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AKR1C3-mediated adipose androgen generation drives lipotoxicity in women with polycystic ovary syndrome

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Context

Polycystic ovary syndrome (PCOS) is a prevalent metabolic disorder, occurring in up to 10% of women of reproductive age. PCOS is associated with insulin resistance and cardiovascular risk. Androgen excess is a defining feature of PCOS and has been suggested as causally associated with insulin resistance; however, mechanistic evidence linking both is lacking. We hypothesized that adipose tissue is an important site linking androgen activation and metabolic dysfunction in PCOS.

Methods

We performed a human deep metabolic *in vivo* phenotyping study, examining the systemic and intra-adipose effects of acute and chronic androgen exposure in ten PCOS women, in comparison to ten body mass index-matched healthy controls, complemented by *in vitro* experiments.

Results

PCOS women had increased intra-adipose concentrations of testosterone (p=0.0006) and dihydrotestosterone (p=0.01), with increased expression of the androgen-activating enzyme aldo-ketoreductase type 1 C3 (AKR1C3, p=0.04) in subcutaneous adipose tissue. Adipose glycerol levels in subcutaneous adipose tissue microdialysate supported *in vivo* suppression of lipolysis after acute androgen exposure in PCOS (p=0.04). Mirroring this, non-targeted serum metabolomics revealed pro-lipogenic effects of androgens in PCOS women only. *In vitro* studies showed that insulin increased adipose AKR1C3 expression and activity while androgen exposure increased adipocyte *de novo* lipid synthesis. Pharmacological AKR1C3 inhibition *in vitro* decreased *de novo* lipogenesis.

Conclusions

These findings define a novel intra-adipose mechanism of androgen activation that contributes to adipose remodelling and a systemic lipotoxic metabolome, with intra-adipose androgens driving lipid accumulation and insulin resistance in PCOS. AKR1C3 represents a promising novel therapeutic target in PCOS.

This integrated in vivo and in vitro study examines the role of AKR1C3 in adipose androgen generation in PCOS, and how locally activated androgens exert lipotoxic effects on adipose lipid metabolism.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a prevalent disorder in women of reproductive age, and is defined by androgen excess and anovulatory infertility (1, 2). It affects 6.1-19.9% of women, depending on diagnostic criteria applied (3). In terms of clinical consequences, however, PCOS is primarily a disorder with adverse metabolic risk impact, as evidenced by a high prevalence of insulin resistance (4), dyslipidemia (5), and increased rates of type 2 diabetes mellitus and hypertension (3, 6), over and above those observed in simple obesity. Recent data also highlight increased rates of cardiovascular disease (7) and non-alcoholic fatty liver disease (8, 9) in women affected by PCOS.

Androgen excess is a defining feature of PCOS (10); however, its origins remain insufficiently understood. Although traditionally perceived as a disorder of purely ovarian origin, it is now clear that there are also complex contributions from the adrenal and peripheral tissues to PCOS-related androgen excess (11, 12) (13, 14). The potent androgens testosterone (T) and 5α-dihydrotestosterone (DHT) can be activated from androgen precursors in peripheral target tissues of androgen action (12, 15). The severity of androgen excess and insulin resistance in PCOS are closely correlated, but the direction of causality remains unclear. Insulin resistance due to monogenic insulin receptor mutations has been shown to be associated with a severe androgen excess phenotype (16). Conversely, monogenic causes of androgen excess are associated with adverse metabolic consequences and can present with a PCOS phenotype (17-19).

One of the major metabolic compartments, adipose tissue, is capable of androgen generation, a process that is tightly regulated by a complex network of activating and inactivating enzymes (20). The enzyme aldo-ketoreductase type 1C3 (AKR1C3), which converts androstenedione (A4) to the biologically active androgen T, is abundantly expressed in adipose tissue (21), with increased expression in subcutaneous fat from both women with simple obesity (22) and PCOS (23). Previous work has shown that AKR1C3 is the only enzyme expressed in adipose tissue that is capable of activating A4 to T (22). The enzyme 5α-reductase type 1 (SRD5A1) activates T in peripheral target tissues to the most potent androgen, DHT, and is highly expressed in adipose tissue (24). Studies in rodent and non-human primate models show that pre- and postnatal androgen exposure in females leads to increased adipocyte size, enlarged visceral fat depots and reduced insulin sensitivity (25-27). However, human data on the effects of androgen excess on key processes of adipose biology, such as adipogenesis and lipid metabolism, are lacking.

In this study, we explored the hypothesis that increased intra-adipose androgen generation by AKR1C3 is a central driver of adipose tissue dysfunction and lipotoxicity in PCOS. To test our hypothesis, we undertook a study utilizing innovative *in vivo* physiology tools for deep metabolic phenotyping in a cohort of women with PCOS and age-, sex- and BMI-matched healthy controls, alongside *in vitro* experiments defining the impact of androgens on human adipocyte biology.

METHODS

Study participants

Women with PCOS aged between 18 and 40 years were recruited from outpatient clinics at University Hospitals Birmingham and Birmingham Women's Hospital. Full ethical approval was obtained from Edgbaston Research Ethics Committee (reference 12/WM/0206) and all participants provided written informed consent prior to inclusion in the study. PCOS was

diagnosed according to the Rotterdam European Society of Human Reproduction and Embryology (ESHRE) 2004 criteria, with the presence of two or more of the following: oligo/anovulation (Anov), clinical signs of androgen excess (AE), and polycystic ovaries (PCO) on ultrasound (10); however, only PCOS women with clinical or biochemical signs of androgen excess were recruited to the *in vivo* study (phenotypes AE+Anov+PCO, AE+Anov and AE+PCO). Other potential causes of oligomenorrhea and androgen excess were excluded by history, physical examination and biochemical assessment. All healthy controls were age- and BMI-matched, and recruited via local advertisement, with exclusion of PCOS on clinical and biochemical grounds. Recruitment numbers for each study group were agreed based on *a priori* power calculations; based on estimated detectable differences and standard deviations in adipose microdialysis data and serum androgen profiles across the DHEA challenge, a sample size of 10 PCOS and 10 control patients was deemed sufficient to achieve 80% statistical power for the detection of a difference with a p-value of <0.05. Exclusion criteria for the study were as follows: recent glucocorticoid treatment (within 3 months), pregnancy, age younger than 18 or older than 45 years, recent oral contraceptive use (within 3 months), hyperprolactinemia, thyroid disorders, and dysglycemia (impaired fasting glucose, impaired glucose tolerance or overt diabetes mellitus). PCOS women were also excluded if they had received prior treatment with metformin, insulin sensitizers or androgen receptor blockers. Detailed information on the clinical protocol is provided in **Supplementary Methods** and **Figure 1A**. Subcutaneous abdominal fat samples were obtained by excisional biopsy (2-3cm^3, approximately 3-5g) in both PCOS and control women under local anesthetic and sterile conditions, 5cm lateral to the umbilicus. Excised adipose tissue was immediately placed in RNALater (Life Technologies, Paisley, UK) and stored at -20°C for subsequent RNA extraction.

