystic ovarian syndrome (PCOS). Protein and mRNA expression of androgen synthesis enzymes (Cytochrome P450 17A1 [*CYP17A1*] and Aldo-keto reductase 1C3 [*AKR1C3*]) were measured in SAT biopsies from women with PCOS, diagnosed according to the Rotterdam criteria (n=15) and healthy controls (n=15). Cultured mature adipocytes (differentiated from SAT biopsies) were treated with insulin ± phosphoinositol-3-kinase inhibitor (LY294002) or LH ± insulin. *CYP17A1* and *AKR1C3* mRNA expression and testosterone concentrations were measured in treated and untreated adipocyte cultures. *AKR1C3* mRNA was significantly ($P<0.001$) greater in PCOS versus non-PCOS SAT, but *CYP17A1* was not significantly different between the two groups. AKR1C3 and CYP17A1 protein expression was not significantly different in PCOS versus non-PCOS SAT. In untreated adipocyte cultures, *CYP17A1*, *AKR1C3* and testosterone levels were significantly higher in the PCOS versus the non-PCOS groups. Addition of insulin increased *AKR1C3* mRNA and testosterone levels, but not *CYP17A1* mRNA in non-PCOS with no effect on PCOS adipocytes. The stimulatory effects of insulin were not inhibited by LY294002. Addition of LH increased *CYP17A1*, *AKR1C3* and testosterone in non-PCOS adipocytes with no effect in PCOS adipocytes. In conclusion, SAT of women with PCOS produces excess androgen, which may contribute to PCOS-related hyperandrogenaemia. This SAT androgen excess is independent of obesity and is not directly stimulated by inulin or LH.

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Introduction

Polycystic ovarian syndrome (PCOS) is the most common ovarian endocrinopathy with a prevalence of 6–10% based on National Institutes of Health (NIH) criteria (Fauser *et al.* 2012) and 17% according to Rotterdam consensus criteria (Lauritsen *et al.* 2014). It accounts for 83% of anovulatory infertility (Kousta *et al.* 1999) and 89% of hyperandrogenism (Elhassan *et al.* 2018). It is characterized by a varied combination of clinical (anovulation and hyperandrogenism), biochemical (excess serum luteinizing hormone (LH) and androgen concentrations) and ovarian morphological (polycystic ovaries) features. PCOS is well-known to negatively affect the quality of life and psychological wellbeing of women (Li Y *et al.* 2011) and is associated with significant long-term metabolic and cardiovascular morbidities (Fauser *et al.* 2012).

Despite its high prevalence, the underlying mechanisms of PCOS remain largely uncertain. It is well established that androgen excess plays a central role in PCOS pathogenesis. Current evidence suggests three main cellular sources for this androgen excess including ovarian theca cells (Gilling-Smith *et al.* 2005), adrenal cortical cells (Kumar *et al.* 2005) and adipocytes (Rosenfield *et al.* 2011; O'Reilly *et al.* 2017). Aldo-keto reductase 1C3 (AKR1C3), also known as 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) has been reported as the predominant androgen biosynthesis enzyme in adipose tissue that converts androstenedione to testosterone (Quinkler *et al.* 2004). Recent research has shown evidence of increased SAT *AKR1C3* mRNA expression (Wang *et al.* 2012; O'Reilly *et al.* 2017) and increased intra-adipose concentrations of testosterone in women with PCOS (O'Reilly *et al.* 2017). AKR1C3 expression and activity in omental adipose tissue were found to correlate positively with adiposity (Blouin *et al.* 2005). Furthermore, simple obesity has been associated with increased androgen production in reproductive age women (Samojlik *et al.* 1984; Kirschner *et al.* 1990). Therefore, it remains to be determined whether increased adipose tissue AKR1C3 expression in PCOS women is independent of obesity.

Although, hyperinsulinaemia (due to insulin resistance) and hyperandrogenaemia are closely linked and positively correlated in PCOS women, the exact interaction between them remains unclear. Recently, O'Reilly and co-workers reported that insulin significantly increased *AKR1C3* mRNA expression in

77 differentiated subcutaneous adipocytes and increased testosterone generation in cell media from cultured
78 subcutaneous adipocytes from non-PCOS women (O'Reilly *et al.* 2017). Currently, there are no data on
79 insulin actions in adipose tissue of PCOS women. Furthermore, there are no data on the mechanisms of
80 insulin-mediated effects on androgen production in adipocytes of women with or without PCOS.
81 Currently, there are only limited and conflicting data on the mechanisms of insulin actions in theca cells.
82 One study involving theca cells from normal ovaries has suggested phosphatidylinositol-3-kinase (PI-3k)
83 as a possible insulin signaling mediator for insulin stimulatory effects on *CYP17* (Munir *et al.* 2004). On
84 the other hand, the insulin-mediated increase in steroidogenesis in granulosa lutein cells of normal ovaries
85 was independent of the PI-3K pathway (Poretsky *et al.* 2001). Therefore, the role of the PI-3K pathway in
86 normal and polycystic ovaries remains uncertain. Another area of uncertainty is whether insulin alone is
87 capable of stimulating androgen synthesis or requires LH-induced cAMP activation. Currently there are
88 only few and conflicting data on the interaction between insulin and LH in ovarian tissue (Munir *et al.*
89 2004, Nestler *et al.*1998) with no data for adipose tissue.

