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

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

3

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19

20 **Abstract**

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

35

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

38

## 39 **1. Introduction**

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

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

51 Although studies using animal models of PCOS have also indicated adipose tissue  
52 abnormalities, the data is inconclusive (Keller et al., 2014; Puttabyatappa et al.,  
53 2018; Veiga-Lopez et al., 2013). Insulin and androgens, both typically increased in  
54 PCOS, affect adipogenesis and adipose tissue function (Chazenbalk et al., 2013;  
55 Klemm et al., 2001), and thus are likely to play an important role in adipose tissue  
56 dysfunction in PCOS. White adipose tissue (WAT) is a key metabolic and endocrine  
57 organ regulating energy homeostasis, insulin sensitivity and inflammation, therefore  
58 functional WAT is essential in maintaining body homeostasis (Lafontan, 2014). There  
59 is strong evidence showing differences between subcutaneous adipose tissue (SAT)  
60 and visceral adipose tissue (VAT) biology, including distinct gene expression in VAT  
61 and SAT preadipocytes (Macotela et al., 2012; Tchkonina et al., 2013)

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

64 2013). We have previously documented that ewes exposed to increased androgens  
65 *in utero* manifest ovarian, hormonal and metabolic phenotypes reminiscent of PCOS  
66 (Hogg et al., 2011; 2012; Rae et al., 2013; Ramaswamy et al., 2016), and male  
67 offspring from such pregnancies display similar metabolic disturbances as seen in  
68 sons born to women with PCOS (Siemienowicz et al., 2019). We have reported that  
69 adult prenatally androgen-excess exposed (PA) sheep have increased body weight  
70 and are insulin resistant (Siemienowicz et al., 2020), while in adolescence those  
71 sheep are hyperinsulinemic and have increased fat accumulation in the liver,  
72 independent of body weight or central adiposity (Hogg et al., 2011). We  
73 hypothesised that the metabolic phenotype of PA sheep is associated with aberrant  
74 adipogenesis. Herein we investigate adipose tissue structure and function in  
75 adulthood and highlight aberrant adipogenesis in adolescence and its consequences  
76 in female PA sheep.

77

## 78 **2. Materials and Methods**

### 79 **2.1 Ethics statement**

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

### 84 **2.2 Animals and tissues**

85 Animal husbandry, experimental protocols and tissue collection were performed as  
86 previously described (Hogg et al., 2012; 2011; Rae et al., 2013; Ramaswamy et al.,  
87 2016). Scottish Greyface ewes were housed in groups in spacious enclosures and  
88 fed hay *ad libitum*. Ewes with a healthy body condition score (2.75-3) were  
89 synchronised with Chronogest (flugestone) sponges (Intervet Ltd, UK) and  
90 Estrumate (cloprostenol) injection (Schering Plough Animal Health, UK) and mated  
91 with Texel rams. Pregnancy was suggested by lack of estrous and confirmed by  
92 ultrasound scanning.

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

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

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

103 In the fetal injection cohort (FI), on day 62 and day 82 of gestation, mothers were  
104 randomised and anaesthetised by initial sedation with 10 mg Xylazine (i.m. Rompun;  
105 Bayer PLC Animal Health Division, UK), followed by 2mg/kg Ketamine (i.v, Ketaset;  
106 Fort Dodge Animal Health, UK). All subsequent procedures were conducted under  
107 surgical aseptic conditions. Fetuses were injected via ultrasound guidance into the  
108 fetal flank with 20G Quinke spinal needle (BD Biosciences, UK) with following  
109 according to the treatment group: control (C; n=12), 0.2ml vehicle (vegetable oil);  
110 testosterone propionate (TP; n=7), 20mg TP in 0.2ml vehicle; diethylsilbesterol  
111 (DES; n=8), 4mg DES in 0.2ml vehicle; dexamethasone (DEX; n=11), 100µg DEX in  
112 0.2ml vehicle. Justification of the rationale, timing and treatment doses have been  
113 published previously (Siemienowicz et al., 2019). Immediately after surgical  
114 procedure completion all pregnant ewes were given prophylactic antibiotics  
115 (Streptacare, Animalcare Ltd., UK, 1 ml/25 kg) and were then monitored during  
116 recovery; no adverse effects of these procedures were observed. Lambs were  
117 weaned at 3 months and fed hay or grass ad libitum and sacrificed in adolescence  
118 (11 months of age).

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

### 124 **2.3 Plasma analyte determination**

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

128 Cobas Mira automated analyser (Roche Diagnostics Ltd, UK). For all assays intra  
129 and inter-assay CV's were < 4% CV and < 5% CV, respectively. Fasting plasma  
130 leptin concentration was measured using Bio-Plex Human Diabetes Assay  
131 (171A7001M; Bio-Rad, UK), as per the manufacturer's instruction. All samples were  
132 assayed in duplicate and the results were acquired and calculated using Bio-Plex  
133 200 array reader system with Bio-Plex Manager software (Bio-Rad, UK). The assay  
134 working range for leptin was 0.011-129ng/ml, assay sensitivity 3.1pg/ml, and intra  
135 and inter-assay CVs were 3% and 4%, respectively. Plasma adiponectin was  
136 measured using human Adiponectin ELISA kit (KHP041; Invitrogen, Life  
137 Technologies, UK), as per the manufacturer's instructions. All samples were assayed  
138 in duplicate. The assay sensitivity was 0.1ng/ml intra and inter-assay CVs were <5%  
139 and <6%, respectively.