Biochemical analysis

Insulin (Mercodia) and serum free fatty acids (Zen-Bio) were measured using commercially available assays, according to manufacturer instructions. Plasma glucose was measured using the 2300 STAT PLUS analyzer (YSI Incorporated, Life Sciences, Ohio, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula [fasting glucose (mmol/L)*fasting insulin (pmol/L)/135]. Microdialysate samples were collected in microvials and analyzed using a mobile photometric, enzyme-kinetic analyzer (CMA Iscus Flex) for glucose, pyruvate, lactate and glycerol.

Steroid metabolite analysis (adipose microdialysate, serum and cellular supernatant)

Androgens from adipose microdialysate, serum and cellular supernatant were measured by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a Waters Xevo mass spectrometer with Acquity uPLC system, as described previously (11). Measurement of T, A4 and DHEA was facilitated by serum steroid oxime analysis, and carried out in positive mode. T, A4 and DHEA were extracted from 150µL interstitial fluid, 400µL serum and 1mL cellular supernatant via liquid-liquid extraction with 1, 2 and 4mL of tert-butyl-methyl-ether (MTBE), respectively. This was followed by derivitization into oximes. Serum DHEAS was extracted from 20µL of serum with 100µL acetonitrile, before evaporation under constant nitrogen flow (28).

For each LC-MS/MS assay, chromatography was optimized using a methanol/water (0.1% formic acid) gradient system. Steroids were quantified according to a linear calibration series with appropriate internal standards. Individual steroids were identified by matching retention times and two mass transitions in comparison to a deuterated reference compound.

Measurement of urinary steroid metabolite excretion by GC/MS is described in the **Supplementary Methods**.

Non-targeted serum metabolomics

We carried out mass spectrometry-based, non-targeted metabolome analysis in negative and positive ion mode at the Phenome Centre Birmingham, UK. In-depth methodology for non-targeted serum metabolomic profiling has been published by our group previously (24) and the full protocol is available in the **Supplementary Methods**.

Primary human adipocyte culture

Paired primary subcutaneous preadipocytes were isolated from adipose tissue obtained from healthy, non-diabetic women aged 18-45 years undergoing elective abdominal laparotomies for non-malignant, non-inflammatory disease, using methods described previously (29). Samples from women with diabetes or treated with systemic glucocorticoids in the last 3 months were excluded. Sample collection was facilitated via the University of Birmingham Tissue Biorepository. Briefly, adipose tissue samples were dissected into 2-3mm^3 pieces and digested with type II collagenase (Sigma Aldrich, Dorset, UK) at 37°C for 60 minutes. Samples were then centrifuged at 12,000rpm for 5 minutes. Preadipocytes were extracted from a pellet containing the stromovascular components and resuspended in DMEM/nutrient mixture F12, containing fetal bovine serum (FBS) 10% (Sigma-Aldrich, UK) and penicillin-streptomycin 1% (Applied Biosystems, UK). Preadipocytes were proliferated to confluence before chemically-defined culture media was applied for 14 days to stimulate differentiation into adipocytes. Differentiation was initiated by washing the cells in serum-free media, followed by culture with differentiation media (DMEM-F12 media containing biotin 33μM, pantothenate 17μM, T3 1nM, insulin 167nM, cortisol 1μM and rosiglitazone 1μM. AKR1C3 activity was determined by generation of testosterone after incubation of cells with 200nM of the testosterone precursor androstenedione (A4) in serum-free media for 24 hours.

Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocyte cell line culture

Conditions for culture and differentiation of the human pre-adipocyte SGBS cell line are described in the **Suppl. Appendix**.

Functional studies of lipid metabolism

A detailed description of methodology for *de novo* lipogenesis, β-oxidation and free fatty acid uptake is provided in **Suppl. Appendix**.

Statistics

The Statistical Package for the Social Sciences (SPSS, Version 22, Chicago, Ill., USA) was used for data analysis. Data are presented as mean ± SEM unless otherwise stated. Baseline clinical and biochemical data were not normally distributed and are therefore presented as median and interquartile range. AUC analysis was carried out using the trapezoidal method. For comparison of single variables, *t* tests (paired or unpaired as appropriate) were used, or nonparametric equivalents where data was not normally distributed. One-way ANOVA with post hoc Tukey testing was used for multiple comparisons between different groups. For realtime PCR data, statistical analysis was performed on mean Δct values only. Correlation testing was performed using Pearson's correlation coefficient or Spearman's test as appropriate. Serum metabolomics data was analyzed by applying univariate (Mann-Whitney U test or Wilcoxon signed rank test) analysis after normalization to total peak area per sample.

RESULTS

Systemic androgen concentrations and measures of insulin resistance

Ten women with PCOS and 10 healthy, sex-, age- and body mass index-matched volunteers were recruited to an *in vivo* physiology study for deep metabolic phenotyping (see **Fig. 1A** and for detailed protocol description the **Suppl. Appendix**); clinical and biochemical characteristics of both groups are summarized in **Suppl. Table 1**.

Baseline circulating androgens, measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), were all significantly higher in PCOS than in controls, as was insulin resistance assessed by HOMA-IR (p=0.003) (**Suppl. Table 1**). Similarly, truncal fat mass and volume on body composition imaging by dual x-ray absorptiometry (DXA) were increased in the PCOS group compared to matched controls (**Suppl. Table 1**). These findings demonstrate the expected systemic androgen excess, insulin resistance, and increased visceral adiposity in PCOS subjects in comparison to sex-, age- and body mass index-matched controls.

Adipose tissue-specific androgen excess in PCOS

To explore *in vivo* androgen concentrations in the adipose tissue metabolic compartment, we inserted a microdialysis catheter into the abdominal subcutaneous adipose tissue of each participant, which allowed the sampling of adipose tissue interstitial fluid across a semi-permeable membrane. We employed LC-MS/MS to measure androgens in adipose microdialysate in both PCOS and control subjects, representative of *in vivo* intra-tissue androgen homeostasis.

Adipose tissue concentrations of the androgen precursors DHEA (p=0.01) and A4 (p=0.01) and the active androgen T (p=0.0006) were significantly increased in PCOS subjects (**Fig. 1B-D**). The most potent androgen DHT was quantifiable in adipose interstitial fluid in all 10 PCOS patients and in six of the 10 control patients, with significantly higher concentrations in PCOS (4.5±1.7 *vs* 0.9±0.3nmol/l, p=0.01, **Fig. 1E**). Adipose tissue microdialysate concentrations of DHEA, A4 and DHT all correlated positively with HOMA-IR (R=0.51, p=0.02, R=0.54, p=0.01 and R=0.44, p=0.04, respectively), while intra-adipose T correlated positively with fasting insulin levels (R=0.49, p=0.04) (for complete correlation analysis, see **Suppl. Table 2**).

Of note, concentrations of the androgen precursors DHEA and A4 were higher in serum, i.e. in systemic circulation, than in adipose tissue in both PCOS and control women (**Fig. 1B+C**). By contrast, intra-adipose concentrations of the androgen receptor-activating androgens T and DHT were significantly higher than serum levels in PCOS women (p<0.0001, **Fig. 1D+E**). Androgen concentrations in control women did not differ between circulation and adipose tissue.