90 Based on the above, we hypothesized that hyperinsulinaemia with or without excess LH in women with
91 PCOS could augment androgen production in peripheral adipose tissue through a mechanism involving
92 the PI-3K insulin signaling pathway. This hypothesis has never previously been tested in PCOS. The aim
93 of this *in-vitro* study was to measure expression of the main androgen synthesizing enzymes (AKR1C3
94 and Cytochrome P450 17A1 (CYP17A1), also called 17α-hydroxylase, 17,20-lyase) in SAT from women
95 with and without PCOS. We also measured expression of these enzymes and testosterone concentrations
96 in mature adipocyte cultures (prepared from SAT) incubated with different concentrations of insulin ± PI-
97 3K inhibitor (LY294002) or LH ± insulin.

98

99 Methods

100 This study was approved by the Derbyshire Ethics Committee (Ref: 09/H0401/27) and all participants
101 gave written informed consent.

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Subjects

The study included a group of PCOS women and a control group of age and BMI-matched healthy women who were scheduled to undergo elective gynaecological surgery at Royal Derby Hospital. All participants were of reproductive age (20-45) with a BMI of 20-35k/m^2.

PCOS was diagnosed according to Rotterdam consensus criteria, by at least two of the following three features: 1) oligo-/anovulation, 2) clinical and/or biochemical hyperandrogenaemia (testosterone≥2.5 nmol/l or FAI≥5%), or 3) sonographic appearance of polycystic ovaries (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group 2004).

Healthy controls (non-PCOS) included women with regular menstrual cycles, normal serum testosterone levels <2.5 nmol/l and normal FAI<5%.

Exclusion criteria were endocrine or metabolic disorders e.g. thyroid disease, diabetes or hyperprolactinaemia, concurrent hormonal therapy e.g. contraceptive pill, anti-androgenic medication and corticosteroids or any weight loss medicines. Women unable to provide written informed consent were excluded.

Adipose tissue biopsies

Approximately 5g of subcutaneous adipose tissue (SAT) samples were obtained from abdominal incisions during elective gynaecological procedures including ovarian drilling, salpingectomy, myomectomy and diagnostic laparoscopy. The biopsy was divided into two parts, one immediately snap frozen and stored at -80°C until use for expression analyses. The second part was transferred into a sterile container containing Hanks' balanced salt solution (HBSS) for primary cell culture on the same day.

SAT primary cell culture

These were prepared as described in our previous publication (Cadagan *et al.* 2014). Briefly, SAT biopsies were cut into small pieces and enzymatically dispersed using collagenase (1mg/ml) prepared in divalent cation-free HBSS. The preparation was incubated at 37°C for no more than 60 minutes then filtered

through a 250μm nylon mesh. This resulted in a solution with two phases with the lower phase containing the stromal vascular function which includes preadipocytes. The lower phase was removed and centrifuged at 1000rpm for 5 minutes. The pellet (containing preadipocytes) was collected and resuspended in high glucose (4500mg/L) Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for culturing in a separate T25flask. Cells were maintained at 37°C in an air/5% CO_2 environment, until confluent (4-6 days).

To initiate in-vitro differentiation into mature adipocytes, preadipocytes were seeded at a density of $3x10^5$ cells/ml in a 24-well plate. Differentiation was induced (Day 0) with the addition of a hormone cocktail (denoted IDM) comprising 50nM insulin, 0.25mM 3-isobutyl-1-methylxanthine (IBMX) and 100nM dexamethasone. After 2 days, IDM was replaced with maintenance differentiation medium (MDM), similar to IDM but without IBMX. Triacylglycerol droplets became visible on Day 5 and cells were fully differentiated into adipocytes on Day 14. For negative controls, cells were maintained in DMEM containing FBS alone.

In-vitro differentiated mature adipocytes were kept for 24hrs in serum-free DMEM to prepare for the experiments. The cultures were then either left untreated (controls) or incubated for 72 hours with insulin (1, 10, 100nM) ± LY294002 (1μg/ml) or LH (10nmol/ml) ± insulin (1, 10, 100nM). The culture media were then removed from each well and testosterone concentrations were measured by a competitive ELISA using acetylcholinesterase (AChE) according to the manufacturer's instructions (Cayman Chemicals, Michigan, USA). This assay is based on the competition between testosterone and the testosterone AChE tracer for the antiserum binding sites and is inversely proportional to the concentration of antigen in the well. The assay was carried out by the addition of 50μl of testosterone standards (3.9-500pg/ml), cell culture supernatants, and media blanks, to precoated wells in a 96-well plate. Also included were wells for total activity and non-specific binding and all were carried out in duplicate. The plate was then incubated at 37°C for two hours, after which the wells were emptied and washed 5 times. Then, Ellman's reagent (200μl) was added to each well and the plate incubated (60-90 minutes) in the

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dark, with shaking before reading at 405nm. Cells remaining in wells were washed, harvested and stored at -80^{0}C for qRT-PCR.

RNA isolation and qRT-PCR

Frozen SAT biopsies (100-120mg) were homogenised (Janke and Kunkel, IKA Labortechnik, Germany) in TRI Reagent (Sigma Aldrich, Dorset, UK). The homogenate was then transferred to an Eppendorf tube and centrifuged at 12,000g for 15 minutes. The resulting fat layer overlying the homogenate was removed to avoid any carryover. Chloroform was then added to the homogenate (0.2ml of chloroform for every 1ml TRI Reagent), mixed thoroughly by manual shaking for 15 seconds, incubated at room temperature for 3 minutes then centrifuged for 15 minutes at 12,000g at 4°C. The resulting upper aqueous layer containing RNA was transferred to a new RNAse-free 1.5ml microcentrifuge tube. Total RNA was precipitated with isopropyl alcohol followed by incubation at room temperature for 10 minutes then centrifugation at 12,000g for 10 minutes at 4°C. The RNA pellet produced was washed with 75% ethanol, centrifuged at 7,500g for 5 minutes at 4 °C before dissolving the RNA pellet in RNase free water.

