

Title: Aberrant subcutaneous adipogenesis precedes adult metabolic dysfunction in an ovine model of polycystic ovary syndrome (PCOS)

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Abstract

Polycystic ovary syndrome (PCOS) affects over 10% of women. Insulin resistance, elevated free fatty acids (FFAs) and increased adiposity are key factors contributing to metabolic dysfunction in PCOS. We hypothesised that aberrant adipogenesis during adolescence, and downstream metabolic perturbations, contributes to the metabolic phenotype of adult PCOS. We used prenatally androgenized (PA) sheep as a clinically realistic model of PCOS. During adolescence, but not during fetal or early life of PA sheep, adipogenesis was decreased in subcutaneous adipose tissue (SAT) accompanied by decreased leptin, adiponectin, and increased FFAs. In adulthood, PA sheep developed adipocyte hypertrophy in SAT paralleled by increased expression of inflammatory markers, elevated FFAs and increased expression of genes linked to fat accumulation in visceral adipose tissue. This study provides better understanding into the pathophysiology of PCOS from puberty to adulthood and identifies opportunity for early clinical intervention to normalise adipogenesis and ameliorate the metabolic phenotype.

Keywords: polycystic ovary syndrome, adipose tissue, adipogenesis, prenatal programming, metabolism, androgens

1. Introduction

Polycystic ovary syndrome (PCOS) is a common disorder, affecting up to 10% of women of reproductive age (Fauser et al., 2012). Women with PCOS have increased risks of hyperinsulinemia, insulin resistance, obesity, dyslipidemia and fatty liver (Fauser et al., 2012; Moran et al., 2015; Teede et al., 2010). Metabolic comorbidities associated with the syndrome worsen with age and pose a significant health and economic burden (Jason, 2011; Teede et al., 2010).

Overweight/obese women with PCOS have increased abdominal adiposity when compared with BMI-matched controls, correlated with an adverse metabolic profile (Puder et al., 2005; Yildirim et al., 2003). In addition, women with PCOS have adipose tissue dysfunction, independent of obesity (Carmina et al., 2005; Echiburú et al., 2018; Manneras-Holm et al., 2010; Seow et al., 2009; Wang et al., 2012).

Although studies using animal models of PCOS have also indicated adipose tissue abnormalities, the data is inconclusive (Keller et al., 2014; Puttabyatappa et al., 2018; Veiga-Lopez et al., 2013). Insulin and androgens, both typically increased in PCOS, affect adipogenesis and adipose tissue function (Chazenbalk et al., 2013; Klemm et al., 2001), and thus are likely to play an important role in adipose tissue dysfunction in PCOS. White adipose tissue (WAT) is a key metabolic and endocrine organ regulating energy homeostasis, insulin sensitivity and inflammation, therefore functional WAT is essential in maintaining body homeostasis (Lafontan, 2014). There is strong evidence showing differences between subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) biology, including distinct gene expression in VAT and SAT preadipocytes (Macotela et al., 2012; Tchkonja et al., 2013)

Animal models of PCOS are instrumental in dissecting mechanisms underlying the pathophysiology of PCOS (Abbott et al., 2013; Padmanabhan and Veiga-Lopez,

2013). We have previously documented that ewes exposed to increased androgens *in utero* manifest ovarian, hormonal and metabolic phenotypes reminiscent of PCOS (Hogg et al., 2011; 2012; Rae et al., 2013; Ramaswamy et al., 2016), and male offspring from such pregnancies display similar metabolic disturbances as seen in sons born to women with PCOS (Siemienowicz et al., 2019). We have reported that adult prenatally androgen-excess exposed (PA) sheep have increased body weight and are insulin resistant (Siemienowicz et al., 2020), while in adolescence those sheep are hyperinsulinemic and have increased fat accumulation in the liver, independent of body weight or central adiposity (Hogg et al., 2011). We hypothesised that the metabolic phenotype of PA sheep is associated with aberrant adipogenesis. Herein we investigate adipose tissue structure and function in adulthood and highlight aberrant adipogenesis in adolescence and its consequences in female PA sheep.

2. Materials and Methods

2.1 Ethics statement

All studies were approved by the UK Home Office and conducted under approved Project Licence PPL60/4401. The Animal Research Ethics Committee of The University of Edinburgh approved this study. The study was carried out in accordance with the relevant guidelines.

2.2 Animals and tissues

Animal husbandry, experimental protocols and tissue collection were performed as previously described (Hogg et al., 2012; 2011; Rae et al., 2013; Ramaswamy et al., 2016). Scottish Greyface ewes were housed in groups in spacious enclosures and fed hay *ad libitum*. Ewes with a healthy body condition score (2.75-3) were synchronised with Chronogest (flugestone) sponges (Intervet Ltd, UK) and Estrumate (cloprostenol) injection (Schering Plough Animal Health, UK) and mated with Texel rams. Pregnancy was suggested by lack of estrous and confirmed by ultrasound scanning.

In the maternal injection cohort (MI) pregnant ewes were randomised to twice weekly IM 100mg testosterone propionate (TP) in 1ml vegetable oil from day (D)62 to D102 of D147 pregnancy or 1ml vegetable oil (control (C)).

In pregnancies where fetal tissue was collected (D112: C=9; PA=4), ewes were sacrificed on D112 of gestation via barbiturate overdose. The gravid uterus was immediately removed, fetal sex and weight recorded, and tissue of interest snap frozen and stored at -80C.

In pregnancies carried to term, lambs were weaned at 3 months and fed hay or grass *ad libitum* until sacrifice at 11 weeks [juvenile (C=8; PA=8)]; 11 months, [adolescent (C=5; PA=9)] or 30 months [adult (C=11; PA=4)].

In the fetal injection cohort (FI), on day 62 and day 82 of gestation, mothers were randomised and anaesthetised by initial sedation with 10 mg Xylazine (i.m. Rompun; Bayer PLC Animal Health Division, UK), followed by 2mg/kg Ketamine (i.v, Ketaset; Fort Dodge Animal Health, UK). All subsequent procedures were conducted under surgical aseptic conditions. Fetuses were injected via ultrasound guidance into the fetal flank with 20G Quinke spinal needle (BD Biosciences, UK) with following according to the treatment group: control (C; n=12), 0.2ml vehicle (vegetable oil); testosterone propionate (TP; n=7), 20mg TP in 0.2ml vehicle; diethylsilbesterol (DES; n=8), 4mg DES in 0.2ml vehicle; dexamethasone (DEX; n=11), 100µg DEX in 0.2ml vehicle. Justification of the rationale, timing and treatment doses have been published previously (Siemienowicz et al., 2019). Immediately after surgical procedure completion all pregnant ewes were given prophylactic antibiotics (Streptacare, Animalcare Ltd., UK, 1 ml/25 kg) and were then monitored during recovery; no adverse effects of these procedures were observed. Lambs were weaned at 3 months and fed hay or grass ad libitum and sacrificed in adolescence (11 months of age).

Animals were sacrificed as described before (Hogg et al., 2011), omental tissue was carefully removed and weighed, and tissues of interest were fixed in Bouins solution for 24h, transferred to 70% ethanol and processed into paraffin wax and/or snap frozen and stored at -80C. Visceral adipose tissue was collected from omentum and subcutaneous adipose tissue from the groin area.

2.3 Plasma analyte determination

Concentrations of fasting plasma free fatty acids (FFAs), triglycerides (TGs), total cholesterol and high-density lipoprotein (HDL) were obtained using commercial assay kits (Alpha Laboratories Ltd., UK) as per manufacturer's instruction, using a

Cobas Mira automated analyser (Roche Diagnostics Ltd, UK). For all assays intra and inter-assay CV's were < 4% CV and < 5% CV, respectively. Fasting plasma leptin concentration was measured using Bio-Plex Human Diabetes Assay (171A7001M; Bio-Rad, UK), as per the manufacturer's instruction. All samples were assayed in duplicate and the results were acquired and calculated using Bio-Plex 200 array reader system with Bio-Plex Manager software (Bio-Rad, UK). The assay working range for leptin was 0.011-129ng/ml, assay sensitivity 3.1pg/ml, and intra and inter-assay CVs were 3% and 4%, respectively. Plasma adiponectin was measured using human Adiponectin ELISA kit (KHP041; Invitrogen, Life Technologies, UK), as per the manufacturer's instructions. All samples were assayed in duplicate. The assay sensitivity was 0.1ng/ml intra and inter-assay CVs were <5% and <6%, respectively.

2.4 Adipocyte morphometric analysis

For adipocyte morphometric analysis, two 5 µm sections were cut per adipose tissue sample, a minimum of 100 µm apart, and mounted on positively charged slides (Superfrost Plus Gold, ThermoScientific, UK). Sections were then stained with haematoxylin and eosin following standard protocol. Two randomly selected fields per section were captured at X4 magnification using Olympus Provis BX2 microscope (Olympus America Inc., USA) attached to a Canon EOS 30D Microcam camera (Canon Inc, Japan). Images were analysed using Adiposoft, ImageJ Software (Galarraga et al., 2012) in a blinded manner. Results were then manually corrected as per Adiposoft instructions and confirmed on a graticuled microscope.

2.5 Western Blotting

For western blotting all controls (C; n=5) and 5 samples from PA group were randomly selected for the analysis. Samples were homogenised with Soniprep150 (MSE UK

Ltd.) in RIPA buffer (ab156034, Abcam, UK) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Scientific Pierce, USA) and centrifuged at 17500g at 4°C for 10 min to pellet cellular debris. Protein concentration was measured using the Bradford Assay on Cobas Fara centrifugal analyser (Roche Diagnostics, UK). Samples were diluted in RIPA buffer and combined with equal volume of 1X Laemmli buffer (0.1M Tris-HCl pH 6.8, 20% glycerol, 2% (w/v) SDS, 0.16% (w/v) bromophenol blue and 3% β-mercaptoethanol). After heat denaturation (95°C for 5 min), samples (20 µg total protein) underwent electrophoresis, alongside full-range PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific Pierce, USA), on 4-20% Tris-HEPES-SDS Precast Polyacrylamide Mini Gels (Thermo Scientific Pierce, USA) and were transferred to a PVDF membrane (IPFL00010; Immobilon-FL PVDF; Merck Millipore, Germany) using fast semi-dry blotter (Thermo Scientific Pierce, USA). Membranes were blocked in Odyssey Blocking Buffer (Li-Cor, USA) overnight at 4°C. Membrane was probed with primary antibody raised against STAT1 (1:1000; SC-346, Santa Cruz Biotechnology) for 2h at RT, washed and probed with a primary antibody raised against PPARγ (1:500; SC-1984 Santa Cruz Biotechnology) for 4h at RT. After washing, membranes were incubated with two different fluorescently-labelled secondary antibodies at the concentration 1:10000 IRDye 680RD (926-68074; Li-Cor) and IRDye 800CW (926-32213; Li-Cor), both for 1h at RT. After final washing, membranes were visualised on the Odyssey Imager (Li-Cor, USA). The size of the visualised protein band was confirmed with reference to the molecular weight markers. Protein densitometry was analysed with Image Studio Lite Software (Li-Cor, USA) with STAT1 protein levels used as the loading control.