The intra-adipose ratio of T to A4, reflective of AKR1C3 activity, was significantly higher than in serum (p<0.0001, **Fig. 1F**) in the PCOS patients but not in the BMI-matched controls (data not shown). Similarly, in the PCOS women, the intra-adipose ratio of DHT to T, reflecting 5α-reductase activity, was significantly higher than in serum (p<0.0001, **Fig. 1G**).

Subcutaneous adipose tissue biopsies from PCOS and control women were used for mRNA expression analysis by realtime qPCR. This showed significantly increased AKR1C3 mRNA expression in subcutaneous fat from PCOS women (mean Δct PCOS 12.1±0.2 *vs* 13.1±0.4 in controls, p=0.04, **Fig. 1H**). Conversely, we found significantly decreased intra-adipose expression of SRD5A1 in PCOS women (mean Δct PCOS 16.1±0.4 *vs.* 14.3±0.4 in controls, p=0.005, **Fig. 1I**), indicating that AKR1C3 is the major driver of intra-adipose androgen excess in PCOS.

An acute oral androgen challenge suppresses in vivo lipolysis in PCOS adipose tissue

To examine the effect of androgens on *in vivo* adipose tissue metabolism, study participants received an oral dose of the androgen precursor DHEA at baseline, followed by sampling of

serum and adipose tissue microdialysate every 30 minutes for 4 hours. We have previously shown that this DHEA challenge test results in rapid generation of downstream androgens that can be detected in serum and urine (15, 30). Following the oral ingestion of 100mg DHEA, we measured circulating androgens by LC-MS/MS and documented significantly higher areas under the curve (AUC; mean±SEM nmol/L.min) in women with PCOS for serum DHEA (7437±720 *vs* 4317±654 in controls, p=0.005), A4 (258±65 *vs* 63±25 in controls, p=0.01), and T (2507±237 *vs* 1556±303 in controls, p=0.01). Increased circulating concentrations of T sulphate and DHT glucuronide after DHEA exposure in both controls and PCOS (all p<0.0001, **Fig. 1J-K**) further documented the extent of acute androgen generation following DHEA administration. This demonstrates that we exposed both groups to an equivalent acute androgen challenge on the background of chronic androgen excess (PCOS) or normal circulating androgen concentrations (controls).

To examine the *in vivo* metabolic impact of this androgen load, we measured markers of insulin sensitivity and lipid metabolism at baseline and after acute DHEA exposure, looking at both systemic (serum) and tissue-specific (adipose microdialysate) outcomes (**Fig. 2**). Plasma glucose levels were similar at baseline and across the DHEA challenge in both PCOS and controls (**Fig. 2A**). Baseline fasting insulin was significantly increased in PCOS (**Suppl. Table 1**), but AUC values for insulin across the DHEA challenge (pmol/L.min) did not differ significantly between PCOS and controls (14672±4120 *vs* 8043±2958, p=0.21, **Fig. 2B**). There was no difference in baseline or AUC values for serum free fatty acids between the two groups (**Fig. 2C**).

Assessing the adipose tissue-specific *in vivo* metabolic response, we measured metabolic markers in adipose microdialysate. Adipose pyruvate, lactate and glucose did not differ significantly between PCOS women and controls at baseline and in response to DHEA (**Fig. 2D-F**). However, AUC values for glycerol (μM.min) significantly decreased in PCOS patients after DHEA (PCOS 35347±4781 *vs* 22310±3577 in controls, p=0.04) (**Fig. 2G-H**), consistent with decreased lipid mobilization in PCOS in response to acute androgen exposure.

Androgen excess induces distinct changes in the circulating metabolome in PCOS

Mass spectrometry-based non-targeted metabolome analysis was employed to determine differences in global metabolic phenotype in PCOS serum compared to controls before and after DHEA administration. At baseline, we identified 119 metabolites that were statistically different (p<0.01) when comparing PCOS to control subjects. Significantly perturbed metabolite classes in PCOS included diacylglycerides, fatty acids and oxidized fatty acids, sterol and steroid metabolites, as well as glycerophospholipids (GPL) and lysoglycerophospholipids (LGPL) (**Fig. 3A+B**); other classes included aromatic amino acid metabolism, ceramides and sphingolipids, and ubiquinone/quinone metabolism. Both oxidized and non-oxidized fatty acids and the majority of diacylglycerides showed higher concentrations in control subjects, while GPL and LGPL concentrations were higher in PCOS subjects.

In response to DHEA administration, i.e. acute androgen exposure, the metabolome of PCOS patients (with a background of chronic androgen excess) and that of BMI-matched controls (with normal baseline androgens) showed distinct changes (**Fig. 3C-F, Suppl. Table 3**). DHEA induced highly significant changes in 15 diacylglycerides, 32 fatty acids and 16 oxidized fatty acids in the control group; in PCOS women, changes were observed in 16 diacylglycerides, 31 fatty acids and 19 oxidized fatty acids. In all cases, DHEA reduced circulating concentrations of diacylglcyerides and oxidized and non-oxidized fatty acids compared to baseline, in both

PCOS and controls, a demonstration of the potent effects of androgens in the regulation of lipid metabolism.

Of note, the majority of GPLs and LGPLs, which were significantly increased in PCOS at baseline (**Fig. 3B**), showed a diametrically opposed metabolic response to the acute androgen challenge, with significant further upregulation in PCOS and downregulation in the BMI-matched health controls (**Fig. 3E-F, Suppl. Table 3**), indicating a distinctly different metabolic response to androgens in PCOS.

Androgens promote adipose lipid accumulation in vitro

To dissect the observed effects in further detail, we utilized a human pre-adipocyte cell line, SGBS, previously validated as a model of human subcutaneous adipocyte biology (31). To determine the role of androgens in adipose lipid metabolism, differentiated adipocytes were treated with increasing concentration of T and DHT. Androgen treatment for 24 hours resulted in a dose-dependent increase in mRNA expression of acetyl-CoA-carboxylase (ACC1), the rate-limiting step in lipogenesis, but not ACC2 (fold change vs. untreated control: T 20nM 1.3±0.1, p=0.02; T 40nM 1.6±0.1, p<0.001; DHT 10nM 1.8±0.3, p=0.006; DHT 20nM 2.2±0.8, p=0.01, **Figure 4A**).

Consistent with upregulation of ACC1 mRNA expression, T 40nM significantly increased *de novo* lipogenesis in SGBS cells in the absence of insulin, as measured by uptake of 1-[^{14}C]-acetate incorporation into lipid (150.4±7.3% of untreated cells, p=0.02, **Figure 4B**). This effect was also observed with DHT 20nM (121.9±8.4%, p=0.03), confirming it as androgen-mediated as DHT, in contrast to T, cannot be converted to oestrogens via aromatase activity. Insulin (5nM) expectedly resulted in a significant increase of *de novo* lipogenesis compared to control incubations without insulin (250.0±19.1%, p=0.009). However, there was no additional effect of insulin when added to the incubations with androgens (p=0.9 for both T and DHT).