Total RNA from cultured mature adipocytes was extracted as described above except that TRI Reagent was added at 1ml for every 10cm^2 of the culture plate and cells lysed directly.

Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, Vilnius, Lithuania); 1200ng of total RNA was used to produce 20µl of cDNA. Reverse transcription was performed at 25^{0}C for 10 minutes, 37^{0}C for 2 hours, and 85^{0}C for 5 minutes. Each reaction was performed in duplicate: one containing the reverse transcriptase (+RT) and other one without RT (–RT) in which reverse transcriptase was substituted with water.

qRT-PCR for *CYP17A1* (17,20 alpha hydroxylase) and *AKR1C3*, (17B HSD5) was performed alongside three selected reference genes (*GAPDH, ACTB* and *LRP10*). The assay was performed using 2µl cDNA template and 18µl TaqMan Universal PCR Master Mix (Applied Biosystems, California, USA) containing the specific TaqMan expression assay for the selected gene (Applied Biosystems, California, USA). Each reaction was carried out in triplicate on +RT samples, and –RT samples as well as a no template control

(NCT). qPCR was carried out on a Chromo 4 (BioRad, UK) utilising an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute for annealing and extension. At the end of each cycle the plate was read and fluorescence intensity recorded.

The efficiency and optimization of the qRT-PCR reaction was evaluated by generating standard curves for three selected reference genes and target genes using a 10-fold dilution of a cDNA template. Each dilution was assayed in triplicate. PCR was carried out on a Chromo 4TM System (BioRad, UK).

Western blot (WB) for AKR1C3 and CYP17A1 proteins

Western immunoblot analysis of AKR1C3 and CYP17A1 protein expression in non-PCOS (n=7) and PCOS (n=4) patients was carried out by extracting total proteins from frozen SAT samples as previously described (Cadagan *et al.* 2014). Proteins were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Following blocking with 5% Marvel in TBS, immune detection of AKR1C3 and CYP17A1 proteins was performed by incubating overnight with rabbit anti-human AKR1C3 polyclonal antibodies (Antibody Registry ID, AB_2753336) a 1:1000 dilution or CYP17A1 (ID, AB_1603486), at a 1:100 dilution. After further washing, goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase was added to blots that were left to incubate at room temperature for two hours with gentle rocking. Molecular weight markers were used to facilitate protein size detection. Visualisation of bands was carried out using Immunstar reagent (BioRad, UK) and Chemidoc V4.2 (BioRad, UK). Each blot was then stripped and re-probed using β-actin to control for loading of the protein. Protein was quantified by densitometry and relative expression levels adjusted for β-actin.

Data analysis

qRT-PCR data were expressed as comparative threshold (Ct) values and analysed using GenEx software. The mean delta Ct (ΔCt) was calculated by using the Ct method to compare the relative amount of the target sequence to the values of the chosen reference genes (Vandesompele *et al.* 2002). The two-tailed,

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unpaired t-test was used to compare expression levels of target genes in SAT of PCOS and non-PCOS groups.

For ELISA data, values obtained were interpolated against the standard curve to determine the testosterone concentration. Students Unpaired t-test was used to compare between the PCOS and non-PCOS groups. Comparison of multiple means was performed using one-way analysis of variance (ANOVA) with post hoc comparisons employing Tukey's test. Statistical significance was considered when $P<0.05$. Data are expressed as mean ± SEM.

Results

Table 1 shows characteristics of the study participants inclining PCOS women (n=15) and age- and BMI-matched healthy controls (n=15).

Reference genes

Of the reference genes tested, *GAPDH, ACTB,* and *LPR10* were consistently expressed and stable across PCOS and non-PCOS groups with no statistically significant ($P>0.05$) difference between the two groups.

CYP17A1 and *AKR1C3* mRNA expression in SAT

CYP17A1 and *AKR1C3* mRNA were expressed in SAT of both non-PCOS (n=8) and PCOS (n=8) women (Figure 1). *ACTB, GAPDH,* and *LPR10* were the references genes used to normalise gene analysis. Data analysis showed no statistically significant ($P>0.05$) difference in *CYP17A1* mRNA in PCOS (12.25±4.31) versus non-PCOS (8.40±4.53) SAT (Figure 1a). On the other hand, *AKR1C3* mRNA expression in PCOS SAT (15.12±2.00) was significantly ($P<0.001$) higher than that (3.30±1.03) of the non-PCOS SAT (Figure 1b).

AKR1C3 & CYP17A1 protein expression in SAT

Western immunoblotting showed expression at 36kDa and 57kDa suggestive of the presence of AKR1C3 and CYP17A1 respectively in SAT of both groups (non-PCOS, n=7; PCOS, n=4) (Figure 1c,d). The relative intensity (normalized to β-actin) of AKR1C3 and CYP17A1 proteins expression were similar in

PCOS compared to non-PCOS. There was no significant difference in the protein expression between non-PCOS and PCOS (AKR1C3, P=0.6989; CYP17A1, P>0.05) (Figures 1e,f).

CYP17A1 mRNA expression in *in-vitro* differentiated mature adipocytes

Figure 2 shows *CYP17A1* mRNA expression in untreated and hormone-treated mature adipocyte cultures prepared from SAT of PCOS (n=5) *vs.* non-PCOS (n=5) women. The expression in untreated adipocytes was significantly (P<0.0001) higher in PCOS (17.98±1.30) versus non-PCOS (1.12±0.17) adipocytes (Figure 2). Addition of increasing concentrations of insulin (1, 10, and 100nM) to the adipocyte cultures did not result in any statistically significant (P>0.05) change in the *CYP17A1* mRNA expression in either of the two groups (Figure 2a). On the other hand, addition of LH (10nmol/L) resulted in a statistically significant (P<0.001) increase in *CYP17A1* mRNA expression in non-PCOS, but not in PCOS adipocytes (Figure 2b). Adding increasing concentrations of insulin with LH did not have any additional effect on the expression of *CYP17A1* mRNA in either of the two groups (Figure 2b).