2.5 Quantitative (q)RT-PCR

RNA was extracted from adipose tissue with a combination of TRI Reagent with the RNeasy Mini Kit (Qiagen Ltd.) and from liver using RNeasy Mini Kit following the manufacturer's instructions. On-column DNase digestion was performed using RNase-Free DNase set (Qiagen Ltd.) and RNA concentration and purity was assessed using a NanoDrop One spectrometer (ThermoFisher Scientific, UK). Complimentary DNA was synthesised using TaqMan Reverse Transcription Kit (Applied Biosystems, UK) as described previously (Hogg et al., 2012). To select the most stable housekeeping genes the geNorm Reference Gene Selection Kit (Primerdesign Ltd., UK) was used, identifying in visceral adipose tissue the suitability of the geometric mean of *RPS26* and *18S*, and for subcutaneous adipose tissue and liver the geometric mean of *ACTB* and *MDH1* was utilised. Primers (Table 1) were designed and synthesised as described previously (Siemienowicz et al., 2020). Real time RT-PCR was performed on 384-well plate format (Applied Biosystems) with all samples assayed in duplicate and housekeeping control genes included in each run, as well as template, RNA and RT-negative controls, using the ABI 7900HT Fast Real Time PCR system (Applied Biosystems) as described previously (Hogg et al., 2012). The transcript abundance of target gene relative to the housekeeping genes was quantified using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

2.6 Statistical analysis

All data sets were normality tested prior to further analysis (Shapiro-Wilk test), and logarithmically transformed if necessary. For comparing means of two treatment groups with equal variances, unpaired, two-tailed Student's t test was used accepting $P < 0.05$ as significant. For more than two comparisons ANOVA was used with Dunnett's post hoc test. Correlation was assessed by calculation of Pearson

202 product-moment co-efficient. Statistical analysis was performed using GraphPad
203 Prism 8.0 software (GraphPad Prism Software, San Diego, CA, USA). Asterisks
204 were used to indicate level of significance based on the following criteria: * $P < 0.05$,
205 ** $P < 0.01$.
206

3. Results

3.1 *PA sheep have structurally altered subcutaneous adipose tissue in adulthood*

Female PA sheep prenatally programmed to develop a PCOS-like condition show no difference in birth weight or body weight during the first year of life, through puberty and adolescence, however, in adulthood they have increased body weight as compared to controls (Siemienowicz et al., 2020). Adipocyte morphology was assessed by histological morphometric analysis (Fig. 1A). In VAT there was no difference in the numbers of adipocytes per mm² (Fig. 1B), no change in the mean size of adipocytes (Fig. 1C) and no alteration the number of adipocytes of different sizes (Fig. 1D). In contrast, adipocyte number was decreased in SAT (Fig. 1E; $P<0.05$), with a trend to an augmented mean size of adipocytes (Fig. 1F; $P=0.06$) as a result of an increased number of large adipocytes (defined as larger than 5000 μm^2) (Fig. 1G; $P<0.05$). The increased number of large SAT adipocytes in PA sheep was not a consequence of increased body weight as the difference was also observed between a weight-matched subset of controls ($n=4$) and PA sheep ($n=4$) (Fig. 1H; $P<0.05$). Key transcriptional regulators of adipogenesis, *PPARG*, *CEBPA*, *CEBPB* and *CEBPD* were not altered in adult VAT or SAT (Fig. 1I). There are fewer, but larger adipocytes in SAT in PA sheep but this was not associated with alterations in adipogenesis in adulthood.

3.2 *Prenatal androgen excess is associated with adipose tissue dysfunction in adulthood*

There were no differences in the transcript abundance of markers of mature adipocytes (*LEP* and *ADIPOQ*) in VAT or SAT (Fig. 2A) in adult PA sheep. Furthermore, there was no difference in plasma concentrations (Fig. 2B,C), although

there was a trend towards decreased adiponectin level (Fig. 2C; $P=0.08$) in PA sheep when compared to controls. There were no differences in transcript abundance of inflammatory markers in VAT (Fig. 2D) while in SAT PA sheep had increased *TNF* ($P<0.05$), *IL6* ($P<0.05$) and *CCL2* ($P<0.05$) when compared to controls (Fig. 2E; $P<0.05$). Although the PA sheep have normal levels of TGs, cholesterol and HDL cholesterol (Fig. 3A-C) they have increased circulating FFAs (Fig. 3D; $P<0.05$). There was negative correlation ($r= -0.66$) between the total number of adipocytes per mm^2 and the levels of circulating FFAs (Fig. 3E; $P<0.05$). Although at this time there was no difference in omental fat (Fig. 3F) there was increased transcript abundance of genes involved in fat uptake and accumulation in VAT (*SLC27A1*, *CAV1*, *CAV2*, *FABP5* and *LPIN2*) (Fig. 3G-K; $P<0.05$). Alterations in adipose tissue associated with inflammation is seen in SAT only and this is associated with increased FFAs. In summary, we observed structurally altered subcutaneous adipose tissue, with attendant dysfunction in adulthood attributable to prenatal androgen excess.

3.3 Altered adipogenesis is observed during adolescence

We next assessed expression of transcription factors involved in adipogenesis at 11 months of age when there was no difference in body weight (Siemienowicz et al., 2020) and omental fat weight (Hogg et al., 2011). In VAT there was no difference in the transcript abundance of *PPARG*, *CEBPA* and *CEBPB*, although *CEBPD* was increased ($P<0.05$) in adolescent PA sheep (Fig. 4A). However, in SAT there was decreased transcript abundance of *PPARG*, *CEBPA*, *CEBPB* (Fig. 4B; $P<0.05$) but no difference in *CEBPD* (Fig. 4B). We confirmed decreased level of *PPARG* in SAT (Fig. 4C; $P<0.05$) in adolescent PA females, by Western blot. In addition, adolescent PA sheep had decreased expression of markers of mature adipocytes in SAT,

SLC2A4 and *PLIN1* (Supplementary Figure 1; $P<0.05-0.01$). In order to determine the specificity of prenatal androgens in the regulation of adolescent adipogenesis (Fig. 4D) we directly injected various steroids into the mid-gestation fetus and assessed adipogenesis markers in SAT in adolescence. Reduced adipogenesis-associated gene expression in SAT was only associated with prenatal androgen excess (TP) and neither estrogenic (DES) excess nor glucocorticoid (DEX) excess showed similar effects (Fig. 4D; $P<0.05$).

3.4 Adolescent SAT transcriptional alterations are associated with increased circulating FFA

Fasting FFAs were increased in adolescent PA sheep when compared to controls (Fig. 5A; $P<0.05$). Their concentrations were negatively correlated with *PPARG* ($r=-0.55$) in SAT (Fig. 5B; $P<0.05$) but not with *PPARG* in VAT (Fig. 5C). These sheep have early accumulation of fat in the liver (Hogg et al., 2011) with increased transcript abundance of genes involved in FFAs uptake and deposition in the liver (Fig. 5D-F; $P<0.05$).

3.5 Adiponectin is a biomarker of reduced SAT adipogenesis during adolescence

To investigate potential biomarkers of altered adipogenesis during adolescence the transcript abundance of markers of mature adipocytes was investigated in adipose tissue at 11 months of age in control and PA animals. In VAT there was no changes in *LEP* or *ADIPOQ* expression (Fig. 6A,B). In contrast *LEP* ($P<0.01$) and *ADIPOQ* ($P<0.05$) were reduced in SAT in adolescent PA sheep when compared to controls (Fig. 6C,D). This was reflected by a reduction in circulating leptin (Fig 6E; $P<0.05$) and adiponectin (Fig. 6F; $P<0.05$) in the plasma. Circulating adiponectin correlated ($r=0.79$) to *ADIPOQ* in SAT (Fig. 6G; $P<0.01$) but not VAT (Fig. 6H), and there was

282 no correlation identified between circulating leptin and SAT *LEP* gene expression.

283 Collectively, in female PA offspring, we observed altered adipogenesis during
284 adolescence, with increased circulating FFA, and this response was specific to
285 androgenic excess during development. Reduced circulating adiponectin has
286 potential as a biomarker of reduced adipogenesis in SAT during adolescence.

287 **3.6 *There is no evidence of altered adipogenesis before puberty***

288 Altered transcript abundance for transcription factors involved in adipogenesis was
289 not observed pre-pubertally at 11 weeks of age or in fetal life (Table 1).

290

4. Discussion

We demonstrated, using an ovine model of PCOS, that during adolescence, but not fetal or prepubertal life, there was decreased adipogenesis in SAT in PA sheep. This was accompanied by decreased concentrations of leptin and adiponectin, and increased concentrations of FFAs, likely underpinning the observation of upregulated expression of FFAs transporters in liver. Adult consequences of such altered adolescent adipogenesis were that PA female offspring displayed adipocyte hypertrophy in SAT paralleled by increased expression of inflammatory markers and increased expression of genes linked to fat accumulation in VAT.

In fetal life pre-adipocyte differentiation into adipocytes occurs between the 14th and the 16th weeks of gestation in humans (Poissonnet et al., 1983) and in the third month of gestation in sheep (Wensvoort, 1967). In late gestation adipocyte proliferation decreases, and until adolescence, increased adiposity primarily occurs via filling of predetermined adipocytes. During puberty another window of adipocyte proliferation occurs (Rosen and Spiegelman, 2014) setting the total number of adipocytes. Whilst differentiation potential of pre-adipocytes into mature adipocytes is present throughout life, dependent upon energy status and storage needs (Rosen and Spiegelman, 2014), in adult life, the capacity of preadipocytes to become fully functional mature adipocytes declines (Tchkonia et al., 2010). Hence increased or decreased adult body weight, with corresponding fluctuations in body fat mass, is reflective of changes in adipocyte volume but not number (MacLean et al., 2015; Spalding et al., 2008). In our study altered adipogenesis in SAT of PA females was only evident during adolescence (11 months).

316 During adipogenesis, pre-adipocyte terminal differentiation to mature adipocytes
317 involves accruing lipid transport and synthesis capacity, insulin responsiveness and
318 synthesis of adipokines, regulated by PPARG and the family of CCAAT/enhancer
319 binding protein transcription factors (C/EBP) (Cristancho and Lazar, 2011). SAT
320 expansion improves lipid buffering and metabolic profile (Kim et al., 2007) while
321 inability to increase adipose cell number results in hypertrophic adipocytes,
322 increasing risk of metabolic diseases (Dubois et al., 2006). Impaired SAT
323 adipogenesis results in decreased storage capacity, and accumulation of lipids in
324 non-adipose tissues, lipotoxicity and metabolic perturbations (Carobbio et al., 2017).
325 Furthermore, decreased subcutaneous pre-adipocyte differentiation and defective
326 storage capacity of SAT are associated with increased visceral adiposity (Alligier et
327 al., 2013; Lessard et al., 2014), suggesting increased visceral fat accumulation might
328 be a compensatory adaptation to limitations in SAT expandability (Britton and Fox,
329 2011).

330

331 Decreased expression of PPARG, *CEBPA* and *CEBPB* in SAT, in PA sheep
332 suggests decreased differentiation of pre-adipocytes into mature adipocytes and
333 indicates lower capacity of SAT to safely store fat, which combined with observations
334 of increased levels of FFAs in PA adolescent sheep supports suggestions of
335 decreased lipid storage volume in SAT. This may also contribute to fatty liver
336 development due to increased release of FFAs into circulation and increased hepatic
337 uptake (Hogg et al., 2011). Morphometric analysis of the SAT in adolescent sheep
338 was not performed due to lack of histological samples, as the aim of the study was to
339 determine adult outcomes in terms of adipose structure, and examine the earlier life
340 mechanistic antecedents of any altered adult structure observed; we acknowledge

this limitation of the data presented here. Veiga-Lopez *et al.* previously reported that postpubertal, (21 months old), prenatally androgenised female sheep showed reduction in adipocyte cell size in both VAT and SAT and concluded failure of a subset of small adipocytes to differentiate. (Veiga-Lopez *et al.*, 2013). We did not observe any effect on VAT in our adolescent animals. The discrepancies between studies may be attributed to different timing of the prenatal testosterone treatment and different age at study.