In SGBS cells, exposure to DHT 20nM significantly decreased β-oxidation of fatty acids compared to untreated control cells (83.2±5.9%, p=0.03) (**Figure 4C**) while we did not observe androgen effects on free fatty acid uptake (**Figure 4D**).

AKR1C3 links adipose androgen excess and lipid accumulation in PCOS

To examine the role of insulin in AKR1C3-mediated androgen generation, we examined expression and activity of AKR1C3 in primary human adipocytes and SGBS cells before and after insulin treatment. AKR1C3 mRNA expression increased significantly across differentiation from pre-adipocyte (day 0) to the mature adipocyte (day 14) phenotype in both subcutaneous and omental female adipocytes (mean Δct for subcutaneous fat: 16.4±0.5 [preadipocytes] *vs* 11.7±1.3 [adipocytes], p=0.03; mean Δct for omental fat: 19.5±0.1 [preadipocytes] *vs* 11.4±1.1 [adipocytes], p=0.001). AKR1C3 activity, as determined by generation of T after incubation of cells with 200nM A4 for 24 hours, was significantly upregulated across adipocyte differentiation in both subcutaneous and omental fat depots (p=0.02 and p=0.04, respectively).

AKR1C3 mRNA expression increased significantly in both SGBS and primary female subcutaneous adipocytes (n=3) after exposure to insulin 20nM (p=0.03 and p=0.04, respectively, **Figure 5A+C**), but not in primary female omental adipocytes (n=3, p=0.18). Similarly, AKR1C3 activity was significantly increased by insulin exposure at doses in SGBS cells and primary female subcutaneous adipocytes (**Figure 5B+D**), but not in primary omental adipocytes.

Selective pharmacological AKR1C3 inhibition with 3-4-trifluoromethyl-phenylamino-benzoic acid (10μM for 24 hours) significantly reduced generation of active androgen T from the androgen precursor A4. This was observed in both control adipocytes and those treated with 10nM insulin (100% [control] *vs* 59 ± 3% [inhibitor], p<0.0001; 131±10% [10nM insulin] *vs*

77±16% [inhibitor], p=0.02, **Figure 5E**). Adipocytes treated with 100nM A4 showed significantly increased *de novo* lipogenesis (179.4±21.5%, p=0.002); this effect was significantly attenuated by co-incubation with the selective AKR1C3 inhibitor (p=0.04, **Figure 5F**). Hence, disruption of AKR1C3-mediated androgen generation ameliorates androgen-mediated adverse effects on adipocyte lipogenesis, thereby mechanistically linking androgen excess and insulin resistance.

DISCUSSION

Utilizing a human *in vivo* physiology approach, we have demonstrated an adipose tissue-driven mechanistic link between androgen excess and lipid metabolism in PCOS. We used mass spectrometry to determine both systemic and adipose tissue androgen concentrations, revealing significantly increased androgen synthesis in PCOS adipose tissue, alongside increased expression and activity of the androgen-activating enzyme AKR1C3. This demonstrates the significant capacity of adipose tissue to act as a source of androgen excess in PCOS. Furthermore, androgens promoted *in vitro* lipid accumulation in human adipocytes, and suppressed *in vivo* lipolysis in women with PCOS. The net effect of this is promotion of adipocyte hypertrophy. Enlarged, 'lipid-overloaded' adipocytes are insulin resistant (32), fueling the adverse metabolic phenotype observed in PCOS, with lipid overspill and lipotoxicity in other organs such as liver. We could show that *in vitro* inhibition of AKR1C3 decreased androgen excess and subsequently *de novo* lipogenesis, identifying AKR1C3 as a novel target for therapeutic intervention in PCOS.

Several 17β-hydroxysteroid dehydrogenase enzymes are capable of activating androstenedione (A4) to testosterone (T), but of those only AKR1C3 is expressed in human adipose tissue (Quinkler et al., 2004). Interestingly, expression of the androgen-inactivating enzyme HSD17B2, which is capable of inactivating T to A4 and has been detected in omental adipose tissue (33), was not detectable in subcutaneous adipose tissue in the current study or in previous work by our group (22). Our studies reveal the androgen-activating enzyme AKR1C3 as the predominant driver of active androgen generation in adipose tissue of PCOS patients. AKR1C3 is a crucial enzyme in androgen biosynthesis in the adrenal, ovary, prostate and adipose tissue (34), and *AKR1C3* single nucleotide polymorphisms have been reported as associated with an increased prevalence of PCOS and hyperandrogenism in women (35). BMI differed slightly between the control and PCOS groups in this study (medians 33.1 v 28.6kg/m^2), although this difference did not show statistical significance, possibly due to the size of the cohorts. With this minor caveat in mind, our finding of increased AKR1C3 expression and activity confirm and expand the significance of results from a previous study describing increased AKR1C3 mRNA expression in subcutaneous adipose tissue of PCOS women (23).

In our *in vitro* studies, we conclusively demonstrated that AKR1C3 expression is regulated by insulin, mechanistically linking androgen excess and insulin resistance, the two major metabolic features in PCOS. We found that insulin increased AKR1C3 expression and T generation *in vitro*, and *in vivo* we found a significant correlation between adipose tissue T and circulating insulin. Some previously published evidence had provided preliminary evidence for the potential regulation of adipose androgen metabolism by insulin. The fasting induced transcription factor Kruppel-like factor 15 (KLF15) is crucially required for adipocyte differentiation (36), represents a key regulator of gluconeogenesis (37) and has been recently shown to represent a molecular link between glucose metabolism and lipogenesis (38). The AKR1C3 promoter contains a KLF15 binding site and *in vitro* overexpression of KLF15 results

in increased AKR1C3 promoter activity and T formation by adrenal NCIH295R cells (39). Intriguingly, we found increased expression of both AKR1C3 and KLF15 in the subcutaneous adipose tissue biopsies in our PCOS patients.

In this study, we demonstrated the functional impact of chronic and acute androgen exposure on the regulation of adipose tissue lipid metabolism, demonstrating suppressed lipolysis *in vivo* and increased lipogenesis *in vitro*. Data from non-targeted serum metabolomics support this observation of enhanced lipogenesis in PCOS. Serum metabolomics also revealed significantly increased concentrations of glycerophospholipids (GPL) and lysoglycerophospholipids (LGPL) in women with PCOS but not in controls; the acute androgen challenge yielded a further increase of these metabolites in PCOS patients, whereas concentrations in BMI-matched controls decreased. This demonstrates distinctly different metabolic responses to androgens in PCOS and controls and suggests that androgen excess plays a distinct in role in creating a lipotoxic metabolic environment. Both GPL and LGPL previously identified as markers of risk and progression in non-alcoholic fatty liver disease (40), which is more prevalent in women with PCOS.