AKR1C3 mRNA expression in *in-vitro* differentiated mature adipocytes

Figure 3 shows *AKR1C3* mRNA expressions in untreated and hormone-treated mature adipocytes. The level in untreated mature adipocytes of PCOS women (5.19±1.27) was significantly (P=0.01) higher than that (0.97±0.21) of non-PCOS women (Figure 3). Only insulin at 100nM resulted in a statistically significant (P<0.001) increase in the expression of *AKR1C3* mRNA in adipocytes of non-PCOS, but not of PCOS women (Figure 3a). With regards to the lower insulin concentrations, there was a trend towards a dose-dependent rise in *AKR1C3* mRNA expression in non-PCOS adipocytes, but this did not reach statistical significance. Addition of LH (10nmol/L) resulted a significant (P<0.01) increase in *AKR1C3* mRNA expression in adipocytes of non-PCOS, but not of PCOS women (Figure 3b). Adding increasing concentrations of insulin with LH, did not have any additional effect on the expression of *AKR1C3* mRNA in either of the two groups.

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Testosterone concentration in differentiated adipocytes

Testosterone concentration in the supernatant of untreated cultured PCOS adipocytes (mean±SEM, 129.27±2.54 pg/ml) was significantly (P<0.0001) higher than that (33.67±4.56 pg/ml) of non-PCOS adipocytes (Figure 4). Addition of insulin (1, 10 and 100nM) resulted in a significant dose-dependent increase in testosterone concentrations in non-PCOS adipocyte cultures but had no effect on PCOS adipocytes (Figure 4a). Adding LH (10nm/ml) to cultured adipocytes had a much more potent effect than insulin with a significant (P<0.0001) increase in testosterone concentration from adipocytes of non-PCOS women (n=5) but had no effect in PCOS adipocytes (Figure 4b). Addition of increasing insulin concentrations to LH had no additional effect on testosterone concentrations.

The Role of PI3-K in insulin-induce augmentation of *AKR1C3* and testosterone in non-PCOS adipocytes

Addition of the PI-3K inhibitor (LY294002, 1μm/ml) with increasing concentrations of insulin to cultured non-PCOS adipocytes did not inhibit the stimulatory effect of insulin on *AKR1C3* mRNA expression or testosterone concentration in adipocyte culture (Figure 5a, 5b).

Discussion

In this *in-vitro* study we investigated the dynamics of androgen production and its interactions with insulin and LH in SAT biopsies obtained from women with and without PCOS. To the best of our knowledge, this is the first report on *CYP17* expression, insulin actions on *CYP17* and *AKR1C3* and the role of the PI-3k insulin signalling pathway in adipocytes derived from SAT of PCOS women. The results show upregulated *AKR1C3* and *CYP171A* mRNA expression in SAT and in cultured subcutaneous adipocytes of PCOS women. In addition, testosterone concentrations were markedly higher in cultured adipocytes derived from SAT of PCOS women. Whilst, exposure to insulin had no effect on *CYP17A1* mRNA expression, it resulted in a dose-dependent augmentation of *AKRC13* expression and testosterone secretion in cultured non-PCOS adipocytes, with no effect on PCOS cultured adipocytes. These stimulatory effects of insulin are independent of the PI-3k signalling pathway. Notably, LH alone resulted in a marked increase of *CYP17A1*, *AKR1C3* and testosterone levels in non-PCOS, but not in PCOS adipocytes.

In our experiments, we used insulin concentrations of 1, 10 and 100nM, which are equivalent to 5.7, 57.3 and 573.4 ng/mL respectively. These concentrations correspond to physiological (1-30 ng/mL), moderately supraphysiological (50-100 ng/mL) and markedly supraphysiological (500-1000ng/ml) in-vivo insulin levels (Poretsky et al. 2001; Munir *et al.* 2004). The moderately supraphysiological levels represent insulin concentrations in women with PCOS. With regards to the LH concentration, in the absence of any previous similar studies, we used 10nmol/l, which is thought to mimic in-vivo physiological levels. However, our LH experiment should be considered preliminary and future studies should use more accurately measured concentrations to represent in-vivo physiological and supraphysiological levels.

As stated above, there are no previous studies on *CYP17* mRNA expression in SAT of PCOS women. Our data are consistent with previous studies on non-PCOS SAT confirming the presence of *CYP17* either directly by measuring *CYP17* mRNA using qRT-PCR (Puche et al, 2002) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kinoshita et al, 2014) or indirectly by measuring 17-OH-Progesterone and A-dione, which require CYP17. In contrast, other studies failed to detect *CYP17* mRNA in SAT of healthy women (Wang et al, 2012; Dalla Valle et al, 2006; Mackenzie et al, 2008). This may be due to an undetectable *CYP17* isozyme or instability of CYP17 in adipose tissue (Kinoshita et al, 2014).

Our SAT *AKR1C3* data are consistent with two recent similar studies reporting increased *AKR1C3* mRNA expression, measured by qRT-RT in SAT biopsies from PCOS women compared with healthy controls (O'Reilly el al, 2017; Wang et al, 2012).