Adipogenesis in PCOS patients has not been thoroughly investigated, however, increased abdominal adipose stem cell commitment to preadipocytes *in vitro*, as measured by the expression of ZFP423 protein, have been found in adult, normal weight women with PCOS (Fisch *et al.*, 2018). Increased *ZFP423* gene expression was also reported in abdominal SAT of prenatally androgenised female rhesus monkeys (Keller *et al.*, 2014). We did not study the preadipocyte commitment but rather, the differentiation of preadipocytes into mature adipocytes. In agreement with our study, it was reported that women with PCOS have decreased expression and increased DNA methylation of *PPARG* in SAT and *PPARG* expression positively correlated with insulin sensitivity and negatively with adipocyte size and testosterone levels (Kokosar *et al.*, 2016). Furthermore, PCOS-like monkeys were also have decreased abdominal SAT *CEBPA* expression, reduced numbers of mature SAT adipocytes and increased FFAs indicating impaired adipogenesis (Keller *et al.*, 2014). In addition, there is growing evidence of decreased adipogenesis in SAT from insulin resistant individuals and subjects with abdominal obesity (Heilbronn *et al.*, 2004; Permana *et al.*, 2004; Yang *et al.*, 2004). Our data is in broad agreement with such observations, lending translational confidence to our findings.

Further translational relevance in terms of PCOS is derived from observations that obese adolescent girls with PCOS have persistently elevated FFAs when compared with obese controls (King et al., 2017). Since SAT represents approximately 80% of total body adipose tissue, decreased SAT adipogenesis in adolescence may predispose to decreased storage capacity in adulthood and increased adipocyte hypertrophy. In support of this, our adult PA sheep have decreased SAT adipocyte numbers and corresponding increased numbers of large, hypertrophic adipocytes, and adult PCOS women have hypertrophic adipocytes in SAT (Echiburú et al., 2018; Faulds et al., 2003; Manneras-Holm et al., 2010).

Hypertrophic expansion of SAT is associated with altered adipokine secretion, inflammation and fibrosis (Longo et al., 2019). Adipocyte hypertrophy in adult PA sheep was paralleled by increased expression of *TNF*, *IL6* and *CCL2* in SAT. Enlarged adipocytes overexpress MCP-1 (encoded by *CCL2* gene) and induce increased recruitment of proinflammatory M1 macrophages culminating in increased production of pro-inflammatory cytokines $TNF\alpha$ and IL-6, promoting altered gene expression and insulin resistance in adipocytes (Weisberg et al., 2003).

Adolescent PA sheep had also decreased expression of markers of mature adipocytes *LEP* and *ADIPOQ* in SAT, functionally realised by decreased circulating leptin and adiponectin concentrations. There was positive correlation of *ADIPOQ* mRNA in SAT, with circulating adiponectin, reminiscent of that observed in women with PCOS (Echiburú et al., 2018; Lecke et al., 2013), and suggesting the likelihood that decreased adiponectin levels in adolescent PCOS-like sheep is a consequence of decreased adipogenesis in SAT. Adiponectin, primarily secreted from SAT, is an

insulin sensitising adipokine, promoting lipid oxidation and reducing plasma concentration of FFAs (Achari and Jain, 2017), and stimulates glucose uptake in adipocytes by increasing expression of GLUT4 (Achari and Jain, 2017; Fu et al., 2005).

Adiponectin is also an autocrine factor promoting and regulating adipocyte differentiation (do Carmo Avides et al., 2008; Fu et al., 2005). Decreased circulating adiponectin levels, independent of adiposity, are consistently reported in adolescent and adult PCOS patients (Cankaya et al., 2014; Escobar-Morreale et al., 2006; Maliqueo et al., 2012; Mirza et al., 2014; Sepilian and Nagamani, 2005), suggesting potential utility as a biomarker of PCOS (Al-Awadi et al., 2016; Saray et al., 2015).

Whilst androgens decrease plasma adiponectin *in vitro* and *in vivo* (Frederiksen et al., 2012; Nishizawa et al., 2002; Xu et al., 2005), and this is inversely correlated with testosterone levels, (Böttner et al., 2004; Riestra et al., 2013), to our knowledge this is the first demonstration of prenatal androgen excess being associated with decreased SAT and circulating adiponectin. It is noteworthy in the context of prenatal androgenic programming of PCOS that adiponectin decreases ovarian thecal androgen synthesis and expression of adiponectin receptors is decreased in theca cells from polycystic ovaries; consequently decreased adiponectin may also contribute to hyperandrogenism in PCOS women (Comim et al., 2013; Lagaly et al., 2008).

Inhibitory effects of androgens on adipogenesis is well documented (Zerradi et al., 2014). Numerous studies demonstrate androgens prevent *in vitro* differentiation and proliferation of murine preadipocyte cell lines (Fujioka et al., 2012; Singh et al., 2003;

2005) and human preadipocytes from both sexes, and from different fat depots (Blouin et al., 2010; Chazenbalk et al., 2013; Gupta et al., 2008; McNelis et al., 2013). To determine steroid specificity of androgenic excess in programming adipose tissue function *in vivo*, we assessed SAT adipogenesis in adolescent, 11 months old female sheep, that were directly injected *in utero* with androgen, estrogen (DES as surrogate estrogen) or glucocorticoid (DEX as surrogate, active glucocorticoid). We found that only androgen treatment, but not estrogen or glucocorticoid, decreased adipogenesis in SAT in adolescence, identifying that the responses measured throughout the study were androgenic.

In summary, we have shown that during adolescence, altered adipogenesis in SAT of PA sheep occurs, with decreased beneficial adipokines, independent of central adiposity. Decreased adipocyte differentiation during adolescence resulted in hypertrophy and inflammation of adult SAT. This decreased capacity of SAT to safely store fat may explain metabolic perturbations observed in PA female sheep, shedding light upon new investigatory, and interventional avenues in clinical PCOS research.

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Declaration of interest

The authors have no conflicts of interest to declare.

References

- Abbott, D.H., Nicol, L.E., Levine, J.E., Xu, N., Goodarzi, M.O., Dumesic, D.A., 2013. Nonhuman primate models of polycystic ovary syndrome. *Mol. Cell. Endocrinol.* 373, 21–28. doi:10.1016/j.mce.2013.01.013
- Achari, A.E., Jain, S.K., 2017. Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. *Int. J. Mol. Sci.* 18, 1321. doi:10.3390/ijms18061321
- Al-Awadi, A.M., Sarray, S., Arekat, M.R., Saleh, L.R., Mahmood, N., Almawi, W.Y., 2016. The high-molecular weight multimer form of adiponectin is a useful marker of polycystic ovary syndrome in Bahraini Arab women. *Clin. Nutr. ESPEN* 13, e33–e38. doi:10.1016/j.clnesp.2016.03.078
- Alligier, M., Gabert, L., Meugnier, E., Lambert-Porcheron, S., Chanseaume, E., Pilleul, F., Debard, C., Sauvinet, V., Morio, B., Vidal-Puig, A., Vidal, H., Laville, M., 2013. Visceral fat accumulation during lipid overfeeding is related to subcutaneous adipose tissue characteristics in healthy men. *J. Clin. Endocrinol. Metab.* 98, 802–810. doi:10.1210/jc.2012-3289
- Blouin, K., Nadeau, M., Perreault, M., Veilleux, A., Drolet, R., Marceau, P., Mailloux, J., Luu-The, V., Tchernof, A., 2010. Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women. *Clin. Endocrinol.* 72, 176–188. doi:10.1111/j.1365-2265.2009.03645.x

473 Böttner, A., Kratzsch, J., Müller, G., Kapellen, T.M., Blüher, S., Keller, E., Blüher, M.,
 474 Kiess, W., 2004. Gender Differences of Adiponectin Levels Develop during the
 475 Progression of Puberty and Are Related to Serum Androgen Levels. *J. Clin.*
 476 *Endocrinol. Metab.* 89, 4053–4061. doi:10.1210/jc.2004-0303
 477
 478 Britton, K.A., Fox, C.S., 2011. Ectopic Fat Depots and Cardiovascular Disease.
 479 *Circulation* 124, e837-41. doi:10.1161/CIRCULATIONAHA.111.077602
 480
 481 Cankaya, S., Demir, B., Aksakal, S.E., Dilbaz, B., Demirtas, C., Goktolga, U., 2014.
 482 Insulin resistance and its relationship with high molecule weight adiponectin in
 483 adolescents with polycystic ovary syndrome and a maternal history. *Fertil. Steril.*
 484 102, 826–830. doi:10.1016/j.fertnstert.2014.05.032
 485
 486 Carmina, E., Orio, F., Palomba, S., Cascella, T., Longo, R.A., Colao, A.M.,
 487 Lombardi, G., Lobo, R.A., 2005. Evidence for altered adipocyte function in polycystic
 488 ovary syndrome. *Eur. J. Endocrinol.* 152, 389–394. doi:10.1530/eje.1.01868
 489
 490 Carobbio, S., Pellegrinelli, V., Vidal-Puig, A., 2017. Adipose Tissue Function and
 491 Expandability as Determinants of Lipotoxicity and the Metabolic Syndrome. *Adv.*
 492 *Exp. Med. Biol.* 960, 161–196. doi:10.1007/978-3-319-48382-5_7
 493
 494 Chazenbalk, G., Singh, P., Irge, D., Shah, A., Abbott, D.H., Dumesic, D.A., 2013.
 495 Androgens inhibit adipogenesis during human adipose stem cell commitment to
 496 preadipocyte formation. *Steroids* 78, 920–926. doi:10.1016/j.steroids.2013.05.001

497 Comim, F.V., Hardy, K., Franks, S., 2013. Adiponectin and Its Receptors in the
 498 Ovary: Further Evidence for a Link between Obesity and Hyperandrogenism in
 499 Polycystic Ovary Syndrome. PLoS ONE 8, e80416.
 500 doi:10.1371/journal.pone.0080416
 501
 502 Cristancho, A.G., Lazar, M.A., 2011. Forming functional fat: a growing understanding
 503 of adipocyte differentiation. Nat. Rev. Mol. Cell Biol. 12, 722–734.
 504 doi:10.1038/nrm3198
 505
 506 do Carmo Avides, M., Domingues, L., Vicente, A., Teixeira, J., 2008. Differentiation
 507 of human pre-adipocytes by recombinant adiponectin. Protein Expr. Purif. 59, 122–
 508 126. doi:10.1016/j.pep.2008.01.012
 509
 510 Dubois, S.G., Heilbronn, L.K., Smith, S.R., Albu, J.B., Kelley, D.E., Ravussin, E.,
 511 Look AHEAD Adipose Research Group, 2006. Decreased expression of adipogenic
 512 genes in obese subjects with type 2 diabetes. Obesity 14, 1543–1552.
 513 doi:10.1038/oby.2006.178
 514
 515 Echiburú, B., Pérez-Bravo, F., Galgani, J.E., Sandoval, D., Saldías, C., Crisosto, N.,
 516 Maliqueo, M., Sir-Petermann, T., 2018. Enlarged adipocytes in subcutaneous
 517 adipose tissue associated to hyperandrogenism and visceral adipose tissue volume
 518 in women with polycystic ovary syndrome. Steroids 130, 15–21.
 519 doi:10.1016/j.steroids.2017.12.009
 520