In conclusion, we have shown evidence that intra-adipose androgen excess and dysfunctional adipose lipid metabolism are causally linked drivers of adverse metabolic risk in PCOS. We have described putative novel mechanisms by which androgens regulate adipose tissue function, lipid metabolism and fat mass, and have highlighted, at the level of the adipocyte, the enzyme AKR1C3 as a key regulator linking insulin resistance and androgen excess in PCOS. We postulate that a vicious circle in adipose tissue drives metabolic risk in PCOS (**Fig. 6A**), consisting of increased androgen generation in adipose tissue by AKR1C3, subsequent lipid accumulation and increased fat mass, resulting in systemic insulin resistance and lipotoxic organ damage, with androgen generation further exacerbated by hyperinsulinemia. We have shown that selective *in vitro* inhibition of AKR1C3 significantly reduced androgen-induced *de novo* lipogenesis. This suggests that local generation of T from A in subcutaneous adipose tissue by AKR1C3 is an important driver of androgen-mediated lipid accumulation.

The disruption of this vicious circle should now be studied in patients with PCOS, employing selective pharmacological inhibition of AKR1C3. Murine models are not suitable for the exploration of human androgen synthesis and metabolism as steroidogenic enzymes such as the 17β-hydroxysteroid dehydrogenase family are very differently organized in mice and men. In humans, AKR1C3 is a central gatekeeper enzyme not only in classic androgen synthesis, but also in the alternative pathway to DHT synthesis (41) and the 11-oxygenated androgen pathway (42). Recent work (43) has shown that 11-oxygenated androgens represent the majority of circulating androgens in PCOS, including 11-keto-testosterone, which has 3-4 fold higher serum concentrations than T in PCOS patients, while activating the androgen receptor with similar potency. Therefore, selective AKR1C3 inhibition is likely to provide most efficient control of PCOS-related androgen excess (**Fig. 6B**). In addition, based on our findings, inhibition of AKR1C3 is likely to exert beneficial effects on the adverse metabolic phenotype in PCOS, through amelioration of both circulating androgen burden and abnormal adipose tissue biology. In conclusion, AKR1C3 represents a highly promising, novel therapeutic target in women with PCOS.

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Disclosure summary: W.A. serves as a scientific consultant to Bayer AG, all other authors have nothing to declare

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Figure 1: Circulating androgens and *in vivo* intra-adipose tissue androgen synthesis in PCOS (n=10) and age- and body mass-index healthy controls (n=10). (A) Deep metabolic *in vivo* phenotyping protocol with serum and adipose microdialysate sampling every 30 minutes for 4 hours. Following baseline sampling, an acute androgen challenge was administered by oral intake of a 100mg dose of the androgen precursor dehydroepiandrosterone (DHEA) at 0min; schematic represents the classic androgen synthesis pathway from DHEA to active androgens. **(B-E)** Serum and *in vivo* intra-adipose concentrations of DHEA, androstenedione (A4), testosterone (T) and 5α-dihydrotestosterone (DHT) in controls (white circles) and PCOS women (red circles). Lines represent the median of each group. **(F-G)** Steroid ratios reflective of the conversion of A4 to T (T/A4) and the conversion of T to DHT (DHT/T) as measured in serum and adipose tissue microdialysate of PCOS women. Lines represent the median of each group. **(H-I)** Relative mRNA expression of the androgen-generating enzymes AKR1C3 (converting A4 to T) and SRD5A1 (converting T to DHT) in subcutaneous abdominal adipose tissue from PCOS and controls (n=7 for each). Lines represent the median of each group. **(J-K)** Serum T sulfate and DHT glucuronide concentrations before and 150 min after oral administration of the androgen precursor DHEA. Boxes represent median and 25^{th} to 75^{th} centile, whiskers represent 10^{th} and 90^{th} centile. Significance levels *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (Mann Whitney test). All steroid concentrations were measured by mass spectrometry-based assays.

Figure 2: Impact of acute oral androgen challenge (DHEA 100mg) on *in vivo* metabolic markers in serum and adipose tissue microdialysate. Serum glucose, insulin and free fatty acids **(A-C)** and intra-adipose pyruvate, glucose, lactate and glycerol levels **(D-G)** in PCOS (n=10, dark line, red circles) and control women (n=10, dotted line, white circles across the DHEA challenge test (mean±SEM). Time of oral administration of 100mg DHEA indicated by arrow. **Panel H**, area under the curve (AUC) for glycerol between 120 and 240 minutes after androgen exposure in the PCOS group compared to controls. *p<0.05 (Mann Whitney test). For further details see **Supplemental Methods**.

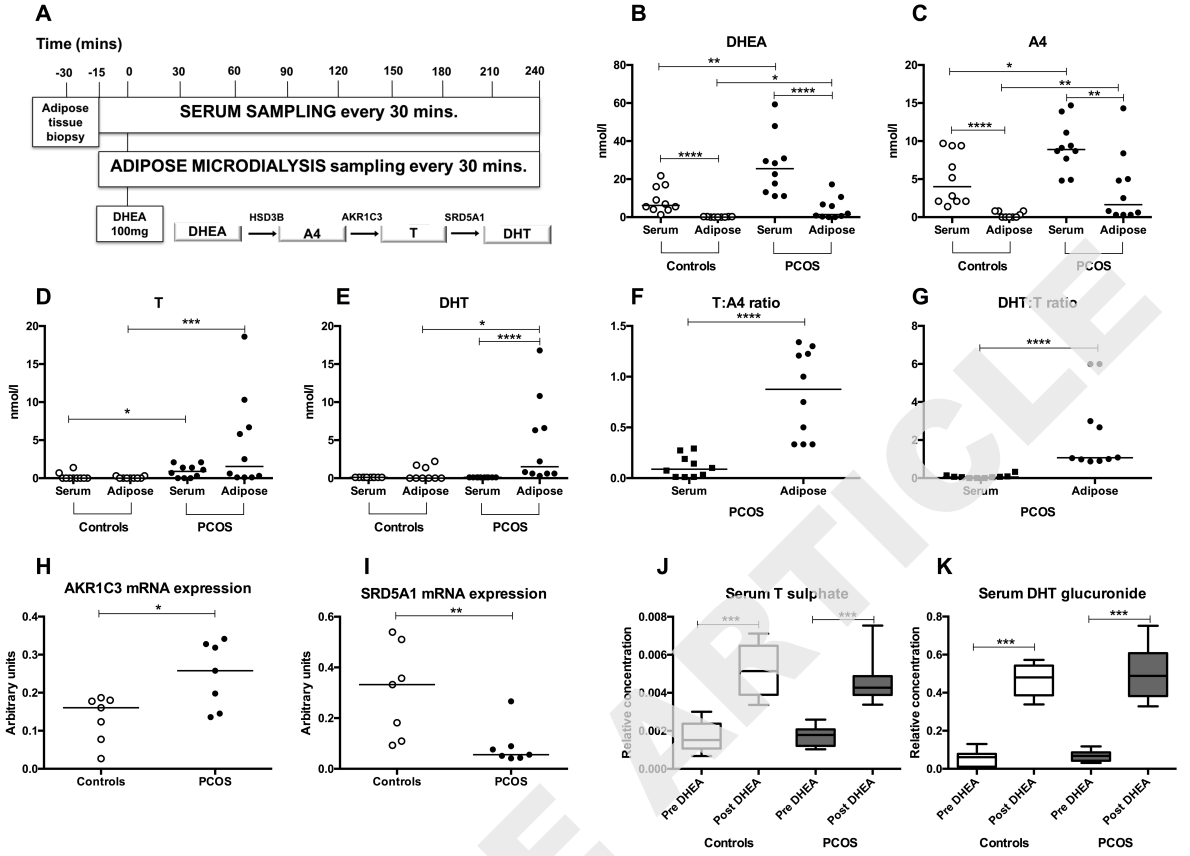
Figure 3: Baseline differences and changes induced in the non-targeted serum metabolome by acute androgen exposure in women with PCOS (n=10) and age- and BMI-matched healthy control women (n=10). Panel A, the number of serum metabolites associated with lipid and steroid metabolism observed to be significantly different (p<0.01) at baseline between control and PCOS subjects. **Panel B**, Significantly different glycerophospholipid (GPL) and lysoglycerophospholipid (LGPL) metabolites between PCOS women and controls at baseline. **Panels C-D**, Metabolic responses lipid and steroid metabolite following exposure of controls and PCOS subjects to an acute androgen challenge (DHEA 100mg administered orally at 0min; serum metabolome analysis carried out with 150min serum sample, representative of the maximum of circulating androgen concentrations after DHEA). **Panels E-F**, Differential response of GPL and LGPL to the acute DHEA challenge in comparison of controls and PCOS women. Data matrix analyzed applying univariate (Wilcoxon signed rank test) after normalization to total ion current per sample. Abbreviations: DAG, diacylglycerides; FA, fatty