#### 140 **2.4 Adipocyte morphometric analysis**

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

#### 150 **2.5 Western Blotting**

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



153 Ltd.) in RIPA buffer (ab156034, Abcam, UK) supplemented with Halt™ Protease  
154 Inhibitor Cocktail (Thermo Scientific Pierce, USA) and centrifuged at 17500g at 4°C  
155 for 10 min to pellet cellular debris. Protein concentration was measured using the  
156 Bradford Assay on Cobas Fara centrifugal analyser (Roche Diagnostics, UK).  
157 Samples were diluted in RIPA buffer and combined with equal volume of 1X Laemmli  
158 buffer (0.1M Tris-HCl pH 6.8, 20% glycerol, 2% (w/v) SDS, 0.16% (w/v) bromophenol  
159 blue and 3% β-mercaptoethanol). After heat denaturation (95°C for 5 min), samples  
160 (20 µg total protein) underwent electrophoresis, alongside full-range PageRuler™  
161 Plus Prestained Protein Ladder (Thermo Scientific Pierce, USA), on 4-20% Tris-  
162 HEPES-SDS Precast Polyacrylamide Mini Gels (Thermo Scientific Pierce, USA) and  
163 were transferred to a PVDF membrane (IPFL00010; Immobilon-FL PVDF; Merck  
164 Millipore, Germany) using fast semi-dry blotter (Thermo Scientific Pierce, USA).  
165 Membranes were blocked in Odyssey Blocking Buffer (Li-Cor, USA) overnight at 4°C.  
166 Membrane was probed with primary antibody raised against STAT1 (1:1000; SC-346,  
167 Santa Cruz Biotechnology) for 2h at RT, washed and probed with a primary antibody  
168 raised against PPARγ (1:500; SC-1984 Santa Cruz Biotechnology) for 4h at RT. After  
169 washing, membranes were incubated with two different fluorescently-labelled  
170 secondary antibodies at the concentration 1:10000 IRDye 680RD (926-68074; Li-Cor)  
171 and IRDye 800CW (926-32213; Li-Cor), both for 1h at RT. After final washing,  
172 membranes were visualised on the Odyssey Imager (Li-Cor, USA). The size of the  
173 visualised protein band was confirmed with reference to the molecular weight markers.  
174 Protein densitometry was analysed with Image Studio Lite Software (Li-Cor, USA) with  
175 STAT1 protein levels used as the loading control.

## 176 **2.5 Quantitative (q)RT-PCR**

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

## 196 **2.6 Statistical analysis**

197 All data sets were normality tested prior to further analysis (Shapiro-Wilk test), and  
198 logarithmically transformed if necessary. For comparing means of two treatment  
199 groups with equal variances, unpaired, two-tailed Student's t test was used  
200 accepting  $P < 0.05$  as significant. For more than two comparisons ANOVA was used  
201 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

## 207 **3. Results**

### 208 **3.1 PA sheep have structurally altered subcutaneous adipose tissue in** 209 **adulthood**

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

### 227 **3.2 Prenatal androgen excess is associated with adipose tissue dysfunction in** 228 **adulthood**

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

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

### 247 **3.3 Altered adipogenesis is observed during adolescence**

248 We next assessed expression of transcription factors involved in adipogenesis at 11  
249 months of age when there was no difference in body weight (Siemienowicz et al.,  
250 2020) and omental fat weight (Hogg et al., 2011). In VAT there was no difference in  
251 the transcript abundance of *PPARG*, *CEBPA* and *CEBPB*, although *CEBPD* was  
252 increased (P<0.05) in adolescent PA sheep (Fig. 4A). However, in SAT there was  
253 decreased transcript abundance of *PPARG*, *CEBPA*, *CEBPB* (Fig. 4B; P<0.05) but  
254 no difference in *CEBPD* (Fig. 4B). We confirmed decreased level of *PPARG* in SAT  
255 (Fig. 4C; P<0.05) in adolescent PA females, by Western blot. In addition, adolescent  
256 PA sheep had decreased expression of markers of mature adipocytes in SAT,

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

### 264 **3.4 Adolescent SAT transcriptional alterations are associated with increased** 265 **circulating FFA**

266 Fasting FFAs were increased in adolescent PA sheep when compared to controls  
267 (Fig. 5A;  $P < 0.05$ ). Their concentrations were negatively correlated with *PPARG* ( $r = -$   
268 0.55) in SAT (Fig. 5B;  $P < 0.05$ ) but not with *PPARG* in VAT (Fig. 5C). These sheep  
269 have early accumulation of fat in the liver (Hogg et al., 2011) with increased  
270 transcript abundance of genes involved in FFAs uptake and deposition in the liver  
271 (Fig. 5D-F;  $P < 0.05$ ).

### 272 **3.5 Adiponectin is a biomarker of reduced SAT adipogenesis during** 273 **adolescence**

274 To investigate potential biomarkers of altered adipogenesis during adolescence the  
275 transcript abundance of markers of mature adipocytes was investigated in adipose  
276 tissue at 11 months of age in control and PA animals. In VAT there was no changes  
277 in *LEP* or *ADIPOQ* expression (Fig. 6A,B). In contrast *LEP* ( $P < 0.01$ ) and *ADIPOQ*  
278 ( $P < 0.05$ ) were reduced in SAT in adolescent PA sheep when compared to controls  
279 (Fig. 6C,D). This was reflected by a reduction in circulating leptin (Fig 6E;  $P < 0.05$ )  
280 and adiponectin (Fig. 6F;  $P < 0.05$ ) in the plasma. Circulating adiponectin correlated  
281 ( $r = 0.79$ ) 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