521 Escobar-Morreale, H.F., Villuendas, G., Botella-Carretero, J.I., Álvarez-Blasco, F.,
 522 Sanchón, R., Luque-Ramírez, M., Millán, J.L.S., 2006. Adiponectin and resistin in
 523 PCOS: a clinical, biochemical and molecular genetic study. *Hum. Reprod.* 21, 2257–
 524 2265. doi:10.1093/humrep/del146
 525
 526 Faulds, G., Ryden, M., Ek, I., 2003. Mechanisms behind lipolytic catecholamine
 527 resistance of subcutaneous fat cells in the polycystic ovarian syndrome. *J. Clin.*
 528 *Endocrinol. Metab.* 88, 2269. doi:10.1210/jc.2002-021573
 529
 530 Fauser BCJM, Tarlatzis BC, Rebar RW, Legro RS, Balen AH, Lobo R, Carmina E,
 531 Chang J, Yildiz BO, Laven JSE *et al.* 2012 Consensus on women's health aspects of
 532 polycystic ovary syndrome (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd
 533 PCOS Consensus Workshop Group. *Hum. Reprod.* 27, 14-24. doi:
 534 10.1093/humrep/der396
 535
 536 Fisch, S.C., Nikou, A.F., Wright, E.A., Phan, J.D., Leung, K.L., Grogan, T.R., Abbott,
 537 D.H., Chazenbalk, G.D., Dumesic, D.A., 2018. Precocious subcutaneous abdominal
 538 stem cell development to adipocytes in normal-weight women with polycystic ovary
 539 syndrome. *Fertil. Steril.* 110, 1367–1376. doi:10.1016/j.fertnstert.2018.08.042
 540
 541 Frederiksen, L., Højlund, K., Hougaard, D.M., Mosbech, T.H., Larsen, R., Flyvbjerg,
 542 A., Frystyk, J., Brixen, K., Andersen, M., 2012. Testosterone therapy decreases
 543 subcutaneous fat and adiponectin in aging men. *Eur. J. Endocrinol.* 166, 469–476.
 544 doi:10.1530/EJE-11-0565
 545

546 Fu, Y., Luo, N., Klein, R.L., Garvey, W.T., 2005. Adiponectin promotes adipocyte
 547 differentiation, insulin sensitivity, and lipid accumulation. *J. Lipid Res.* 46, 1369–
 548 1379. doi:10.1194/jlr.M400373-JLR200
 549
 550 Fujioka, K., Fujioka, K., Kajita, K., Kajita, K., WU, Z., Hanamoto, T., Wu, Z.,
 551 Hanamoto, T., Ikeda, T., Ikeda, T., Mori, I., Okada, H., Mori, I., Okada, H., Yamauchi,
 552 M., Yamauchi, M., Uno, Y., Morita, H., Uno, Y., Nagano, I., Morita, H., Takahashi, Y.,
 553 Nagano, I., Takahashi, Y., Ishizuka, T., Ishizuka, T., 2012. Dehydroepiandrosterone
 554 reduces preadipocyte proliferation via androgen receptor. *Am. J. Physiol. Endocrinol.*
 555 *Metab.* 302, E694–704. doi:10.1152/ajpendo.00112.2011
 556
 557 Galarraaga, M., Campión, J., Muñoz-Barrutia, A., Boqué, N., Moreno, H., Martínez,
 558 J.A., Milagro, F., Ortiz-de-Solórzano, C., 2012. Adiposoft: automated software for the
 559 analysis of white adipose tissue cellularity in histological sections. *J. Lipid Res.* 53,
 560 2791–2796. doi:10.1194/jlr.D023788
 561
 562 Gupta, V., Bhasin, S., Guo, W., Singh, R., Miki, R., Chauhan, P., Choong, K.,
 563 Tchkonina, T., Lebrasseur, N.K., Flanagan, J.N., Hamilton, J.A., Viereck, J.C., Narula,
 564 N.S., Kirkland, J.L., Jasuja, R., 2008. Effects of dihydrotestosterone on differentiation
 565 and proliferation of human mesenchymal stem cells and preadipocytes. *Mol. Cell.*
 566 *Endocrinol.* 296, 32–40. doi:10.1016/j.mce.2008.08.019
 567
 568 Heilbronn, L., Smith, S.R., Ravussin, E., 2004. Failure of fat cell proliferation,
 569 mitochondrial function and fat oxidation results in ectopic fat storage, insulin

570 resistance and type II diabetes mellitus. *Int. J. Obes.* 28, S12–S21.
 571 doi:10.1038/sj.ijo.0802853
 572

573 Hogg, K., Wood, C., McNeilly, A.S., Duncan, W.C., 2011. The in utero programming
 574 effect of increased maternal androgens and a direct fetal intervention on liver and
 575 metabolic function in adult sheep. *PLoS ONE* 6, e24877.
 576 doi:10.1371/journal.pone.0024877
 577

578 Hogg, K., Young, J.M., Oliver, E.M., Souza, C.J., McNeilly, A.S., Duncan, W.C.,
 579 2012. Enhanced Thecal Androgen Production Is Prenatally Programmed in an Ovine
 580 Model of Polycystic Ovary Syndrome. *Endocrinology* 153, 450–461.
 581 doi:10.1210/en.2011-1607
 582

583 Jason, J., 2011. Polycystic ovary syndrome in the United States: clinical visit rates,
 584 characteristics, and associated health care costs. *Arch. Intern. Med.* 171, 1209–
 585 1211. doi:10.1001/archinternmed.2011.288
 586

587 Keller, E., Chazenbalk, G.D., Aguilera, P., Madrigal, V., Elashoff, D., Grogan, T.,
 588 Dumesic, D.A., Abbott, D.H., 2014. Impaired preadipocyte differentiation into
 589 adipocytes in subcutaneous abdominal adipose of PCOS-like female rhesus
 590 monkeys. *Endocrinology* 155, 2696–2703. doi:10.1210/en.2014-1050
 591

592 Kim, J.-Y., van de Wall, E., Laplante, M., Azzara, A., Trujillo, M.E., Hofmann, S.M.,
 593 Schraw, T., Durand, J.L., Li, H., Li, G., Jelicks, L.A., Mehler, M.F., Hui, D.Y.,
 594 Deshaies, Y., Shulman, G.I., Schwartz, G.J., Scherer, P.E., 2007. Obesity-

595 associated improvements in metabolic profile through expansion of adipose tissue. J.
596 Clin. Invest. 117, 2621–2637. doi:10.1172/JCI31021
597
598 King, M., Dorosz, J., Patel, S.S., Kinney, G.L., Cree-Green, M., Wang, H., Ferland,
599 A., Truong, U., Nadeau, K.J., Maahs, D.M., Eckel, R.H., Moreau, K.L., Hokanson,
600 J.E., 2017. Obese adolescents with polycystic ovarian syndrome have elevated
601 cardiovascular disease risk markers. Vasc. Med. 22, 85–95.
602 doi:10.1177/1358863X16682107
603
604 Klemm, D.J., Leitner, J.W., Watson, P., Nesterova, A., Reusch, J., Goalstone, M.L.,
605 Draznin, B., 2001. Insulin-induced adipocyte differentiation - Activation of CREB
606 rescues adipogenesis from the arrest caused by inhibition of prenylation. J. Biol.
607 Chem. 276, 28430–28435. doi:10.1074/jbc.m103382200
608
609 Kokosar, M., Benrick, A., Perfilyev, A., Fornes, R., Nilsson, E., Maliqueo, M., Behre,
610 C.J., Sazonova, A., Ohlsson, C., Ling, C., Stener-Victorin, E., 2016. Epigenetic and
611 Transcriptional Alterations in Human Adipose Tissue of Polycystic Ovary Syndrome
612 SCI. Rep. 6, 22883. doi:10.1038/srep22883
613
614 Lafontan, M., 2014. Adipose tissue and adipocyte dysregulation. Diabetes Metab.
615 40, 16–28. doi:10.1016/j.diabet.2013.08.002
616
617 Lagaly, D.V., Aad, P.Y., Grado-Ahuir, J.A., Hulsey, L.B., Spicer, L.J., 2008. Role of
618 adiponectin in regulating ovarian theca and granulosa cell function. Mol. Cell.
619 Endocrinol. 284, 38–45. doi:10.1016/j.mce.2008.01.007

620 Lecke, S.B., Morsch, D.M., Spritzer, P.M., 2013. Association between adipose tissue
 621 expression and serum levels of leptin and adiponectin in women with polycystic
 622 ovary syndrome. *Genet. Mol. Res.* 12, 4292–4296. doi:10.4238/2013.February.28.16
 623
 624 Lessard, J., Laforest, S., Pelletier, M., Leboeuf, M., Blackburn, L., Tchernof, A.,
 625 2014. Low abdominal subcutaneous preadipocyte adipogenesis is associated with
 626 visceral obesity, visceral adipocyte hypertrophy, and a dysmetabolic state. *Adipocyte*
 627 3, 197–205. doi:10.4161/adip.29385
 628
 629 Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data
 630 Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25, 402–408.
 631 doi:10.1006/meth.2001.1262
 632
 633 Longo, M., Zatterale, F., Naderi, J., Parrillo, L., Formisano, P., Raciti, G.A., Beguinot,
 634 F., Miele, C., 2019. Adipose Tissue Dysfunction as Determinant of Obesity-
 635 Associated Metabolic Complications. *Int. J. Mol. Sci.* 20, 2358.
 636 doi:10.3390/ijms20092358
 637
 638 MacLean, P.S., Higgins, J.A., Giles, E.D., Sherk, V.D., Jackman, M.R., 2015. The
 639 role for adipose tissue in weight regain after weight loss. *Obes. Rev.* 16, 45–54.
 640 doi:10.1111/obr.12255
 641
 642 Macotela, Y., Emanuelli, B., Mori, M.A., Gesta, S., 2012. Intrinsic differences in
 643 adipocyte precursor cells from different white fat depots. *Diabetes* 61, 1691–1699.
 644 doi:10.2337/db11-1753

645

646 Maliqueo, M., Maliqueo, M., Pérez-Bravo, F., Galgani, J.E., Galgani, J.E., Pérez, F.,
647 Echiburú, B., Echiburú, B., Crisosto, N., de Guevara, A.L., Sir-Petermann, T., 2012.
648 Relationship of serum adipocyte-derived proteins with insulin sensitivity and
649 reproductive features in pre-pubertal and pubertal daughters of polycystic ovary
650 syndrome women. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 161, 56–61.
651 doi:10.1016/j.ejogrb.2011.12.012

652

653 Manneras-Holm, L., Leonhardt, H., Jennische, E., Kullberg, J., Oden, A., Holm, G.,
654 Hellstrom, M., Lonn, L., Olivecrona, G., Stener-Victorin, E., Lonn, M., 2010. Adipose
655 tissue has aberrant morphology and function in PCOS: enlarged adipocytes and low
656 serum adiponectin, but not circulating sex steroids, are strongly associated with
657 insulin resistance. *J. Clin. Endocrinol. Metab.* 96, E304–11. doi:10.1210/jc.2010-
658 1290

659

660 McNelis, J.C., Manolopoulos, K.N., Gathercole, L.L., Bujalska, I.J., Stewart, P.M.,
661 Tomlinson, J.W., Arlt, W., 2013. Dehydroepiandrosterone exerts antiglucocorticoid
662 action on human preadipocyte proliferation, differentiation, and glucose uptake. *Am.*
663 *J. Physiol. Endocrinol. Metab.* 305, E1134–44. doi:10.1152/ajpendo.00314.2012