Our testosterone results are supported by a previous in-vivo study by O'Reilly et al (2017) who reported increased testosterone concentrations in SAT microdialysate in PCOS women (n=10) compared to healthy controls (n=10).

We present the first study on the effects of insulin on *AKR1C3* and *CYP17A1* expression in PCOS cultured adipocytes derived from SAT. Our non-PCOS data are consistent with that of O'Reilly *et al* (2017) who

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297 confirmed the stimulatory effect of insulin (20nM) on the expression and activity of *AKR1C3* and
298 *CYP17A1* mRNA in cultured subcutaneous adipocytes from non-PCOS women (n=3).

299 With regards to our PI-3k data, there have been no previous studies on adipocytes. When compared with
300 data from the ovary, our results are consistent with one study showing that insulin-mediated
301 steroidogenesis in granulosa cell culture was not inhibited by PI-3k inhibitor (wortmannin) (Poretsky et al.
302 2001). In contrast, our data disagree with another study on theca cells suggesting PI-3k as a possible
303 mediator for the insulin stimulatory effects on *CYP17* mRNA expression or activity (Munir *et al.* 2004).
304 Further studies are therefore required to address this uncertainty.

305 We present the first data on insulin/LH interactions in adipose tissue. Our findings are consistent with one
306 study on theca cells showing that insulin alone is capable of stimulating testosterone production in women
307 with and without PCOS (Nestler *et al.*1998). In contrast, our data disagree with another study on cultured
308 theca cells reporting that insulin requires LH-induced cAMP activation to stimulate *CYP17* activity
309 (Munir *et al.* 2004).

310 The lack of any stimulatory effects of insulin/LH on androgen production in PCOS adipocytes was
311 surprising. The exact explanation of this phenomenon remains uncertain. It is tempting to postulate that
312 the high androgen levels in PCOS adipocytes may have reached a plateau level beyond which they cannot
313 be augmented any further. However, our data do not support this hypothesis as insulin at high
314 concentration (100nM) increased *AKR1C3* and testosterone in non-PCOS adipocytes to levels higher than
315 those of PCOS adipocytes. This suggests that PCOS *AKR1C3* and testosterone are "potentially" not at a
316 plateau. Another possible mechanism is the presence of a defect in insulin/LH receptors in PCOS
317 adipocytes affecting their sensitivity to insulin/LH. This is supported by previous in-vitro studies reporting
318 post-binding defect in insulin signal transduction in PCOS adipocytes in relation to glucose uptake
319 (Diamanti-Kandarakis and Dunaif 2012; Ciaraldi et al, 1992). This defect has been attributed to serine
320 phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) secondary to increased
321 intracellular serine kinases (Dunaif et al, 1995; Li et al, 2002; Baillargeon et al, 2007, Baptiste et al,

2010). The increase in serine kinases has also been implicated in serine phosphorylation of CYP17, which is known to increase its 17,20-lyase activity with subsequent increase in androgen production. In other words, the same serine kinase, which inhibits insulin signaling, may also increase androgen production in PCOS (Diamanti-Kandarakis and Dunaif 2012).

The apparent discrepancy between the levels of mRNA and protein expressions of AKR1C3 and CYP17A1 in PCOS versus non-PCOS SAT could be due to the small numbers included in the WB experiment. Another possible explanation of the lack of mRNA-protein correlation could be due to the highly variable protein half-life ranging from a few seconds to several days possibly due to variation in protein stability or post-translational processing as well as RNA-silencing mechanisms (Beyer et al, 2004; Wu et al, 2008; Maier *et al.* 2009; Greenbaum *et al.* 2003).

One limitation of our study is the lack of sufficient data on the protein expression of the androgen synthesizing enzymes in SAT. Furthermore, we did not assess if the increased testosterone production in PCOS subcutaneous adipocyte culture is directly linked to *AKR1C* or *CYP17A1* activity. However, evidence for this link has been provided by O'Reilly et al (2017) who reported that inhibition of AKR1C3 activity by 3-4-trifluoromethyl-phenylamino-benzoic acid resulted in reduction of testosterone generation from primary subcutaneous adipocytes obtained from healthy (non-PCOS) women.

Our study has provided an important and new insight into the subcutaneous adipose tissue dynamics that may contribute to the pathogenesis and pathophysiology of PCOS. Our data support the hypothesis that SAT could be an important source of PCOS-related hyperandrogenaemia. The markedly higher testosterone levels in PCOS adipocytes seem to be independent of weight as both groups were weight-matched. Our data suggest that excess androgen production in subcutaneous adipocytes is not directly caused by PCOS-related hyperinsulinaemia. We also confirm that insulin and LH can each independently stimulate SAT androgen production in healthy women and there is no synergism between them.

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In conclusion, subcutaneous adipose tissue of PCOS women produces increased amounts of androgen, which is not directly linked to inulin or LH. This suggests that SAT is an important contributor to the PCOS-related hyperandrogenaemia.