291 **4. Discussion**

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

300

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

315



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

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

348 Adipogenesis in PCOS patients has not been thoroughly investigated, however,  
349 increased abdominal adipose stem cell commitment to preadipocytes *in vitro*, as  
350 measured by the expression of ZFP423 protein, have been found in adult, normal  
351 weight women with PCOS (Fisch *et al.*, 2018). Increased *ZFP423* gene expression  
352 was also reported in abdominal SAT of prenatally androgenised female rhesus  
353 monkeys (Keller *et al.*, 2014). We did not study the preadipocyte commitment but  
354 rather, the differentiation of preadipocytes into mature adipocytes. In agreement with  
355 our study, it was reported that women with PCOS have decreased expression and  
356 increased DNA methylation of *PPARG* in SAT and *PPARG* expression positively  
357 correlated with insulin sensitivity and negatively with adipocyte size and testosterone  
358 levels (Kokosar *et al.*, 2016). Furthermore, PCOS-like monkeys were also have  
359 decreased abdominal SAT *CEBPA* expression, reduced numbers of mature SAT  
360 adipocytes and increased FFAs indicating impaired adipogenesis (Keller *et al.*,  
361 2014). In addition, there is growing evidence of decreased adipogenesis in SAT from  
362 insulin resistant individuals and subjects with abdominal obesity (Heilbronn *et al.*,  
363 2004; Permana *et al.*, 2004; Yang *et al.*, 2004). Our data is in broad agreement with  
364 such observations, lending translational confidence to our findings.

365

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

375

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

383

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

391 insulin sensitising adipokine, promoting lipid oxidation and reducing plasma  
392 concentration of FFAs (Achari and Jain, 2017), and stimulates glucose uptake in  
393 adipocytes by increasing expression of GLUT4 (Achari and Jain, 2017; Fu et al.,  
394 2005).

395

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

402 Whilst androgens decrease plasma adiponectin *in vitro* and *in vivo* (Frederiksen et  
403 al., 2012; Nishizawa et al., 2002; Xu et al., 2005), and this is inversely correlated with  
404 testosterone levels, (Böttner et al., 2004; Riestra et al., 2013), to our knowledge this  
405 is the first demonstration of prenatal androgen excess being associated with  
406 decreased SAT and circulating adiponectin. It is noteworthy in the context of prenatal  
407 androgenic programming of PCOS that adiponectin decreases ovarian thecal  
408 androgen synthesis and expression of adiponectin receptors is decreased in theca  
409 cells from polycystic ovaries; consequently decreased adiponectin may also  
410 contribute to hyperandrogenism in PCOS women (Comim et al., 2013; Lagaly et al.,  
411 2008).

412

413 Inhibitory effects of androgens on adipogenesis is well documented (Zerradi et al.,  
414 2014). Numerous studies demonstrate androgens prevent *in vitro* differentiation and  
415 proliferation of murine preadipocyte cell lines (Fujioka et al., 2012; Singh et al., 2003;

416 2005) and human preadipocytes from both sexes, and from different fat depots  
417 (Blouin et al., 2010; Chazenbalk et al., 2013; Gupta et al., 2008; McNelis et al.,  
418 2013). To determine steroid specificity of androgenic excess in programming  
419 adipose tissue function *in vivo*, we assessed SAT adipogenesis in adolescent, 11  
420 months old female sheep, that were directly injected *in utero* with androgen,  
421 estrogen (DES as surrogate estrogen) or glucocorticoid (DEX as surrogate, active  
422 glucocorticoid). We found that only androgen treatment, but not estrogen or  
423 glucocorticoid, decreased adipogenesis in SAT in adolescence, identifying that the  
424 responses measured throughout the study were androgenic.