664

665 Mirza, S.S., Shafique, K., Shaikh, A.R., Khan, N.A., Anwar Qureshi, M., Qureshi,
666 M.A., 2014. Association between circulating adiponectin levels and polycystic
667 ovarian syndrome. *J. Ovarian Res.* 7, 18. doi:10.1186/1757-2215-7-18

668 Moran, L.J., Teede, H.J., Norman, R.J., 2015. Metabolic risk in PCOS: phenotype
 669 and adiposity impact. *Trends Endocrinol. Metab.* 26, 136–143.
 670 doi:10.1016/j.tem.2014.12.003
 671
 672 Nishizawa, H., Shimomura, I., Kishida, K., Maeda, N., Kuriyama, H., Nagaretani, H.,
 673 Matsuda, M., Kondo, H., Furuyama, N., Kihara, S., Nakamura, T., Tochino, Y.,
 674 Funahashi, T., Matsuzawa, Y., 2002. Androgens decrease plasma adiponectin, an
 675 insulin-sensitizing adipocyte-derived protein. *Diabetes* 51, 2734–2741.
 676
 677 Padmanabhan, V., Veiga-Lopez, A., 2013. Sheep models of polycystic ovary
 678 syndrome phenotype. *Mol. Cell. Endocrinol.* 373, 8–20.
 679 doi:10.1016/j.mce.2012.10.005
 680
 681 Permana, P.A., Nair, S., Lee, Y.-H., Luczy-Bachman, G., Vozarova De Courten, B.,
 682 Tataranni, P.A., 2004. Subcutaneous abdominal preadipocyte differentiation in vitro
 683 inversely correlates with central obesity. *Am. J. Physiol. Endocrinol. Metab.* 286,
 684 E958–62. doi:10.1152/ajpendo.00544.2003
 685
 686 Poissonnet, C.M., Burdi, A.R., Bookstein, F.L., 1983. Growth and development of
 687 human adipose tissue during early gestation. *Early Hum. Dev.* 8, 1–11.
 688 doi:10.1016/0378-3782(83)90028-2
 689
 690 Puder, J.J., Varga, S., Kraenzlin, M., De Geyter, C., Keller, U., Müller, B., 2005.
 691 Central fat excess in polycystic ovary syndrome: relation to low-grade inflammation

692 and insulin resistance. *J. Clin. Endocrinol. Metab.* 90, 6014–6021.
693 doi:10.1210/jc.2005-1002
694
695 Puttabyatappa, M., Lu, C., Martin, J.D., Chazenbalk, G., Dumesic, D.,
696 Padmanabhan, V., 2018. Developmental Programming: Impact of Prenatal
697 Testosterone Excess on Steroidal Machinery and Cell Differentiation Markers in
698 Visceral Adipocytes of Female Sheep. *Reprod. Sci.* 25, 1010–1023.
699 doi:10.1177/1933719117746767
700
701 Rae, M., Grace, C., Hogg, K., Wilson, L.M., McHaffie, S.L., Ramaswamy, S.,
702 MacCallum, J., Connolly, F., McNeilly, A.S., Duncan, C., 2013. The pancreas is
703 altered by in utero androgen exposure: implications for clinical conditions such as
704 polycystic ovary syndrome (PCOS). *PLoS ONE* 8, e56263.
705 doi:10.1371/journal.pone.0056263
706
707 Ramaswamy, S., Grace, C., Mattei, A.A., Siemienowicz, K., Brownlee, W.,
708 MacCallum, J., McNeilly, A.S., Duncan, W.C., Rae, M.T., 2016. Developmental
709 programming of polycystic ovary syndrome (PCOS): prenatal androgens establish
710 pancreatic islet α/β cell ratio and subsequent insulin secretion. *Sci. Rep.* 6, 27408.
711 doi:10.1038/srep27408
712
713 Riestra, P., Garcia-Anguita, A., Ortega, L., Garces, C., 2013. Relationship of
714 Adiponectin with Sex Hormone Levels in Adolescents. *Horm. Res. Paediatr.* 79, 83–
715 87. doi:10.1159/000346898
716

717 Rosen, E.D., Spiegelman, B.M., 2014. What We Talk About When We Talk About
718 Fat. *Cell* 156, 20–44. doi:10.1016/j.cell.2013.12.012
719

720 Sarray, S., Madan, S., Saleh, L.R., Mahmoud, N., Almawi, W.Y., 2015. Validity of
721 adiponectin-to-leptin and adiponectin-to-resistin ratios as predictors of polycystic
722 ovary syndrome. *Fertil. Steril.* 104, 460–466. doi:10.1016/j.fertnstert.2015.05.007
723

724 Seow, K.-M., Tsai, Y.-L., Hwang, J.-L., Hsu, W.-Y., Ho, L.-T., Juan, C.-C., 2009.
725 Omental adipose tissue overexpression of fatty acid transporter CD36 and
726 decreased expression of hormone-sensitive lipase in insulin-resistant women with
727 polycystic ovary syndrome. *Hum. Reprod.* 24, 1982–1988.
728 doi:10.1093/humrep/dep122
729

730 Sepilian, V., Nagamani, M., 2005. Adiponectin levels in women with polycystic ovary
731 syndrome and severe insulin resistance. *J. Soc. Gynecol. Invest.* 12, 129–134.
732 doi:10.1016/j.jsig.2004.09.003
733

734 Siemienowicz, K., Rae, M.T., Howells, F., Anderson, C., Nicol, L.M., Franks, S.,
735 Duncan, W.C., 2020. Insights into manipulating postprandial energy expenditure to
736 manage weight gain in polycystic ovary syndrome (PCOS). *iScience* 101164.
737 doi:10.1016/j.isci.2020.101164
738

739 Siemienowicz, K.J., Filis, P., Shaw, S., Douglas, A., Thomas, J., Mulroy, S., Howie,
740 F., Fowler, P.A., Duncan, W.C., Rae, M.T., 2019. Fetal androgen exposure is a

741 determinant of adult male metabolic health. *Sci. Rep.* 9, 20195–17.
 742 doi:10.1038/s41598-019-56790-4
 743
 744 Singh, R., Artaza, J.N., Taylor, W.E., Bhasin, S., Gonzalez-Cadavid, N.F., 2003.
 745 Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H
 746 10T1/2 pluripotent cells through an androgen receptor-mediated pathway.
 747 *Endocrinology* 144, 5081–5088. doi:10.1210/en.2003-0741
 748
 749 Singh, R., Artaza, J.N., Taylor, W.E., Braga, M., Yuan, X., Gonzalez-Cadavid, N.F.,
 750 Bhasin, S., 2005. Testosterone inhibits adipogenic differentiation in 3T3-L1 cells:
 751 nuclear translocation of androgen receptor complex with beta-catenin and T-cell
 752 factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic
 753 transcription factors. *Endocrinology* 147, 141–154. doi:10.1210/en.2004-1649
 754
 755 Spalding, K.L., Arner, E., Westermark, P.O., Bernard, S., Buchholz, B.A., Bergmann,
 756 O., Blomqvist, L., Hoffstedt, J., Näslund, E., Britton, T., Concha, H., Hassan, M.,
 757 Rydén, M., Frisén, J., Arner, P., 2008. Dynamics of fat cell turnover in humans.
 758 *Nature* 453, 783–787. doi:10.1038/nature06902
 759
 760 Tchkonja, T., Morbeck, D.E., Zglinicki, von, T., van Deursen, J., Lustgarten, J.,
 761 Scrable, H., Khosla, S., Jensen, M.D., Kirkland, J.L., 2010. Fat tissue, aging, and
 762 cellular senescence. *Aging Cell* 9, 667–684. doi:10.1111/j.1474-9726.2010.00608.x
 763

764 Tchkonina, T., Thomou, T., Zhu, Y., Karagiannides, I., Pothoulakis, C., Jensen, M.D.,
 765 Kirkland, J.L., 2013. Mechanisms and Metabolic Implications of Regional Differences
 766 among Fat Depots. *Cell Metab.* 17, 644–656. doi:10.1016/j.cmet.2013.03.008
 767
 768 Teede, H., Deeks, A., Moran, L., 2010. Polycystic ovary syndrome: a complex
 769 condition with psychological, reproductive and metabolic manifestations that impacts
 770 on health across the lifespan. *BMC Med.* 8, 41. doi:10.1186/1741-7015-8-41
 771
 772 Veiga-Lopez, A., Moeller, J., Patel, D., Ye, W., Pease, A., Kinns, J., Padmanabhan,
 773 V., 2013. Developmental Programming: Impact of Prenatal Testosterone Excess on
 774 Insulin Sensitivity, Adiposity, and Free Fatty Acid Profile in Postpubertal Female
 775 Sheep. *Endocrinology* 154, 1731–1742. doi:10.1210/en.2012-2145
 776
 777 Wang, L., Li, S., Zhao, A., Tao, T., Mao, X., Zhang, P., Liu, W., 2012. The
 778 expression of sex steroid synthesis and inactivation enzymes in subcutaneous
 779 adipose tissue of PCOS patients. *J. Steroid Biochem. Mol. Biol.* 132, 120–126.
 780 doi:10.1016/j.jsbmb.2012.02.003
 781
 782 Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., Ferrante,
 783 A.W., Jr., 2003. Obesity is associated with macrophage accumulation in adipose
 784 tissue. *J. Clin. Invest.* 112, 1796–1808. doi:10.1172/JCI200319246
 785
 786 Wensvoort, P., 1967. The development of adipose tissue in sheep fetuses.
 787 *Path.Vet.* 4, 69–78. doi.org/10.1177/030098586700400107
 788

789 Xu, A., Chan, K.W., Hoo, R.L.C., Wang, Y., Tan, K.C.B., Zhang, J., Chen, B., Lam,
 790 M.C., Tse, C., Cooper, G.J.S., Lam, K.S.L., 2005. Testosterone selectively reduces
 791 the high molecular weight form of adiponectin by inhibiting its secretion from
 792 adipocytes. *J. Biol. Chem.* 280, 18073–18080. doi:10.1074/jbc.M414231200
 793
 794 Yang, X., Jansson, P.-A., Nagaev, I., Jack, M.M., Carvalho, E., Sunnerhagen, K.S.,
 795 Cam, M.C., Cushman, S.W., Smith, U., 2004. Evidence of impaired adipogenesis in
 796 insulin resistance. *Biochem. Biophys. Res. Commun.* 317, 1045–1051.
 797 doi:10.1016/j.bbrc.2004.03.152
 798
 799 Yildirim, B., Sabir, N., Sabir, Kaleli, B., 2003. Relation of intra-abdominal fat
 800 distribution to metabolic disorders in nonobese patients with polycystic ovary
 801 syndrome. *Fertil. Steril.* 79, 1358–1364. doi:10.1016/S0015-0282(03)00265-6
 802
 803 Zerradi, M., Dereumetz, J., Boulet, M.-M., Tchernof, A., 2014. Androgens, body fat
 804 Distribution and Adipogenesis. *Curr. Obes. Rep.* 3, 396–403. doi:10.1007/s13679-
 805 014-0119-6
 806