Declaration of interest

We declare that neither of the authors has any conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors' contributions

S Amer: conception of the idea, study design, recruitment, obtaining the fat biopsies, interpretation of results and writing the manuscript

N Alzanati: Laboratory work, data collection, statistical analysis and manuscript review

A Warren: Lab experiment especially adipocyte culture and western blot and manuscript review

R Tarbox: Lab experiments especially PCR and manuscript review

R Khan: supervisor of the laboratory work, validation of laboratory techniques, review of all lab results, writing up and reviewing the manuscript

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Figure legends

Figure 1. *CYP17A1* and *AKR1C3* in SAT of PCOS vs non-PCOS women. a & b) Relative mRNA expression of *CYP17A1* an *AKR1C3* in SAT from non-PCOS (n=8) versus PCOS (n=8) women. Data expressed as mean±SEM, analysed with GenEx and compared using the ΔCt method. c - f) Western immunoblot analysis of AKR1C3 and CYP17A1 protein expression in SAT from non-PCOS (n=7) versus PCOS (n=4) women. C & d) Relative intensity of AKR1C3 and CYP17A1 protein expression. e & f) Relative levels of band intensity in WB of AKR1C3 in both groups. Data presented as mean±SEM. ***P<0.001

Abbreviations: P, PCOS; Non-P, Non-PCOS; SAT, subcutaneous adipose tissue

Figure 2. *CYP17A1* mRNA expression in untreated and hormone treated mature adipocyte cultures of PCOS (n=5) *vs.* non-PCOS (n=5) women. a) Expression in adipocytes treated with different concentrations of insulin (1, 10, 100nM). b) Expression levels in adipocytes treated with LH (10 nmol/ml) ± different concentrations of insulin (1 nM, 10 nM, 100 nM). Data presented as mean ± SEM ΔCt and analysed using one-way ANOVA.

Data presented as mean ± SEM. ***P<0.001; ****P <0.0001

Figure 3. *AKRC13* mRNA expression in untreated and hormone treated mature adipocytes of PCOS (n=5) *vs.* non-PCOS (n=5) women. a) Expression in adipocytes treated with different concentrations of insulin (1, 10, 100nM). b) Expression levels in adipocytes treated with LH (10nmol/ml) ± different concentrations of insulin (1, 10, 100nM). Data presented as mean ±SEM ΔCt and analysed using one-way ANOVA test. *P<0.05; **P <0.01; ***P <0.001.

Figure 4. Testosterone secretion (pg/ml) by untreated and hormone treated mature adipocytes of PCOS (n=5) vs. non-PCOS (n=5) women. a) Levels in adipocytes treated with increasing concentrations of insulin (1, 10, 100nM). c) Levels in adipocytes treated with LH (10nmol/ml) ± insulin in different concentrations. Data presented as mean ± SEM and analysed using one-way ANOVA. **P<0.01; ***P <0.001; ****P <0.0001.

Figure 5. the role of PI-3K inhibitor (LY294002) in insulin mediated actions in non-PCOS adipocytes (n=5). a) Expression of *AKR1C3* in non-PCOS adipocytes treated with insulin (1, 10, 100nM) ± LY294002 (1μg/ml). b) Testosterone levels (pg/ml) in adipocytes treated with insulin (1, 10, 100nM) ± LY294002. Data presented as mean ± SEM and analysed using one-way ANOVA. **P<0.01; ***P <0.001.

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Table 1 Demography of all women included

Parameter	non-PCOS (n=15)	PCOS (n=15)	P value
Age (year)	32.4 (25-45)	30.5 (24-36)	NS
BMI (Kg/m^2)	27.5 (20.0-32.2)	29.4 (20.9-31.62)	NS
Testosterone (nmol/l)	1.5 ± 0.2	2.4± 0.5	0.03
LH (nmol/l)	7.8 ± 1.4	29.8 ± 19.2	<0.01

Data summarised as median (range) and mean± SEM.