425

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

433

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445

446 **Declaration of interest**

447 The authors have no conflicts of interest to declare.

448

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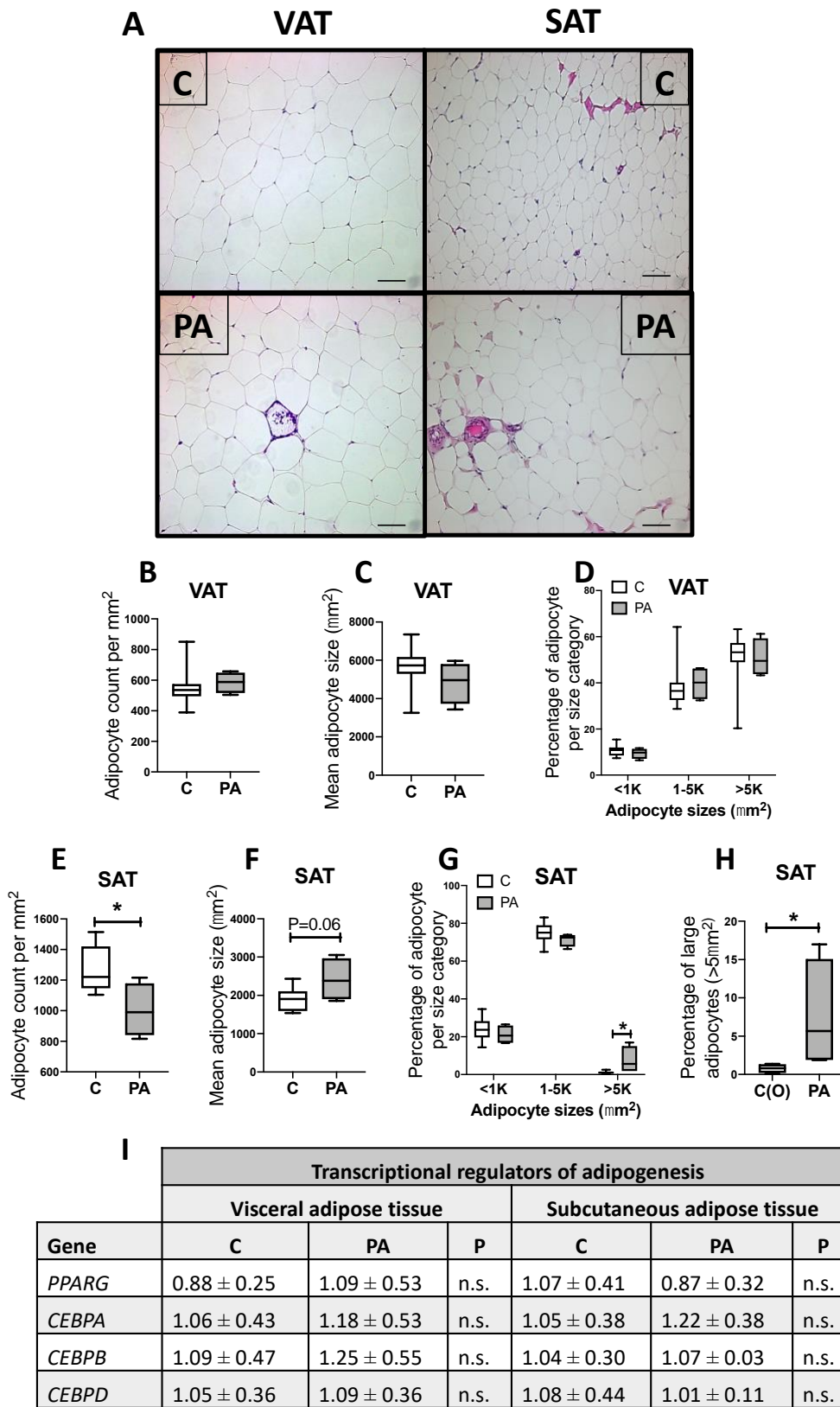
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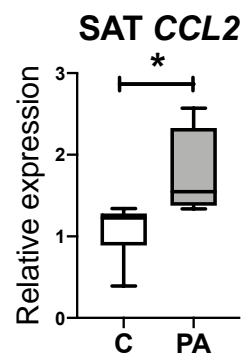
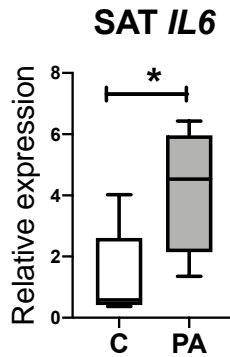
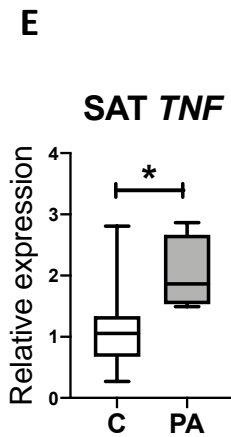
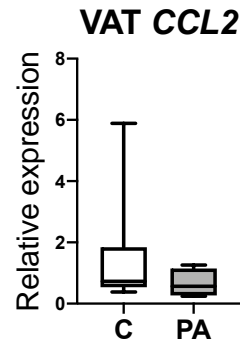
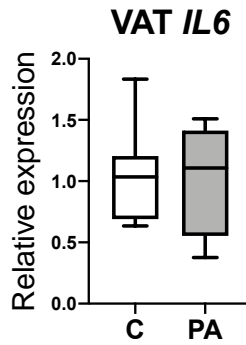
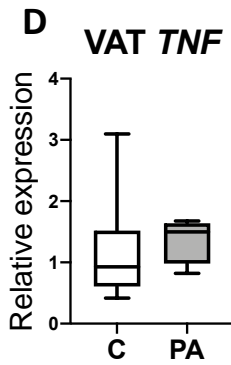
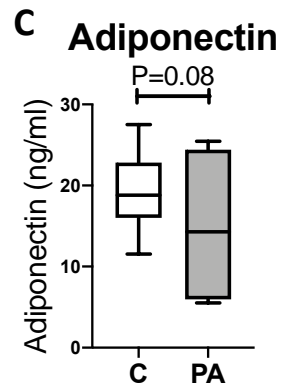
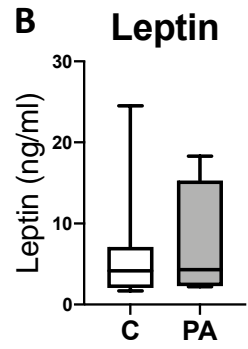
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809 **Figure 1.** Adipose tissue analysis in adult (30M) controls (C; n=11) and prenatally  
810 androgenised sheep (PA; n=4). **(A)** Histological analysis of adipocyte morphology in  
811 VAT and SAT (C=8; PA=4) (scale bars = 100µm). **(B)** In VAT there was no  
812 difference in the numbers of adipocytes per mm<sup>2</sup>, **(C)** no difference in the mean size  
813 of adipocytes and **(D)** no alteration the number of adipocytes of different sizes. **(E)**  
814 PA sheep had decreased number of adipocytes in SAT, **(F)** with a trend to an  
815 augmented mean size of adipocytes and **(G)** increased number of large adipocytes.  
816 **(H)** The increased number of large SAT adipocytes in PA sheep was still seem when  
817 compared to a subset of obese controls (n=4). **(I)** There was no difference in the  
818 expression of *PPARG*, *CEBPA*, *CEBPB* and *CEBPD* in adult VAT or SAT. Box plot  
819 whiskers are lowest and highest observed values, box is the upper and lower  
820 quartile, with median represented by line in box. Data in the table represent mean ±  
821 standard deviation. Unpaired, two-tailed Student's t test was used for comparing  
822 means of two treatment groups with equal variances accepting  $P<0.05$  as significant.  
823 (\* $P<0.05$ ).  
824

**A**

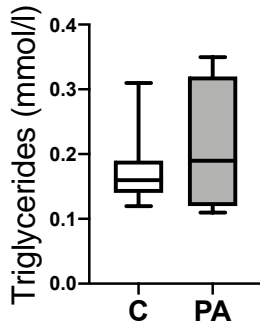
Gene	Markers of mature adipocytes		
	Visceral adipose tissue		
Gene	C	PA	P
<i>LEP</i>	1.07 ± 0.39	1.22 ± 0.57	n.s.
<i>ADIPOQ</i>	1.09 ± 0.52	1.56 ± 0.97	n.s.
Gene	Subcutaneous adipose tissue		
<i>LEP</i>	1.07 ± 0.43	1.29 ± 0.50	n.s.
<i>ADIPOQ</i>	1.08 ± 0.46	1.00 ± 0.25	n.s.