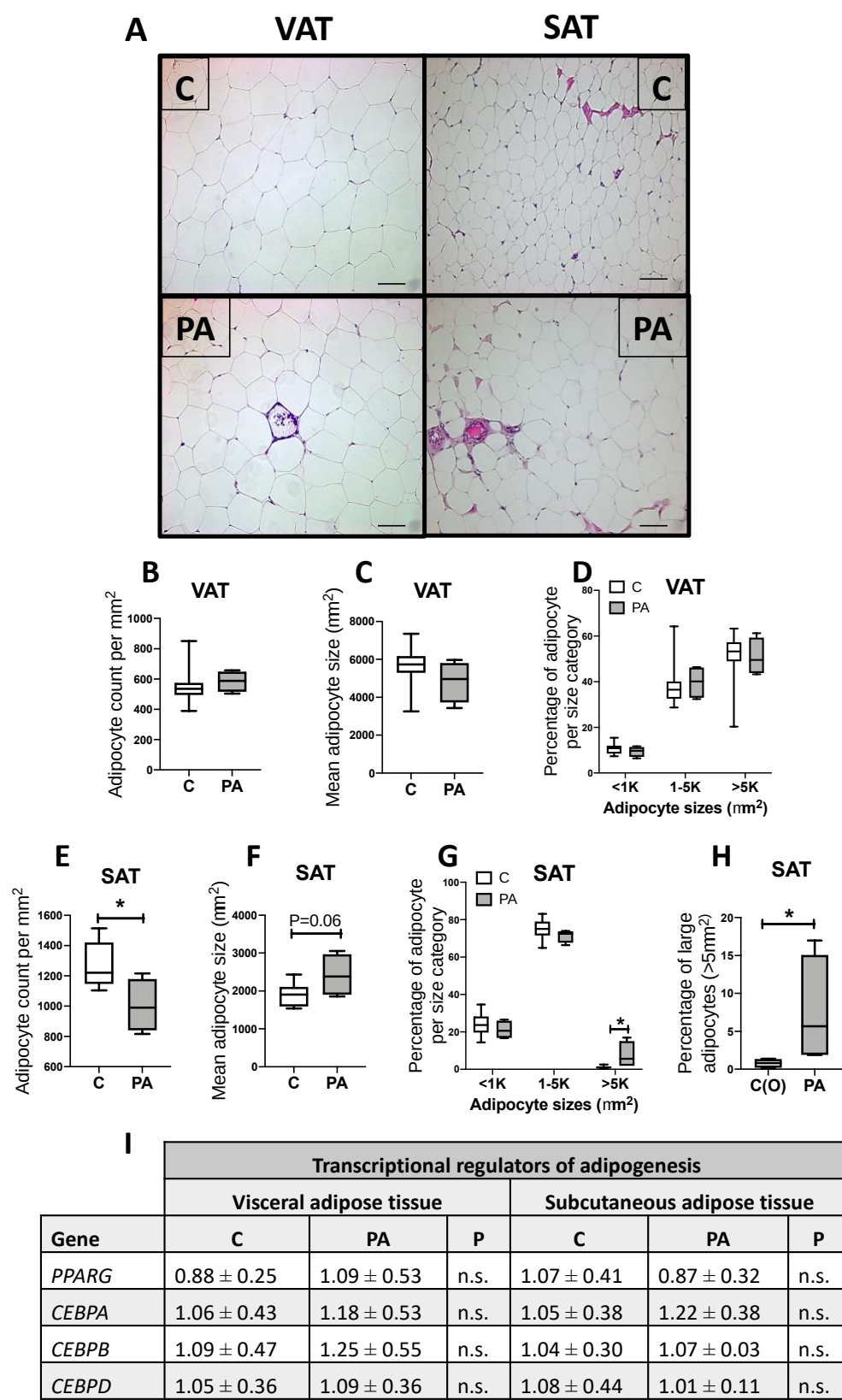
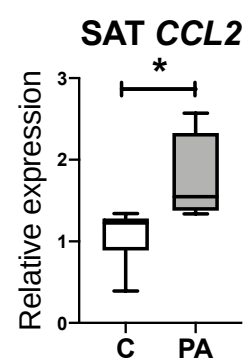
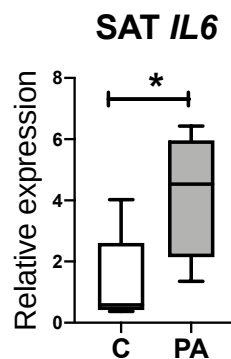
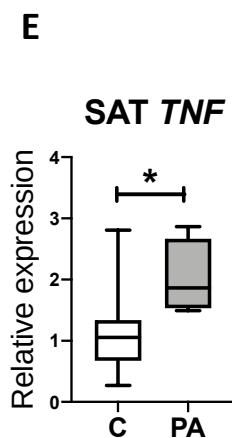
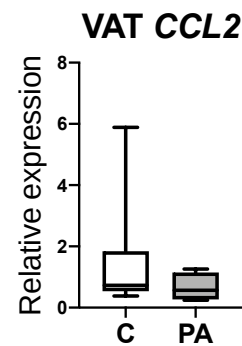
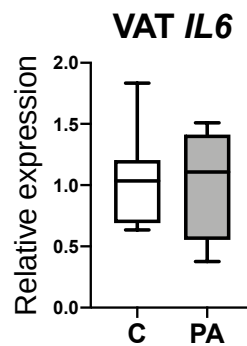
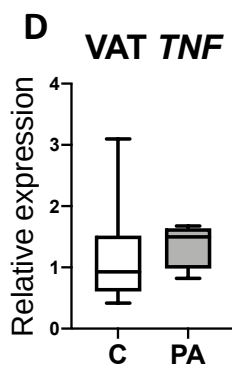
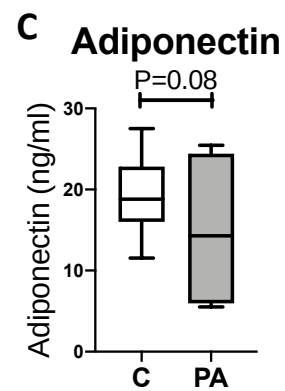
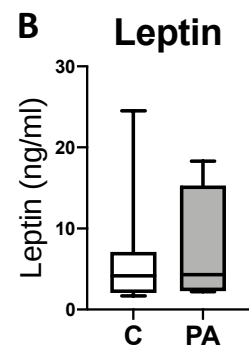


Figure 1. Adipose tissue analysis in adult (30M) controls (C; n=11) and prenatally androgenised sheep (PA; n=4). **(A)** Histological analysis of adipocyte morphology in VAT and SAT (C=8; PA=4) (scale bars = 100µm). **(B)** In VAT there was no difference in the numbers of adipocytes per mm², **(C)** no difference in the mean size of adipocytes and **(D)** no alteration the number of adipocytes of different sizes. **(E)** PA sheep had decreased number of adipocytes in SAT, **(F)** with a trend to an augmented mean size of adipocytes and **(G)** increased number of large adipocytes. **(H)** The increased number of large SAT adipocytes in PA sheep was still seem when compared to a subset of obese controls (n=4). **(I)** There was no difference in the expression of *PPARG*, *CEBPA*, *CEBPB* and *CEBPD* in adult VAT or SAT. Box plot whiskers are lowest and highest observed values, box is the upper and lower quartile, with median represented by line in box. Data in the table represent mean ± standard deviation. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting *P*<0.05 as significant. (**P*<0.05).

A			
Markers of mature adipocytes			
Visceral adipose tissue			
Gene	C	PA	P
<i>LEP</i>	1.07 ± 0.39	1.22 ± 0.57	n.s.
<i>ADIPOQ</i>	1.09 ± 0.52	1.56 ± 0.97	n.s.
Subcutaneous adipose tissue			
Gene	C	PA	P
<i>LEP</i>	1.07 ± 0.43	1.29 ± 0.50	n.s.
<i>ADIPOQ</i>	1.08 ± 0.46	1.00 ± 0.25	n.s.



826 **Figure 2.** Adipose tissue function in adult (30M) controls (C; n=11) and prenatally
827 androgenised sheep (PA; n=4). **(A)** There were no differences in *LEP* and *ADIPOQ*
828 expression in VAT or SAT. **(B)** There was no difference in the circulating leptin
829 between controls and PA sheep. **(C)** There was a trend towards decreased
830 adiponectin level in PA sheep. **(D)** There were no differences in transcript
831 abundance of inflammatory markers in VAT. **(E)** In SAT PA sheep had increased
832 *TNF*, *IL6* and *CCL2* when compared to controls. Box plot whiskers are lowest and
833 highest observed values, box is the upper and lower quartile, with median
834 represented by line in box. Data in the table represent mean $\pm$ standard deviation.
835 Unpaired, two-tailed Student's t test was used for comparing means of two treatment
836 groups with equal variances accepting $P < 0.05$ as significant. (* $P < 0.05$).
837

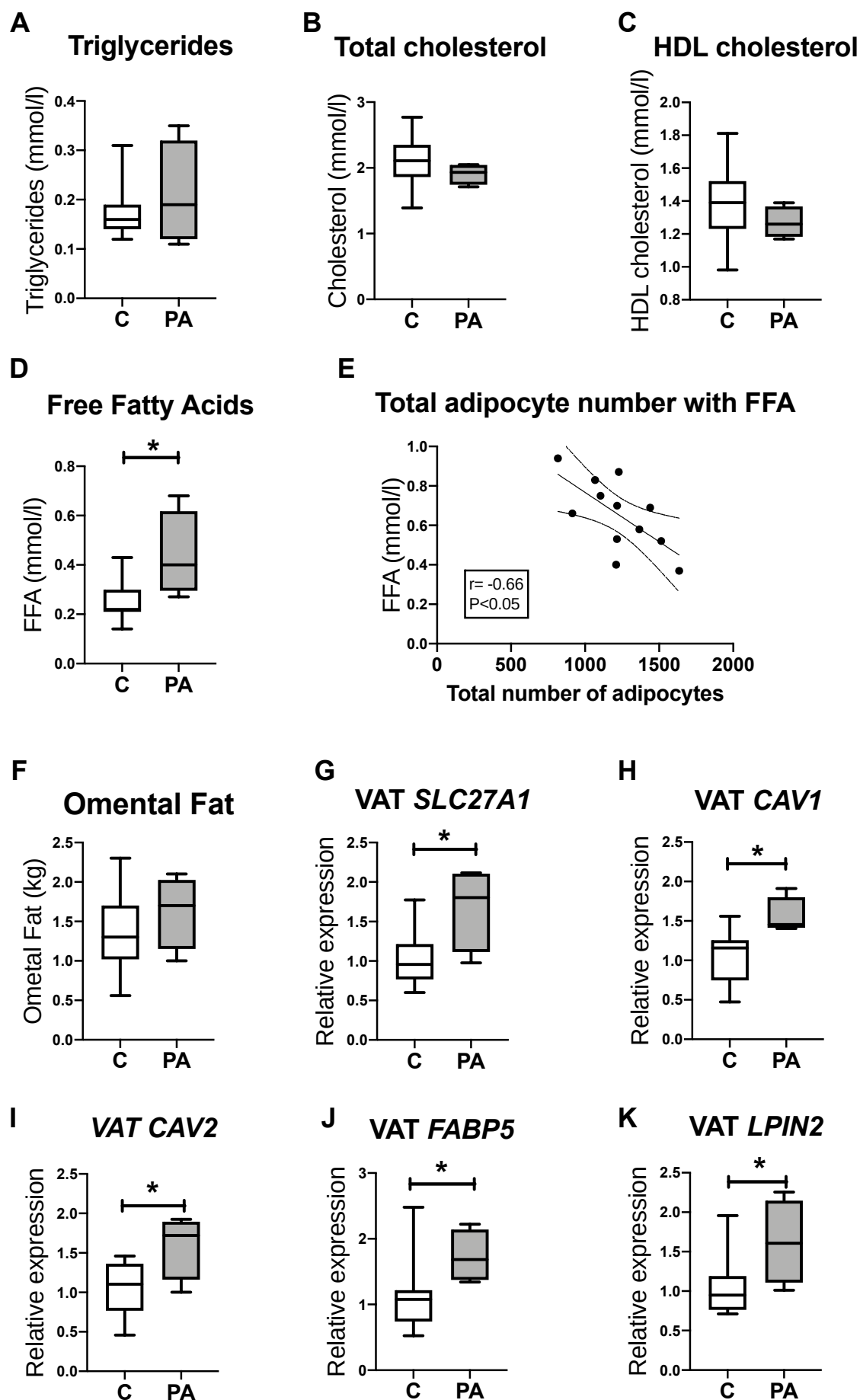


Figure 3. Metabolic function in adult (30M) controls (C; n=11) and prenatally androgenised sheep (PA; n=4). PCOS-like sheep have normal levels of (A) triglycerides, (B) cholesterol and (C) HDL cholesterol and (D) increased circulating FFAs. (E) There was negative correlation between the total number of adipocytes per mm² and the levels of circulating FFAs. (F) At this time there was no difference in omental fat between controls and PA sheep, however there was increased transcript abundance of genes involved in fat uptake and accumulation in VAT (G) *SLC27A1*, (H) *CAV1*, (I) *CAV2*, (J) *FABP5* and (K) *LPIN2*. Box plot whiskers are lowest and highest observed values, box is the upper and lower quartile, with median represented by line in box. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting $P < 0.05$ as significant. Correlation was assessed by calculation of Pearson product-moment coefficient. (* $P < 0.05$).