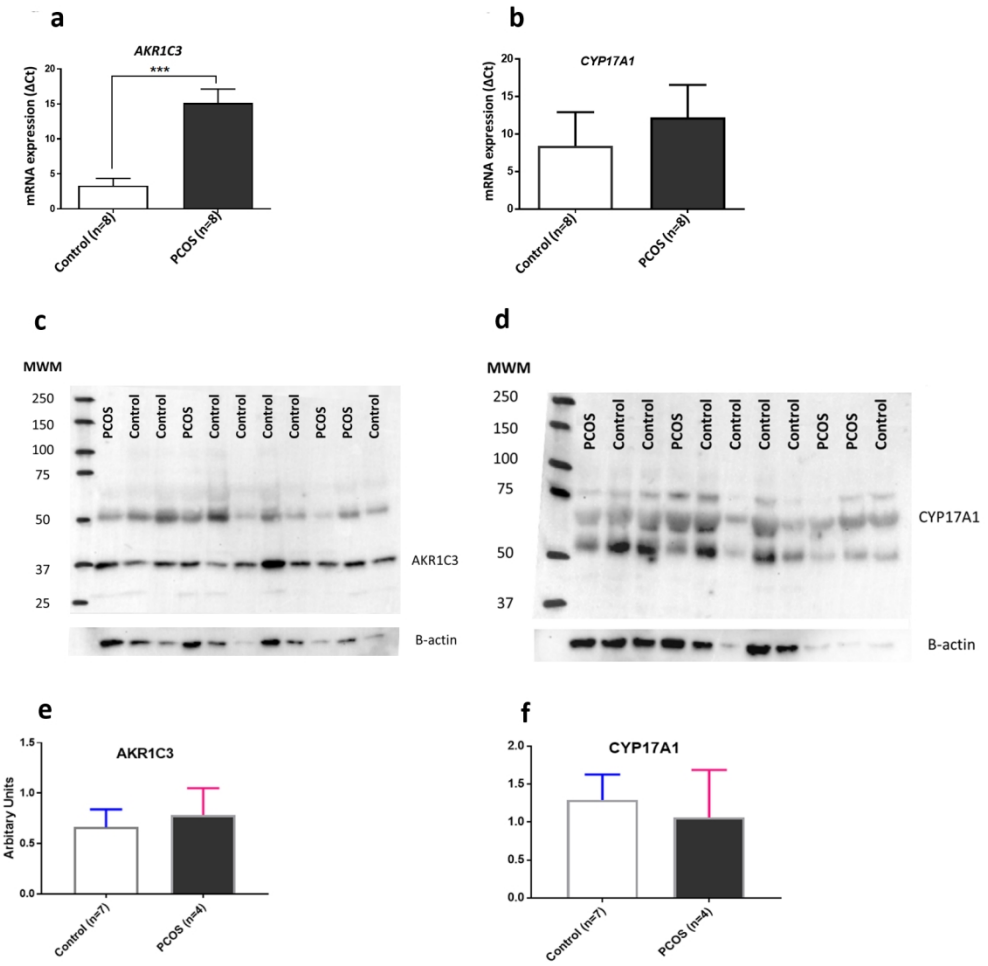

Figure 1. CYP17A1 and AKR1C3 in SAT of PCOS vs non-PCOS women. a & b) Relative mRNA expression of CYP17A1 an AKR1C3 in SAT from non-PCOS (n=8) versus PCOS (n=8) women. Data expressed as mean±SEM, analysed with GenEx and compared using the ΔCt method. c - f) Western immunoblot analysis of AKR1C3 and CYP17A1 protein expression in SAT from non-PCOS (n=7) versus PCOS (n=4) women. C & d) Relative intensity of AKR1C3 and CYP17A1 protein expression. e & f) Relative levels of band intensity in WB of AKR1C3 in both groups. Data presented as mean±SEM. ***P<0.001
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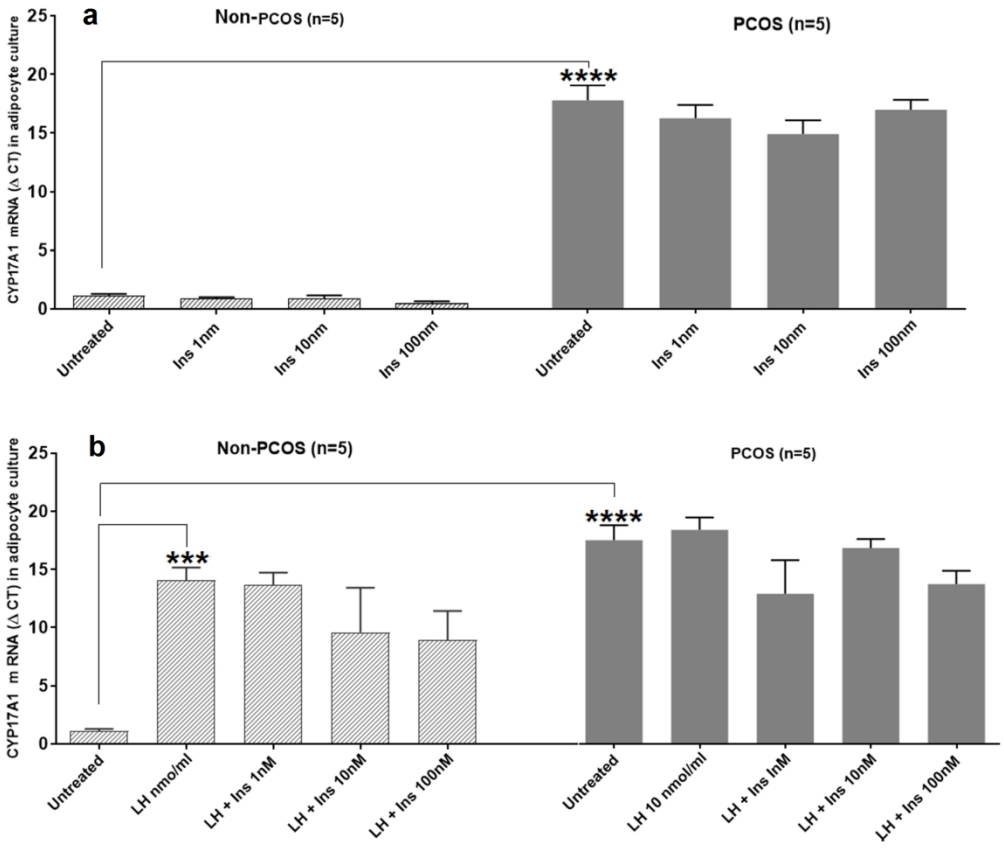

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Data presented as mean ± SEM. ***P<0.001; ****P <0.0001

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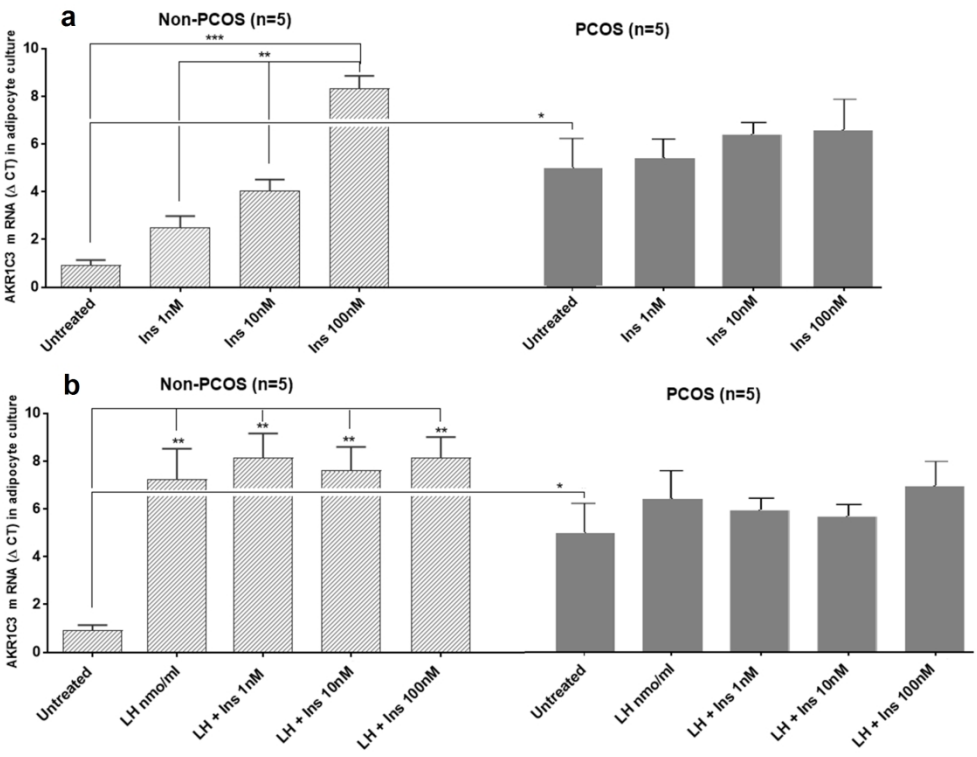