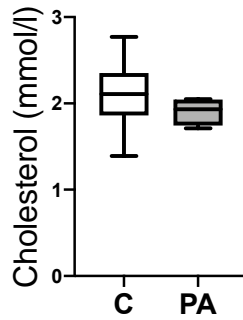


826 **Figure 2.** Adipose tissue function in adult (30M) controls (C; n=11) and prenatally  
827 androgenised sheep (PA; n=4). **(A)** There were no differences in *LEP* and *ADIPOQ*  
828 expression in VAT or SAT. **(B)** There was no difference in the circulating leptin  
829 between controls and PA 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. (\* $P < 0.05$ ).  
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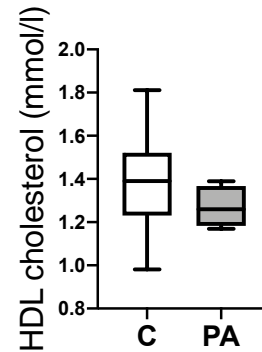
**A** Triglycerides



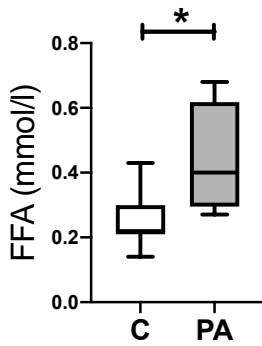
**B** Total cholesterol



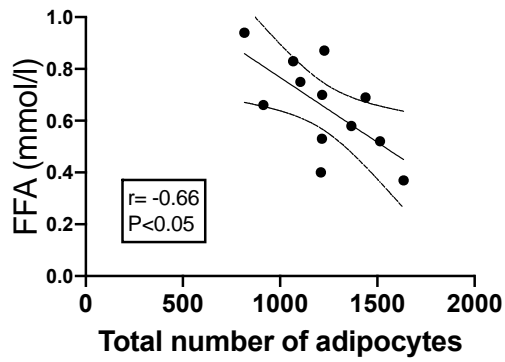
**C** HDL cholesterol



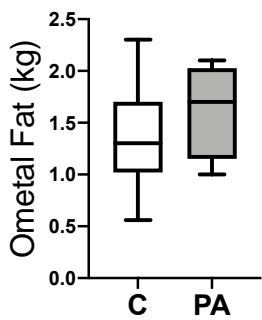
**D** Free Fatty Acids



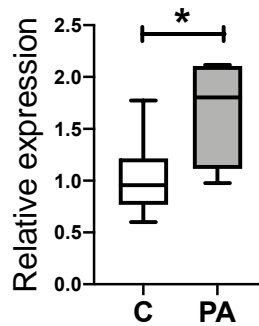
**E** Total adipocyte number with FFA



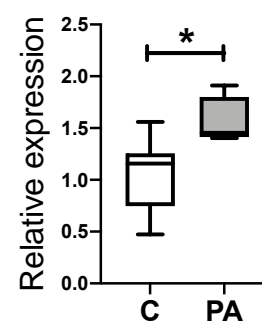
**F** Omental Fat



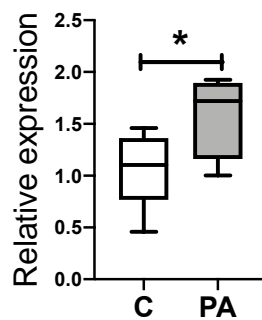
**G** VAT SLC27A1



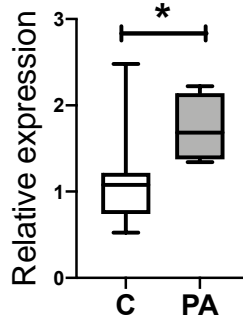
**H** VAT CAV1



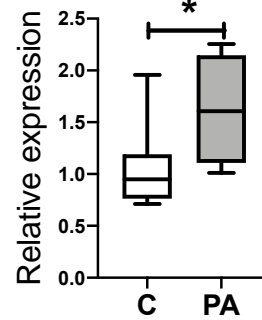
**I** VAT CAV2



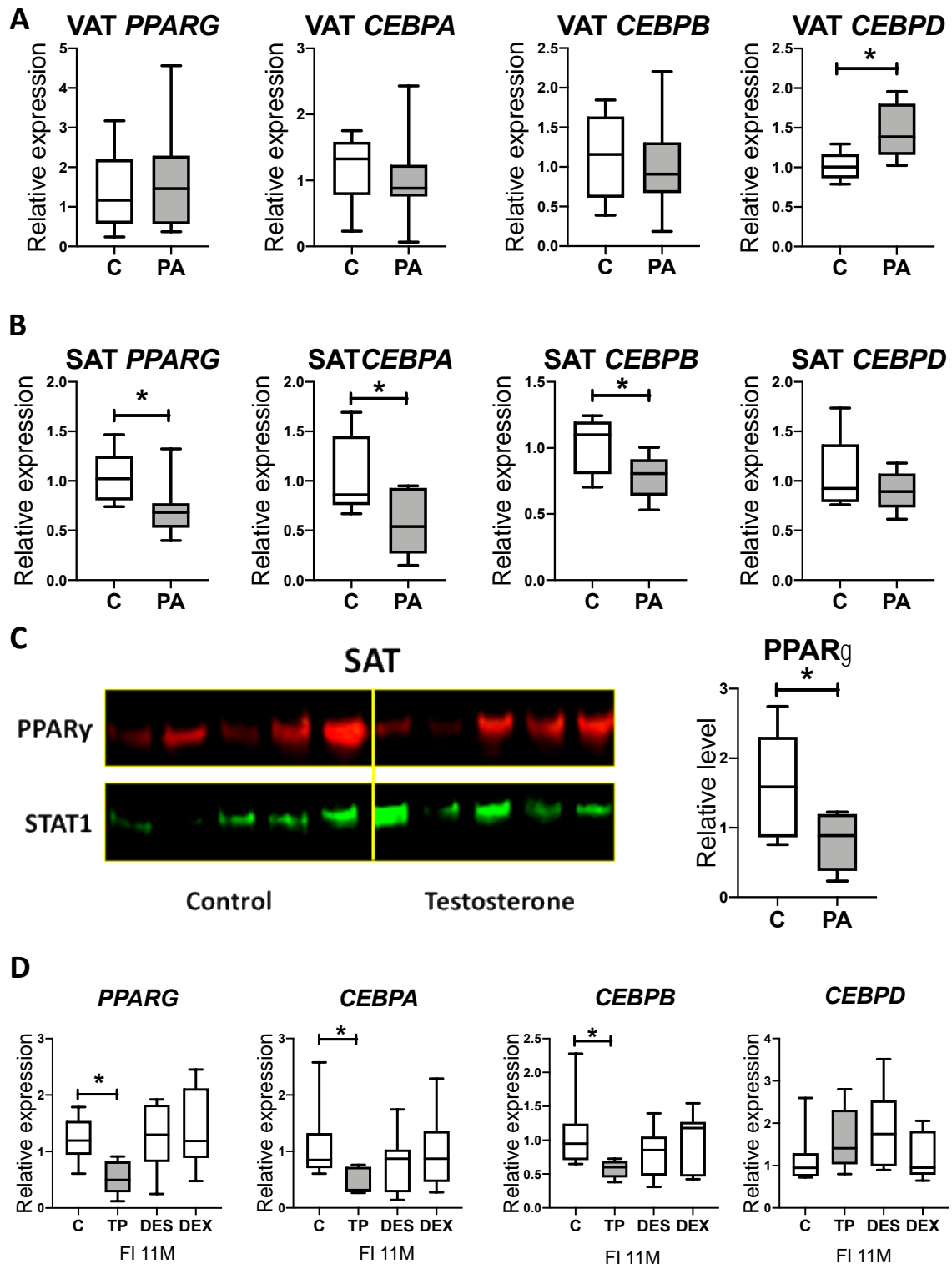