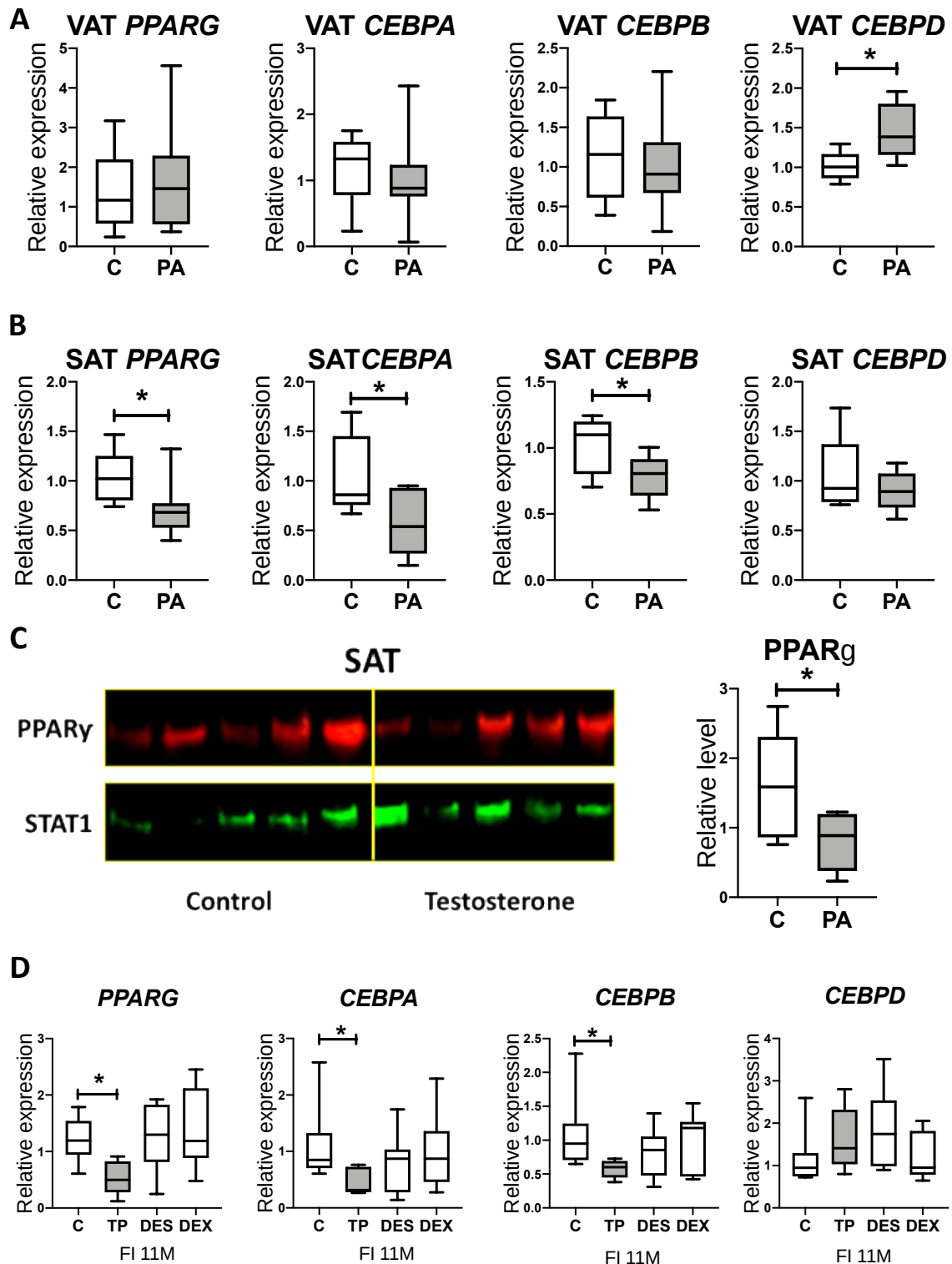


Figure 4. Transcript abundance of adipogenesis markers in adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9). (A) In VAT there was no difference in the transcript abundance of *PPARG*, *CEBPA* and *CEBPB*,

857 although *CEBPD* was increased. **(B)** Prenatally androgenised sheep had decreased
858 adipogenesis in SAT with a reduction in the transcript abundance of *PPARG*,
859 *CEBPA*, *CEBPB* and no difference in *CEBPD*. **(C)** Decreased level of *PPARG* in
860 SAT in prenatally androgenised females was confirmed with western blot. **(D)**. As
861 compared with controls (C; n=12), the transcript abundance of adipogenesis markers
862 in SAT was decreased by directly injected androgens in fetal life (TP; n=7) but not
863 estrogens (DES; n=8) or glucocorticoids (DEX; n=11). Box plot whiskers are lowest
864 and highest observed values, box is the upper and lower quartile, with median
865 represented by line in box. Unpaired, two-tailed Student's t test was used for
866 comparing means of two treatment groups with equal variances accepting $P<0.05$ as
867 significant. Unpaired, one-tailed Student's t test was used for western blot analysis.
868 For more than two comparisons ANOVA was used with Dunnett's post hoc test.
869 (* $P<0.05$; ** $P<0.01$).
870

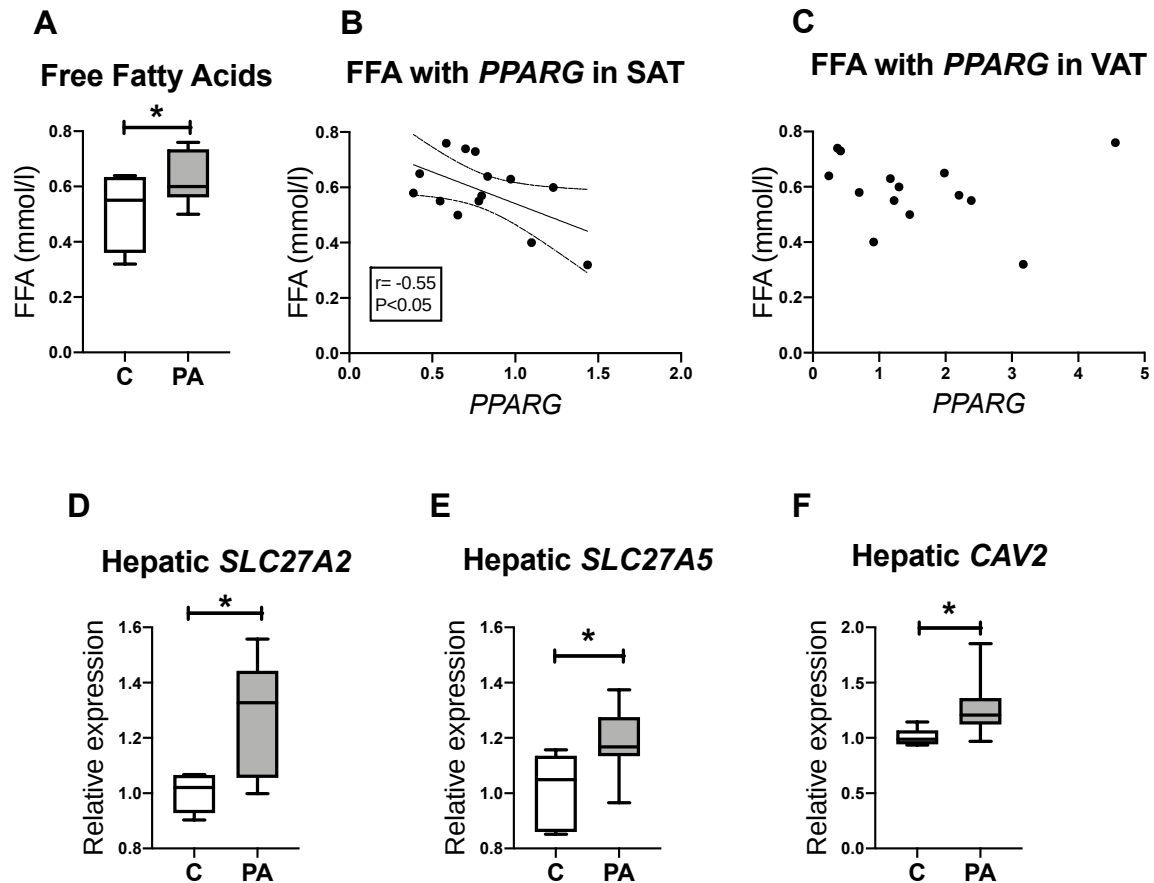


Figure 5. Adipose tissue function in adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9). **(A)** Adolescent prenatally androgenised sheep had increased fasting FFA. **(B)** Concentration of FFAs negatively correlated with *PPARG* ($r=-0.55$) in SAT but not **(C)** with *PPARG* in VAT. There is increased transcript abundance of **(D)** *SLC27A2*, **(E)** *SLC27A5* and **(F)** *CAV2* involved in hepatic fat uptake. Box plot whiskers are lowest and highest observed values, box is the upper and lower quartile, with median represented by line in box. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting $P<0.05$ as significant. Correlation was assessed by calculation of Pearson product-moment co-efficient. (* $P<0.05$).

