Title: Pubertal FGF21 deficit is central in the metabolic pathophysiology of an ovine model of polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS), affecting over 10% of women, is associated with insulin resistance, obesity, dyslipidaemia, fatty liver and adipose tissue dysfunction. Its pathogenesis is poorly understood and consequently treatment remains suboptimal. Prenatally androgenized (PA) sheep, a clinically realistic model of PCOS, recapitulate the metabolic problems associated with PCOS. Fibroblast Growth Factor 21 (FGF21) is a metabolic hormone regulating lipid homeostasis, insulin sensitivity, energy balance and adipose tissue function. We therefore investigated the role of FGF21 in the metabolic phenotype of PA sheep. In adolescence PA sheep had decreased hepatic expression and circulating concentrations of FGF21. Adolescent PA sheep show decreased FGF21 signalling in subcutaneous adipose tissue, increased hepatic triglyceride content, trend towards reduced fatty acid oxidation capacity and increased hepatic expression of inflammatory markers. These data parallel studies on FGF21 deficiency, suggesting that FGF21 therapy during adolescence may represent a treatment strategy to mitigate metabolic problems associated with PCOS.

Keywords: polycystic ovary syndrome, Fibroblast Growth Factor 21 (FGF21), metabolism, prenatal programming, androgens

1. Introduction

Polycystic ovary syndrome (PCOS), affecting over 10% of women, is associated with increased risk of hyperinsulinemia, insulin resistance, obesity, dyslipidemia and non-alcoholic fatty liver disease (NAFLD) (Fauser et al., 2012; Moran et al., 2015; Teede et al., 2010). In addition, PCOS women have enlarged subcutaneous adipose tissue (SAT) (Echiburú et al., 2018; Manneras-Holm et al., 2010), lower levels of circulating adiponectin (Escobar-Morreale et al., 2006; Maliqueo et al., 2012) and increased abdominal adiposity independent of BMI. Taken together, these indicate adipose tissue dysfunction, which further correlates with an adverse metabolic profile (Puder et al., 2005; Yildirim et al., 2003). Metabolic comorbidities associated with the syndrome worsen with age, negatively impacting health and wellbeing of women, and health service resources (Jason, 2011; Teede et al., 2010). The pathogenesis of PCOS remains poorly understood, and, in the absence of mechanistic understanding, treatment remains suboptimal.

Hepatic-derived Fibroblast Growth Factor 21 (FGF21) is a metabolic hormone, regulating glucose and lipid homeostasis, insulin sensitivity, energy balance and adipose tissue function (Fisher and Maratos-Flier, 2016; Lewis et al., 2019). Animals overexpressing FGF21 in the liver have improved insulin sensitivity, reduced triglyceride (TG) concentrations and are resistant to diet-induced obesity (Jimenez et al., 2018; Kharitonenkov et al., 2005). FGF21 knockout (FGF21-KO) mice have hyperinsulinemia with increased proliferation of pancreatic beta cells (So et al., 2015), increased hepatic fat content (Badman et al., 2009; Tanaka et al., 2015), and display delayed weight gain with mild obesity after 24 weeks on standard diet (Badman et al., 2009). FGF21 regulates the activity of PPARG (Dutchak et al., 2012), the master regulator of adipogenesis. FGF21 deficient mice have defects in PPARG signalling and decreased body fat (Dutchak et al.,

2012). In rodents and monkeys, FGF21 treatment improved insulin sensitivity, reduced serum lipids and attenuated hepatic fat accumulation and inflammation (Kharitonenkov et al., 2007; Xu et al., 2009a; 2009b; Zhu et al., 2014). In human clinical trials, though treatment with FGF21 showed only modest improvement in glycaemic control, it consistently improved plasma lipid profiles and decreased hepatic fat content and serum markers of liver fibrosis in patients with NASH (Lewis et al., 2019; Sanyal et al., 2019).