**J** VAT FABP5



**K** VAT LPIN2



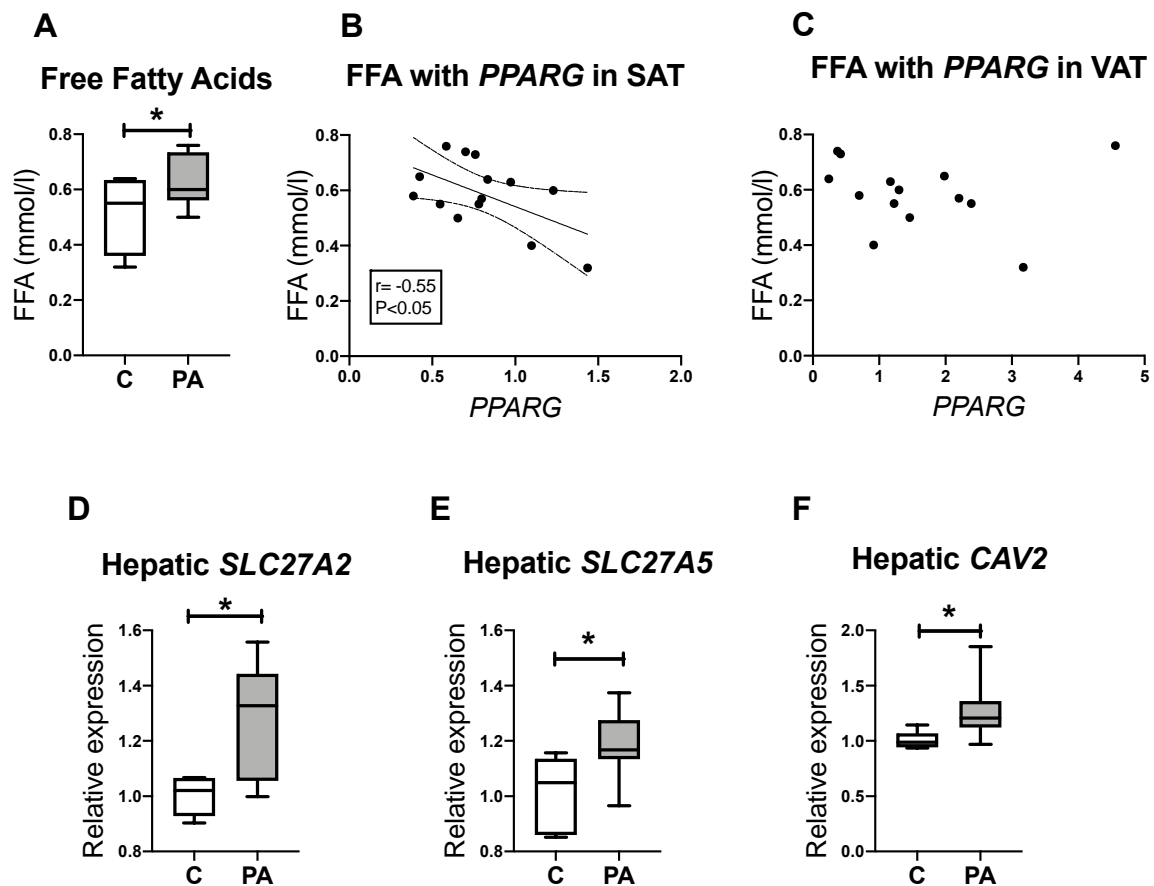
839 **Figure 3.** Metabolic function in adult (30M) controls (C; n=11) and prenatally  
840 androgenised sheep (PA; n=4). PCOS-like sheep have normal levels of (A)  
841 triglycerides, (B) cholesterol and (C) HDL cholesterol and (D) increased circulating  
842 FFAs. (E) There was negative correlation between the total number of adipocytes  
843 per mm<sup>2</sup> and the levels of circulating FFAs. (F) At this time there was no difference in  
844 omental fat between controls and PA sheep, however there was increased transcript  
845 abundance of genes involved in fat uptake and accumulation in VAT (G) *SLC27A1*,  
846 (H) *CAV1*, (I) *CAV2*, (J) *FABP5* and (K) *LPIN2*. Box plot whiskers are lowest and  
847 highest observed values, box is the upper and lower quartile, with median  
848 represented by line in box. Unpaired, two-tailed Student's t test was used for  
849 comparing means of two treatment groups with equal variances accepting  $P<0.05$  as  
850 significant. Correlation was assessed by calculation of Pearson product-moment co-  
851 efficient. (\* $P<0.05$ ).  
852