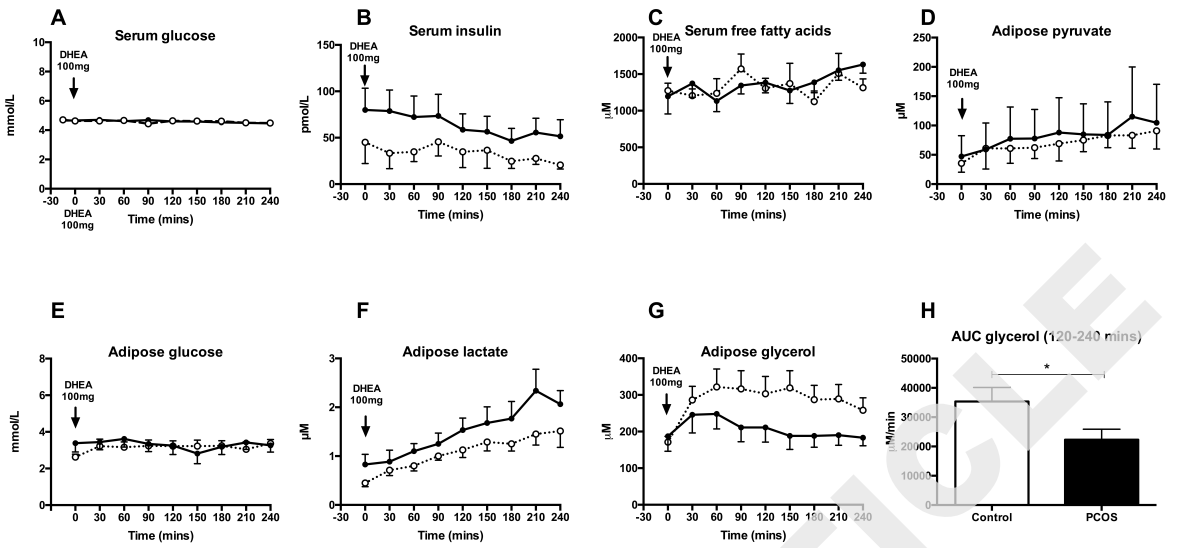
acids; GPL, glycerophospholipids; LGPL, lysoglycerophospholipids; OFA, oxidized fatty acids; SS, sterols and steroid metabolites.

Figure 4: The effects of androgens on adipose lipid metabolism in the human pre-adipocyte SGBS cell line. Panel A, The effect of androgens [testosterone (T) light blue bars, 5α-dihydrotestosterone (DHT) dark blue bars] on the mRNA expression of Acetyl-CoA carboxylase 1 (ACC1), the main provider of malonyl-CoA for fatty acid synthesis. **Panel B,** Effect of T and DHT on *de novo* lipogenesis, determined by incorporation of ^{14}C acetate into lipid. **Panel C,** Effects of T and DHT on β-oxidation, determined by $^{3}H_20$ release from ^{3}H-palmitate. **Panel D,** Effects of androgens on free fatty acid uptake. All data are presented as the mean±SEM of n=3-5 experiments. Significance levels *p<0.05; **p<0.01; ***p<0.001 compared to control (ANOVA with post hoc Tukey test).

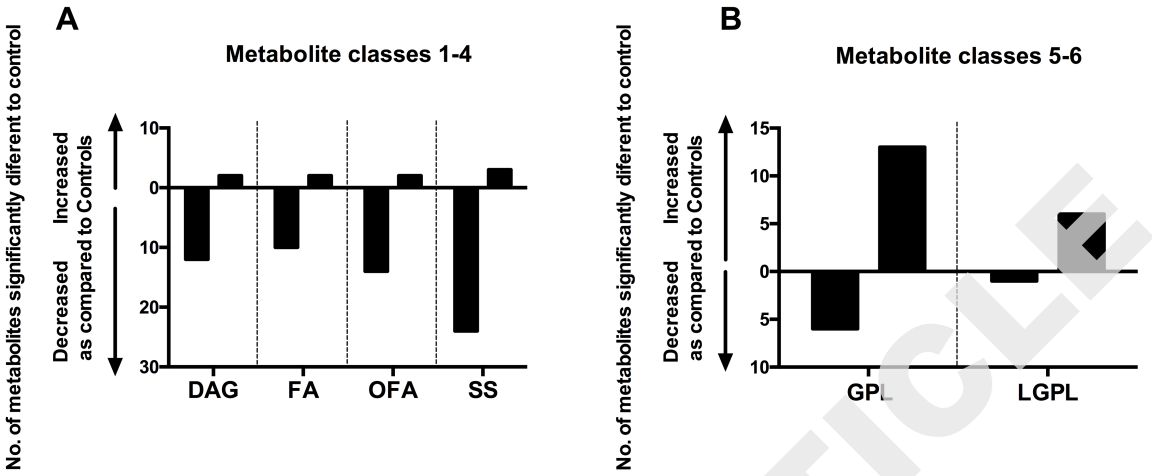
Figure 5: Expression, activity and inhibition of the androgen-activating enzyme aldo-ketoreductase type 1C3 (AKR1C3) in subcutaneous adipose tissue. Panels A-D, Expression and activity of AKR1C3 with and without insulin stimulation in the human pre-adipocyte SGBS cell line and primary subcutaneous adipocytes from women undergoing elective surgery. **Panel E**, Effect of pharmacological AKR1C3 inhibition by 3-4-trifluoromethyl-phenylamino-benzoic acid (10μM) for 24 hours (grey bars) on adipose androgen generation, assessed as conversion of androstenedione to testosterone. **Panel F**, Impact of pharmacological AKR1C3 inhibition on androstenedione-mediated *de novo* lipogenesis. All data are presented as the mean±SEM of n=3-5 experiments. Significance levels *p<0.05; **p<0.01; ***p<0.001 (ANOVA with Tukey post-hoc test).