Prenatal androgen overexposure is associated with a PCOS-phenotype in adult life (Risal et al., 2019). Daughters of women with PCOS have increased cord blood testosterone (Daan et al., 2017) and longer anogenital distance (Barrett et al., 2018) indicating increased *in utero* androgen exposure. Prenatally androgenized sheep is a clinically realistic model of PCOS (Padmanabhan and Veiga-Lopez, 2013), manifesting ovarian, hormonal and metabolic phenotypes reminiscent of PCOS (Connolly et al., 2014; Hogg et al., 2011, 2012; Rae et al., 2013; Ramaswamy et al., 2016), used to provide insights into the molecular pathophysiology of PCOS and to examine therapeutic paradigms (Connolly et al., 2014). We have previously shown, using ovine models of PCOS, that adolescent prenatally androgenized (PA) sheep had hyperinsulinaemia, increased pancreatic beta cell content, fatty liver, diminished adipogenesis in SAT accompanied by decreased levels of leptin and adiponectin, and increased circulating free fatty acids (FFAs), independent of obesity and adiposity (Hogg et al., 2011; Rae et al., 2013; Siemienowicz et al., 2021). Adult PA sheep had decreased postprandial thermogenesis, increased body weight and insulin resistance (Siemienowicz et al., 2020). Decreased adipocyte differentiation during adolescence in PA sheep resulted in hypertrophy and inflammation of adult SAT, paralleled by elevated FFAs concentrations of and increased expression of genes linked to fat accumulation in visceral adipose tissue (VAT) (Siemienowicz et al., 2021). In view of the clinically relevant metabolic

perturbations present in adolescent and adult prenatally androgenized sheep, and intriguing parallels to models of FGF21 manipulation, we hypothesised that dysregulated FGF21 action had a role in the metabolic phenotype in PA sheep. Herein, supporting our hypothesis, we report FGF21 expression, as well adipose tissue and hepatic changes related to FGF21, during the development of metabolic disturbances seen in an ovine model of PCOS.

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*

Animal husbandry, experimental protocols and tissue collection were performed as previously described (Hogg et al., 2011; 2012; 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) then mated with Texel rams. Pregnancy was suggested by lack of estrous, then 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 and 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)].

To further examine the effects of androgen we developed a further cohort where the fetuses were directly injected. In the fetal injection cohort (FI), on day 62 and day 82 of gestation,

mothers were randomised and anesthetised by initial sedation with 10 mg Xylazine (i.m. Rompun; Bayer PLC Animal Health Division, UK), followed by 2mg/kg Ketamine (i.v, Keteset; 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 (PA; n=15), 20mg TP in 0.2ml vehicle; diethylsilbesterol (DES; n=8), 4mg DES in 0.2ml vehicle. In this study we maintained the males until adolescence and could investigate a cohort of males, controls (C; n=14) and testosterone propionate (PA; n=14). 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 and grass *ad libitum* and sacrificed in adolescence (11 months of age for females and 6 months of age for males).

2.3 *Tissue collection*

Fasting blood samples were collected just prior to sacrifice and plasma was separated and stored at -20^{o}C. For adult ewes an additional blood sample was collected at 22 months of age. Liver sampling occurred from the same lobe (right posterior), in approximately the same area. Liver samples from MI cohort were collected from fetuses at D112 of gestation, and from females at 11 weeks, 11 months and 30 months of age. From FI cohort livers were collected from females at 11 months of age and from males at 6 months of age. Subcutaneous adipose tissue (SAT) was collected from the groin region and visceral adipose tissue (VAT) from omentum. Adipose tissue was collected from females from MI

cohort at 11 months and 30 months of age.Tissues were immediately snap frozen, then stored at −80^{o}C until further processing.

2.4 *Plasma analyte determination*

Concentrations of fasting plasma free fatty acids (FFAs) and triglycerides (TGs) 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). Assay intra and inter-assay CV's were < 4% and < 5% respectively. Plasma FGF21 was measured using human FGF21 ELISA kit (ab125966; Abcam Cambridge, UK) as per manufacturer's instructions. All samples were assayed in duplicate. The assay sensitivity was 0.03 ng/ml; intra and inter-assay CVs were 4.7% and 7.2% respectively.