853

854 **Figure 4.** Transcript abundance of adipogenesis markers in adolescent (11M)  
 855 controls (C; n=5) and prenatally androgenised sheep (PA; n=9). (A) In VAT there  
 856 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



871

872

**Figure 5.** Adipose tissue function in adolescent (11M) controls (C; n=5) and

873

prenatally androgenised sheep (PA; n=9). **(A)** Adolescent prenatally androgenised

874

sheep had increased fasting FFA. **(B)** Concentration of FFAs negatively correlated

875

with *PPARG* ( $r=-0.55$ ) in SAT but not **(C)** with *PPARG* in VAT. There is increased

876

transcript abundance of **(D)** *SLC27A2*, **(E)** *SLC27A5* and **(F)** *CAV2* involved in

877

hepatic fat uptake. Box plot whiskers are lowest and highest observed values, box is

878

the upper and lower quartile, with median represented by line in box. Unpaired, two-

879

tailed Student's t test was used for comparing means of two treatment groups with

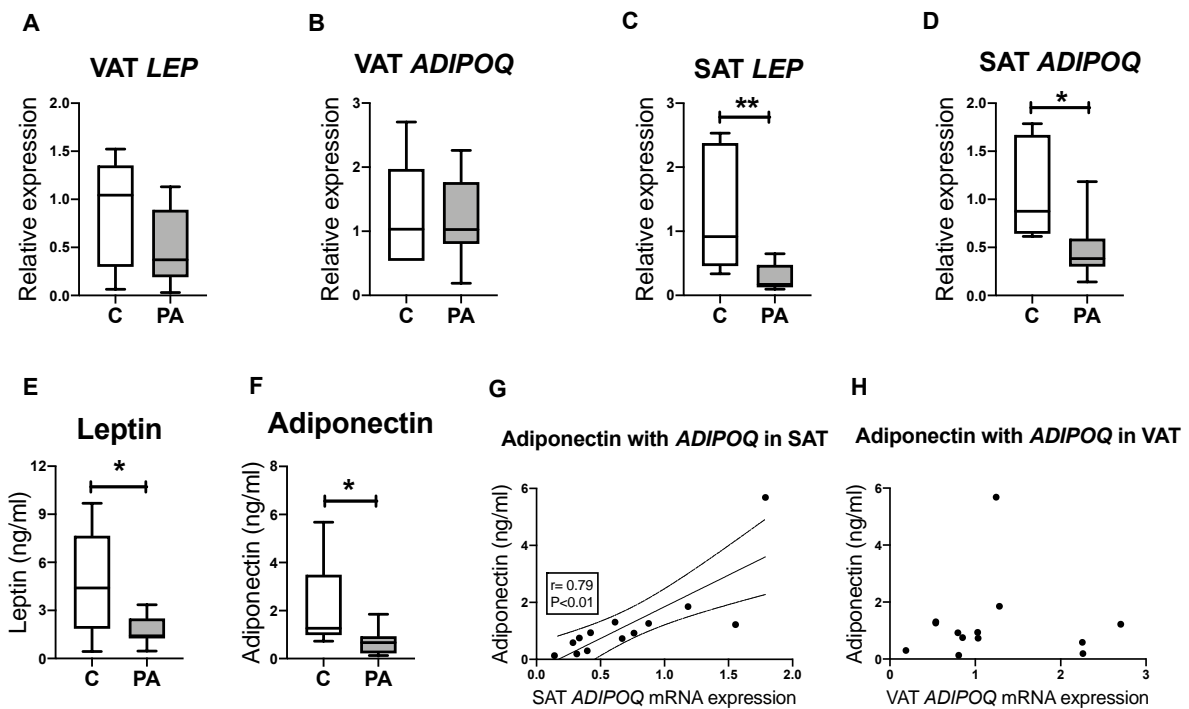
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equal variances accepting  $P<0.05$  as significant. Correlation was assessed by

881

calculation of Pearson product-moment co-efficient. (\* $P<0.05$ ).