Figure 6: Schematic representation of the proposed mechanistic link between androgen excess, insulin resistance and lipotoxicity in polycystic ovary syndrome (A) and graphical representation of the major human androgen biosynthesis pathways (B). AKR1C3 plays a central gatekeeping role in androgen activation in the classic androgen synthesis pathway and the alternative ('backdoor') pathway to 5α-dihydrotestosterone as well as the 11-oxygenated androgen synthesis pathway. Active androgens capable of activating the androgen receptor highlighted in blue boxes and white font.

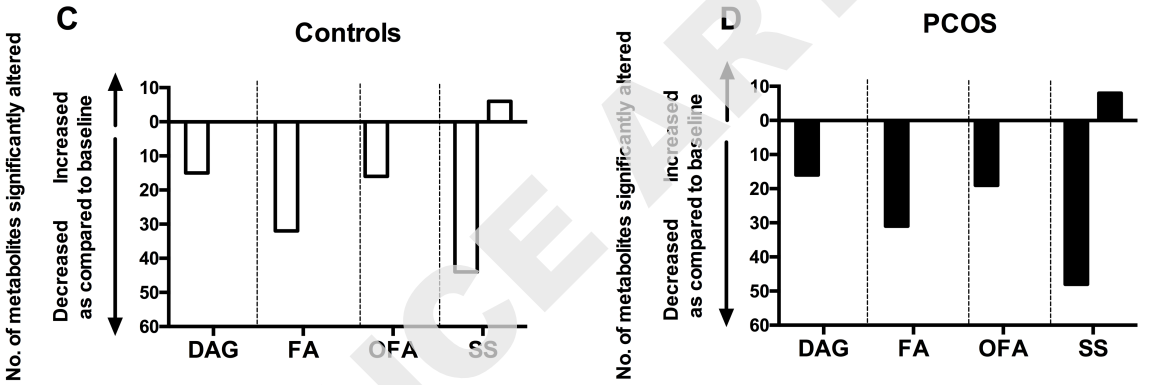




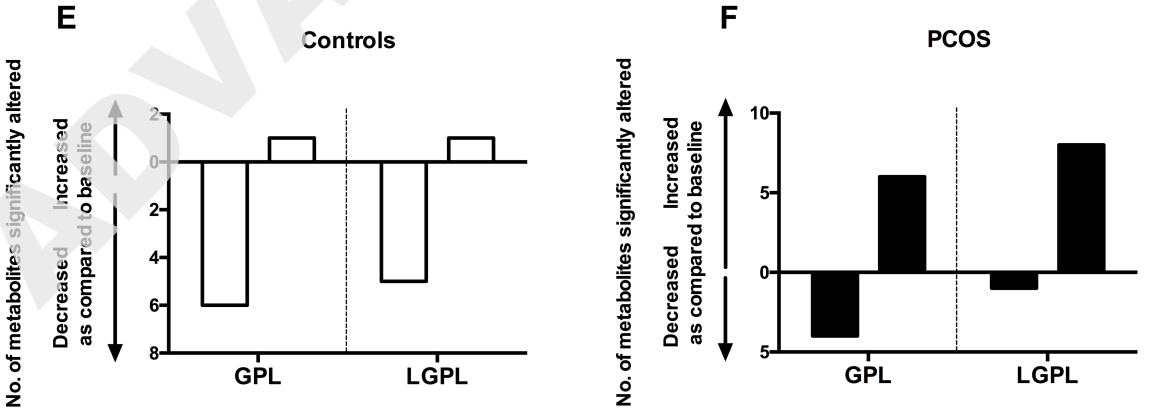
PCOS Baseline Metabolome

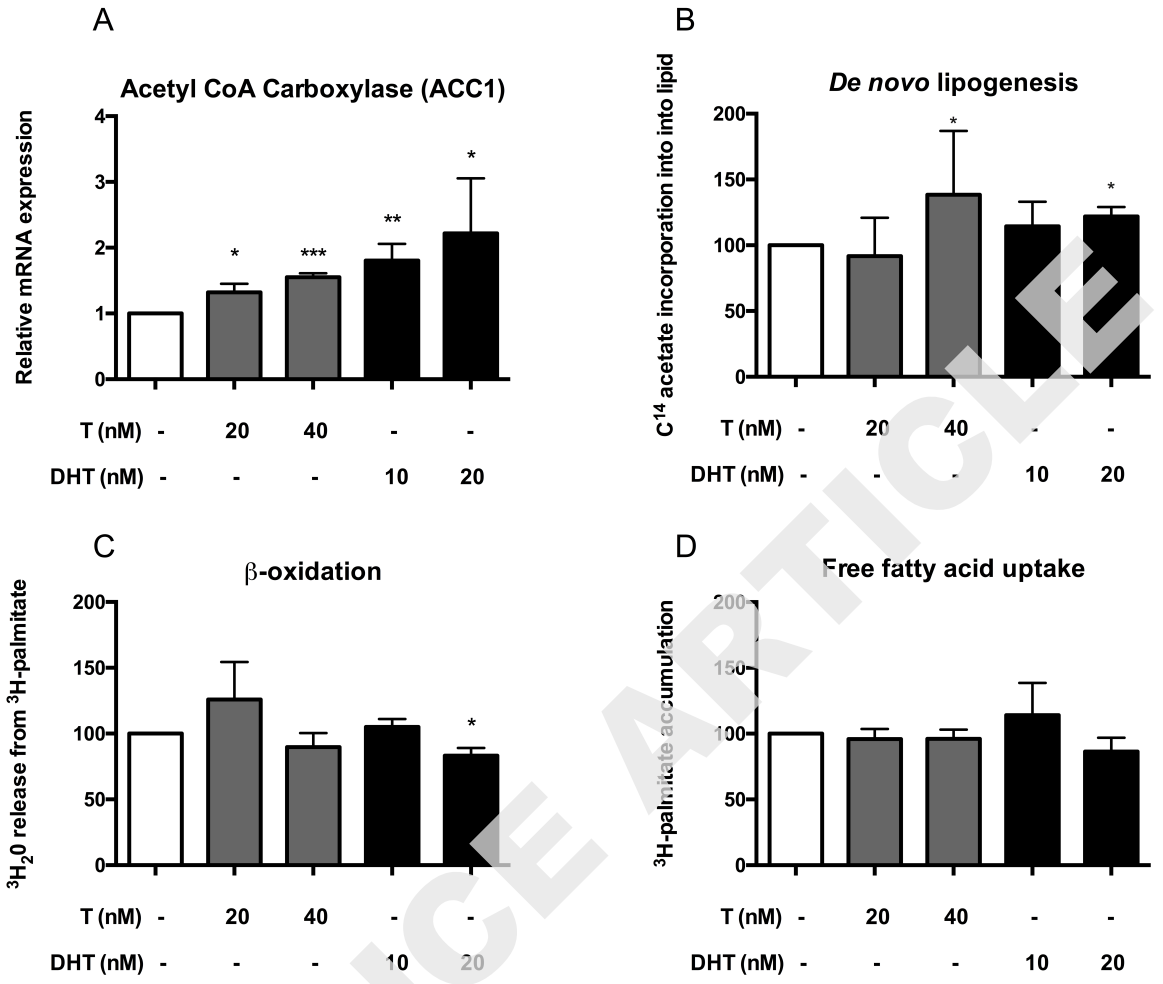


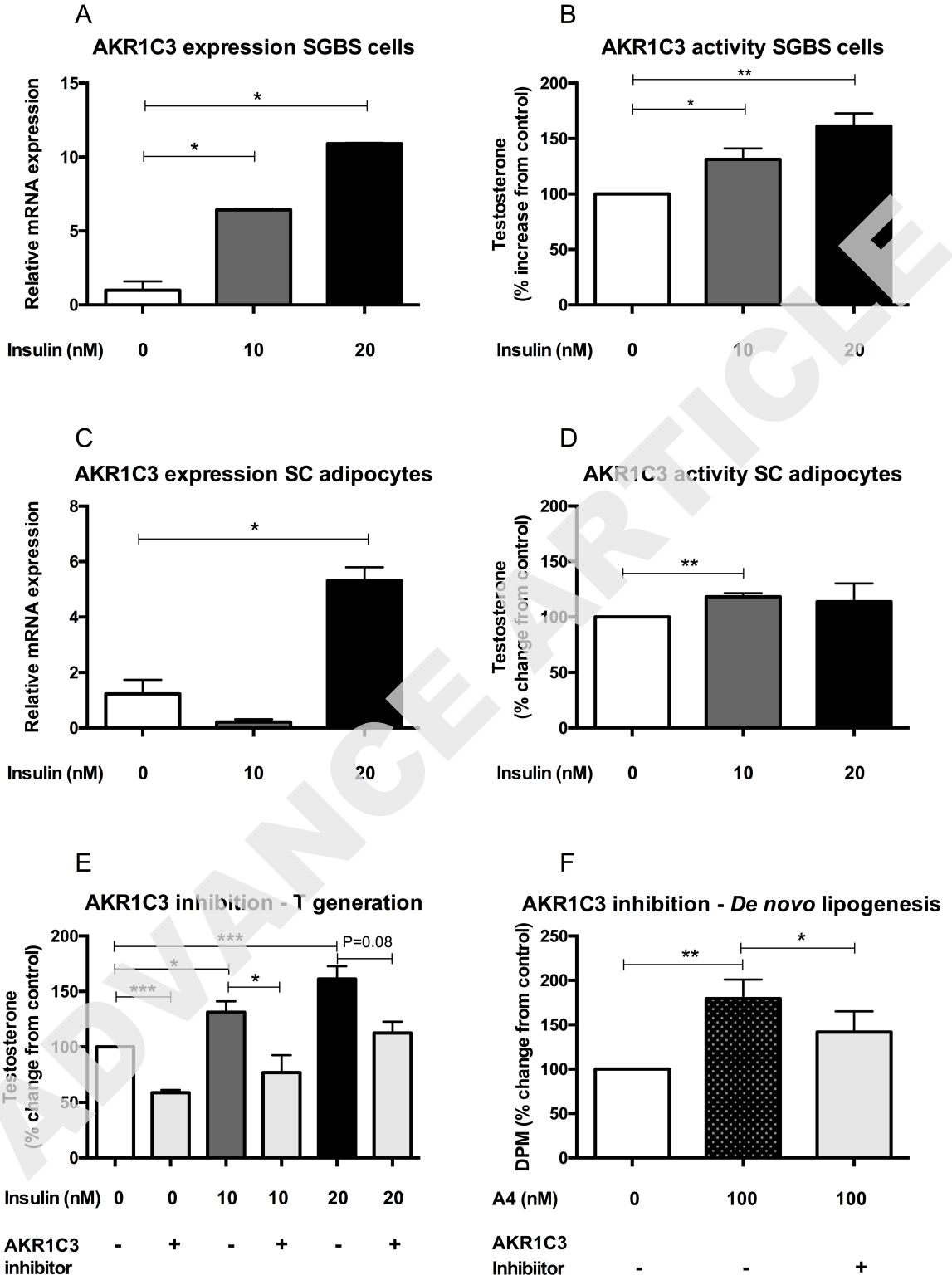
Post-DHEA metabolome: Metabolite classes 1-4



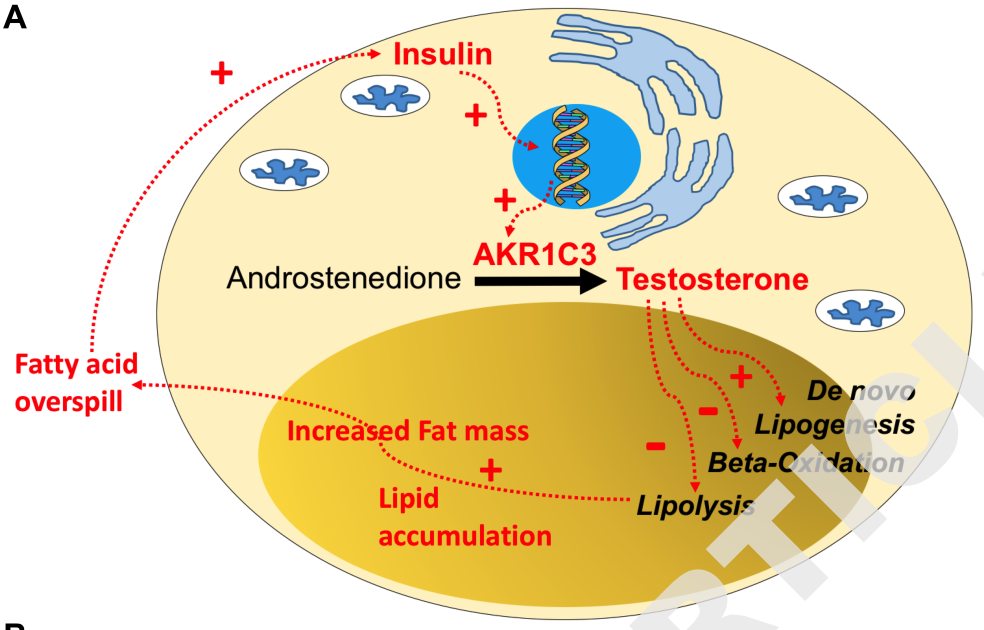
Post-DHEA metabolome: Metabolite classes 5-6







A



B