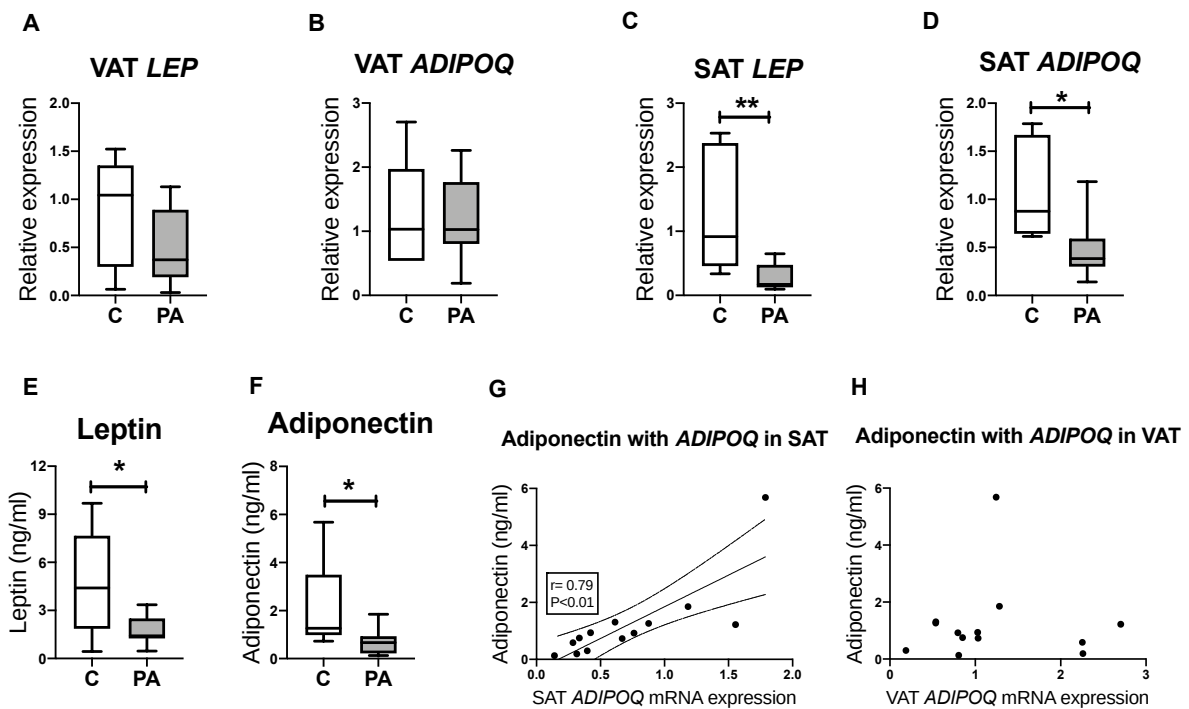
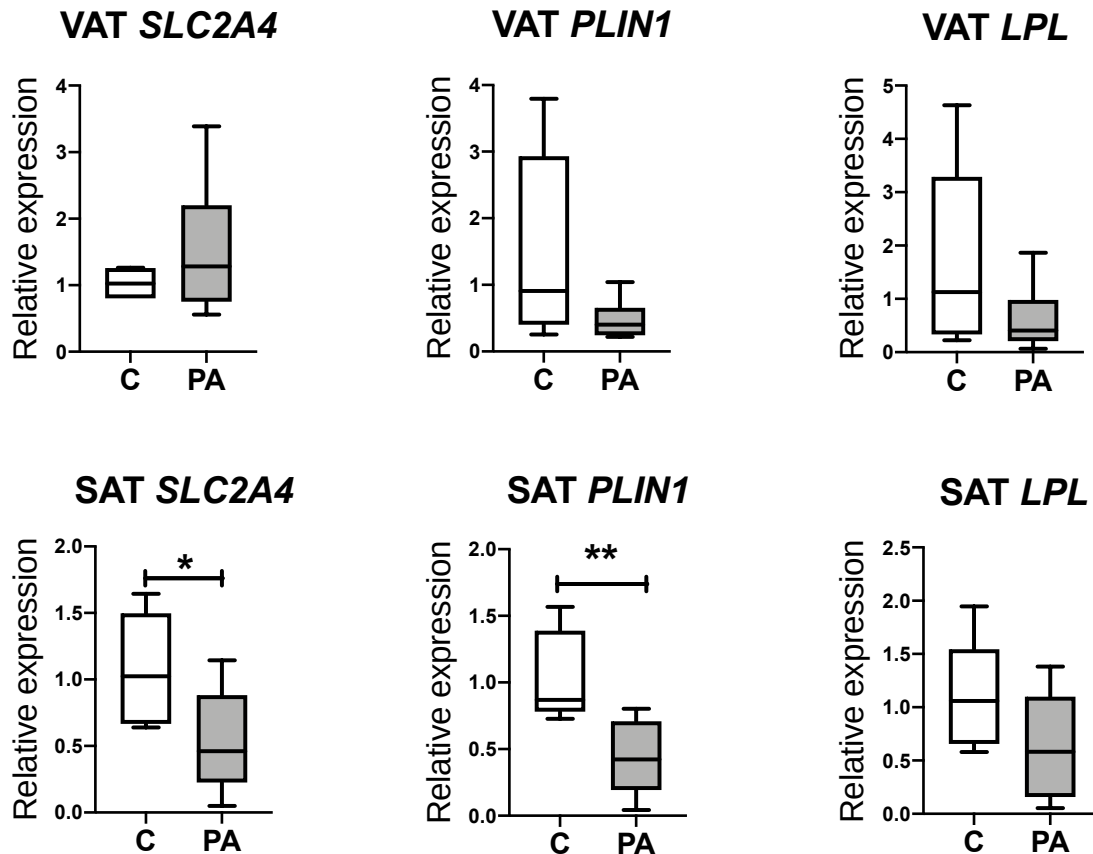


Figure 6. Transcript abundance and circulating levels of leptin and adiponectin in adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9). In VAT there was no difference in the expression of (A) *LEP* or (B) *ADIPOQ*. Adolescent prenatally androgenised sheep had decreased expression of (C) *LEP* and (D) *ADIPOQ* in SAT. This was mirrored by a reduction in circulating (E) leptin and (F) adiponectin in PA sheep. (G) Circulating adiponectin correlated ($r=0.79$) with *ADIPOQ* expression in SAT but not (H) in VAT. Box plot whiskers are lowest and highest observed values, box is the upper and lower quartile, with median represented by line in box. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting $P < 0.05$ as significant. Correlation was assessed by calculation of Pearson product-moment coefficient. (* $P < 0.05$; ** $P < 0.01$).



Supplementary Figure 1. Transcript abundance of markers of mature adipocytes in adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9). (A) In VAT there was no difference in the transcript abundance of *SLC2A4*, *PLIN1* and *LPL*. (B) Prenatally androgenised sheep had decreased expression of *SLC2A4* and *PLIN1* in SAT with no difference in *LPL*. Box plot whiskers are lowest and highest observed values, box is the upper and lower quartile, with median represented by line in box. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting $P < 0.05$ as significant. (* $P < 0.05$; ** $P < 0.01$).

Table 1

Transcriptional regulators of adipogenesis			
Subcutaneous adipose tissue			
Fetal life (D112)			
Gene	C	TP	P
<i>PPARG</i>	1.29 ± 0.85	1.75 ± 0.39	n.s.
<i>CEBPA</i>	1.26 ± 0.79	1.42 ± 0.63	n.s.
<i>CEBPB</i>	1.21 ± 0.72	1.17 ± 0.55	n.s.
<i>CEBPD</i>	1.05 ± 0.35	1.09 ± 0.19	n.s.
Gene	Pre-pubertal (11 weeks)		
<i>PPARG</i>	1.03 ± 0.25	1.30 ± 0.46	n.s.
<i>CEBPA</i>	1.11 ± 0.51	1.47 ± 0.63	n.s.
<i>CEBPB</i>	1.01 ± 0.33	1.34 ± 0.38	n.s.
<i>CEBPD</i>	1.04 ± 0.28	0.90 ± 0.33	n.s.

Table 1. Timing of altered adipogenesis. There was no difference in the expression of transcription factors involved in adipogenesis in fetal life at GD112 (C; n=9; PA; n=4) or in pre-pubertal sheep at 11 weeks of age (C; n=8; PA; n=8). Data in the table represent mean ± standard deviation. Unpaired, two-tailed Student's t test was used for comparing means of two treatment groups with equal variances accepting $P < 0.05$ as significant.

918 **Supplementary Table 1**

Gene	Forward Primer	Reverse Primer
<i>18S</i>	CAACTTTCGATGGTAGTCG	CCTTCCTTGGATGTGGTA
<i>ACTB</i>	ATCGAGGACAGGATGCAGAA	CCAATCCACACGGAGTACTTG
<i>ADIPOQ</i>	AGAGATGGCACCCTGGT	GACCTTCGATCCCAGTGATT
<i>ADIPOR1</i>	TCTCCTGGCTCTTCCACACT	AGTCCCCATGATCAGCA
<i>ADIPOR2</i>	AGGTCTGGGAGCCTCTTGTAG	TGAACCCCTCATCTTCCTGA
<i>CAV1</i>	CATCTCTACACTGTTCCCATCC	ACGTCGTCGTTGAGATGCTT
<i>CAV2</i>	CCACAGCAGCGTCGATTAC	CACTGGCTCTGCAATCACAT
<i>CCL2</i>	GACCCCAACCTGAAATGGGT	GCAGTTAGGGGAAGCCAGAA
<i>CEBPA</i>	GTGGACAAGAACAGCAACGA	CGCAGTGTGTCCAGTTCG
<i>CEBPB</i>	GACAAGCACAGCGACGAGTA	AGCTGCTCCACCTTCTTCTG
<i>CEBPD</i>	CGAGTACCGGCAGCGAC	GTCGCGCAGTCCGGC
<i>FABP5</i>	TTCAGCAGCTGGTAGGAAGA	GCACCTACTTTTCGCAGAGC
<i>IL6</i>	AAATGACACCACCCCAAGCA	CTCCAGAAGACCAGCAGTGG
<i>INSR</i>	CACCATCACTCAGGGGAAAC	CAGGAGGTCTCGGAAGTCAG
<i>IRS1</i>	ATCATCAACCCCATCAGACG	GAGTTTGCCACTACCGCTCT
<i>IRS2</i>	TCCAGAACGGCCTCAACTAC	TCAGGTGATGCGTCAAGAAG
<i>LEP</i>	ATCTCACACACGCAGTCCGT	CCAGCAGGTGGAGAAGGTC
<i>LPIN2</i>	GACGTCACCCTGTCACTCTG	GAGTCCAGGGTTTTCTGCAA
<i>MDH1</i>	TTATCTCCGATGGCAACTCC	GGGAGACCTTCAACAACCTTCC
<i>PPARG</i>	TGCAGTGGGGATGTCTCATA	CAGCGGGAAGGACTTTATGT
<i>RPS26</i>	CAAGGTAGTCAGGAATCGCTCT	TTACATGGGCTTTGGTGGAG
<i>SLC27A2</i>	GTGGAAAGGGGAAAATGTGG	TCAAATTCATGGTCTGCCTTC
<i>SLC27A5</i>	CGGACATCAAGTTGCGAAG	ATCCCTGATACCTGCAGCAC
<i>SLC2A4</i>	CCAGCATCTTTGAGTCAGCA	CAGAAGCAGAGCCACAGTCA
<i>TNF</i>	GGTGCCTCAGCCTCTTCT	GAACCAGAGGCCTGTTGAAG

919

920 **Supplementary Table 1.** Primers for real-time RT-PCR analysis. Forward and
921 reverse primers were designed using Primer3 Input version 0.4 online software
922 (<http://frodo.wi.mit.edu>) with DNA sequences obtained at Ensembl Genome Browser.
923 To confirm the validity of the gene product in the sheep, both conventional PCR and
924 amplicon sequencing were performed. Primer specificity and efficacy for qRT-PCR
925 was evaluated through the generation of standard curves with serial dilutions of

926 cDNA; a standard curve slope of approximately -3.3 was accepted as efficient, and a
927 melt-curve analysis was also performed.