882



883

884 **Figure 6.** Transcript abundance and circulating levels of leptin and adiponectin in

885 adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9). In

886 VAT there was no difference in the expression of (A) *LEP* or (B) *ADIPOQ*.

887 Adolescent prenatally androgenised sheep had decreased expression of (C) *LEP*

888 and (D) *ADIPOQ* in SAT. This was mirrored by a reduction in circulating (E) leptin

889 and (F) adiponectin in PA sheep. (G) Circulating adiponectin correlated ( $r=0.79$ ) with

890 *ADIPOQ* expression in SAT but not (H) in VAT. Box plot whiskers are lowest and

891 highest observed values, box is the upper and lower quartile, with median

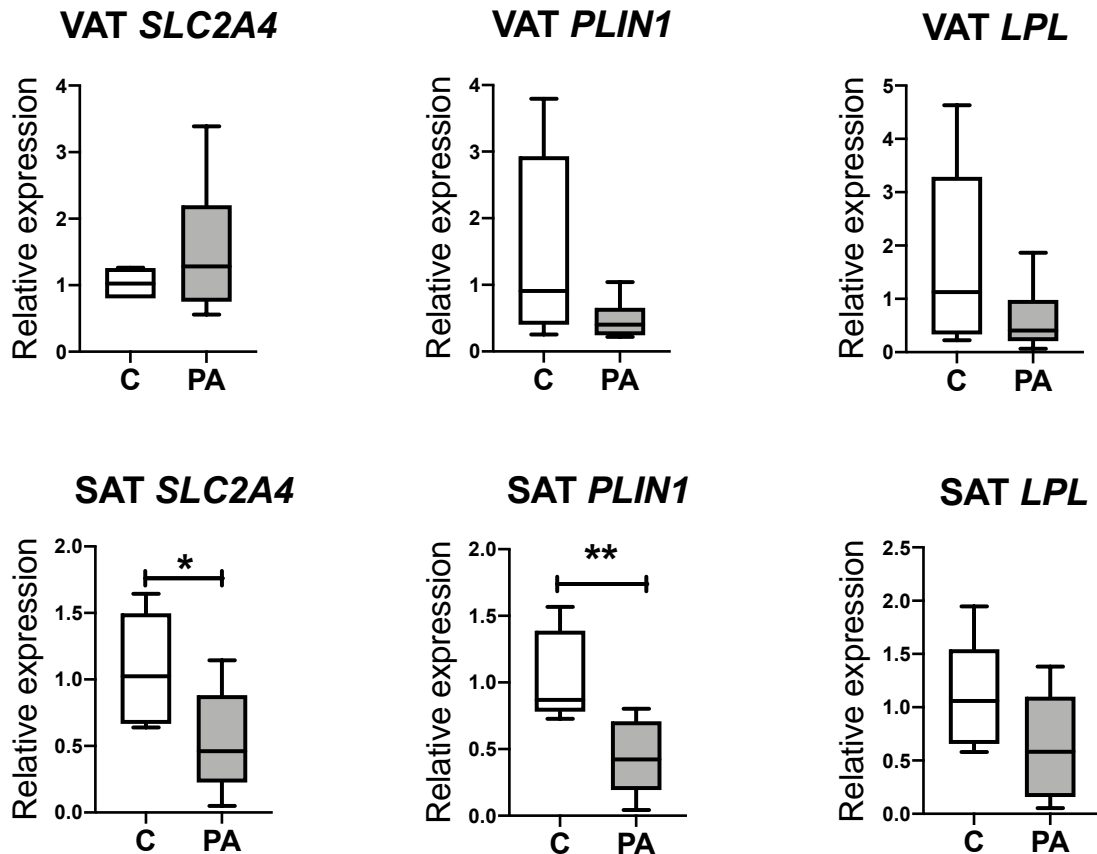
892 represented by line in box. Unpaired, two-tailed Student's t test was used for

893 comparing means of two treatment groups with equal variances accepting  $P < 0.05$  as

894 significant. Correlation was assessed by calculation of Pearson product-moment co-

895 efficient. (\* $P < 0.05$ ; \*\*  $P < 0.01$ ).

896



897

898 **Supplementary Figure 1.** Transcript abundance of markers of mature adipocytes in  
 899 adolescent (11M) controls (C; n=5) and prenatally androgenised sheep (PA; n=9).

900 (A) In VAT there was no difference in the transcript abundance of *SLC2A4*, *PLIN1*

901 and *LPL*. (B) Prenatally androgenised sheep had decreased expression of *SLC2A4*

902 and *PLIN1* in SAT with no difference in *LPL*. Box plot whiskers are lowest and

903 highest observed values, box is the upper and lower quartile, with median

904 represented by line in box. Unpaired, two-tailed Student's t test was used for

905 comparing means of two treatment groups with equal variances accepting  $P < 0.05$  as

906 significant. (\* $P < 0.05$ ; \*\*  $P < 0.01$ ).

907



908 **Table 1**  
 909

<b>Transcriptional regulators of adipogenesis</b>			
<b>Subcutaneous adipose tissue</b>			
<b>Fetal life (D112)</b>			
<b>Gene</b>	<b>C</b>	<b>TP</b>	<b>P</b>
<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.
<b>Gene</b>	<b>Pre-pubertal (11 weeks)</b>		
<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.

910

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

917

918 **Supplementary Table 1**

Gene	Forward Primer	Reverse Primer
<i>18S</i>	CAACTTTCGATGGTAGTCG	CCTTCCTTGGATGTGGTA
<i>ACTB</i>	ATCGAGGACAGGATGCAGAA	CCAATCCACACGGAGTACTTG
<i>ADIPOQ</i>	AGAGATGGCACCCCTGGT	GACCTTCGATCCCAGTGATT
<i>ADIPOR1</i>	TCTCCTGGCTCTTCCACACT	AGCTCCCCATGATCAGCA
<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.