Figure 3. AKRC13 mRNA expression in untreated and hormone treated mature adipocytes of PCOS (n=5) vs. non-PCOS (n=5) women. a) Expression in adipocytes treated with different concentrations of insulin (1, 10, 100nM). b) Expression levels in adipocytes treated with LH (10nmol/ml) ± different concentrations of insulin (1, 10, 100nM). Data presented as mean ±SEM ΔCt and analysed using one-way ANOVA test. *P<0.05; **P <0.01; ***P <0.001.

522x399mm (120 x 120 DPI)

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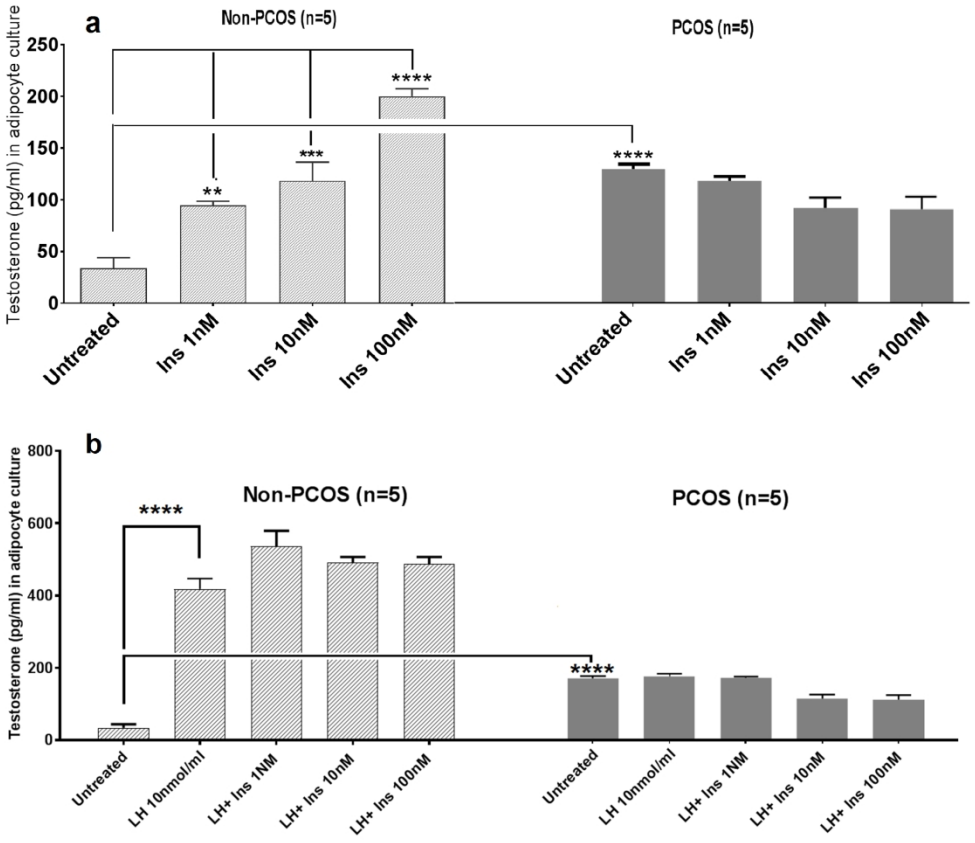

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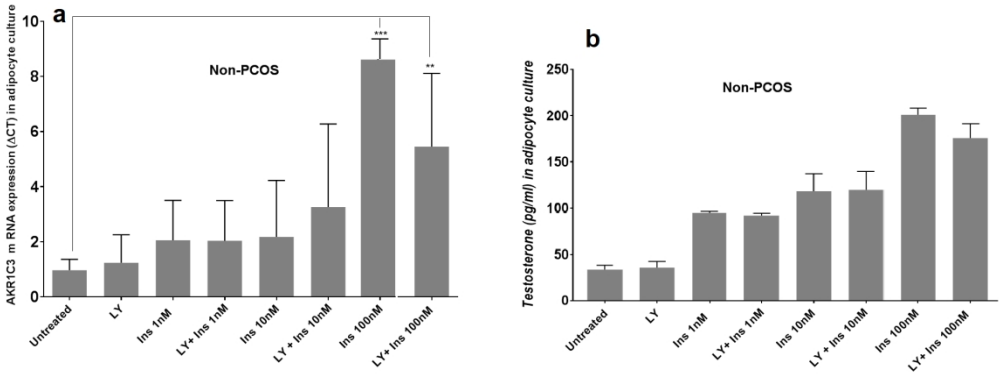

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