2.5 *Hepatic triglyceride determination*

Hepatic triglyceride content was measured using Triglyceride Determination Kit (TR0100, Sigma-Aldrich, Merck, UK). Briefly, liver tissue was cut on dry ice, weighed and homogenized in PBS. Next, samples were centrifuged at room temperature for 30 seconds at 16000g, lipid phase was removed, and all samples were assayed in duplicate, following manufacturer's instructions.

2.6 *Quantitative (q)RT-PCR*

RNA was extracted from adipose tissue with TRI Reagent combined with the RNeasy Mini Kit (Qiagen Ltd.), and from liver using RNeasy Mini Kit following manufacturer's instructions. On-column DNase digestion was performed using RNase-Free DNase set (Qiagen Ltd.), and RNA concentration and purity 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 the suitability of the geometric mean of *ACTB* and *MDH1* for liver and SAT, and *RPS26* and *18S* for VAT.

Primers (Supplementary Table 1) were designed and synthesised as described previously (Siemienowicz et al., 2020). Quantitative 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 ΔΔCt method (Livak and Schmittgen, 2001).

2.7 *RNA sequencing transcriptomic analyses*

RNA sequencing experiment was previously described in detail (Siemienowicz et al., 2019). Briefly, libraries were prepared with the Illumina TruSeq Stranded mRNA kit. Sequencing was performed on the NextSeq 500 High Output v2 kit (75 cycles) on the Illumina NextSeq 500 platform. To assess quality of sequencing data, reads were analysed with FastQC. To remove any lower quality and adapter sequences, TrimGalore! was used. To remove the ERCC reads, all reads were aligned to the ERCC reference genome using HISAT2. These alignments were processed using SAMtools, reads were counted using featureCounts and analysed using the R package erccdashboard. Reads were aligned to reference genome using HISAT2. SAMtools was used to process the alignments and reads were counted at gene locations using featureCounts. Pairwise gene comparisons were carried out using edgeR on all genes with CPM (count per million) value of more than one in six, the remainder removed as low count genes.

2.8 *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

194 with equal variances, unpaired, two-tailed Student's t test was used accepting $P<0.05$ as
195 significant. Correlation was assessed by calculation of Pearson product-moment co-
196 efficient. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad
197 Prism Software, San Diego, CA, USA). Asterisks were used to indicate level of significance
198 based on the following criteria: $*P<0.05$, $**P<0.01$.
199

3. Results

3.1 FGF21 is reduced during adolescence in PA sheep

To determine whether the metabolic disturbances previously reported (Hogg et al., 2011; Rae et al., 2013; Siemienowicz et al., 2020; 2021) in PA sheep from MI cohort were associated with altered FGF21 production, hepatic expression, and circulating concentrations, of FGF21 were assessed. There was no difference in hepatic FGF21 expression in fetal (Fig. 1A), juvenile (pre-pubertal) (Fig. 1B) or in adult life (Fig. 1D). Hepatic *FGF21* was reduced in adolescent PA sheep at 11 months of age by 79% as compared with controls (Fig. 1C; $P<0.01$). The changes in the hepatic *FGF21* expression were mirrored by circulating FGF21, with reduced levels in adolescence (C; 0.9 ± 0.29 ng/ml vs PA; 0.57 ± 0.25 ng/ml) and in the early adulthood at 22 months of age (C; 0.76 ± 0.26 ng/ml vs PA; 0.45 ± 0.13 ng/ml), that normalised in adulthood at 30 months of age (C; 0.87 ± 0.39 ng/ml vs PA; 0.63 ± 0.45 ng/ml) (Fig. 1E; $P<0.05$). Since FGF21 induces PPARGC1A (Potthoff et al., 2009; Ye et al., 2014) we examined hepatic *PPARGC1A* expression and observed that adolescent PA sheep showed a strong trend for decreased *PPARGC1A* (Fig. 1F; $P=0.054$). There was no difference in the expression of *PPARGC1A* between controls and PA sheep in adulthood (Fig. 1G). In addition, we noted a significant correlation between hepatic *FGF21* and *PPARGC1A* expression in the adolescent liver (Fig. 1H; $P<0.001$). There is a window in adolescence in PA sheep where there is reduced FGF21.

3.2 There is decreased FGF21 signalling in the SAT of adolescent PA sheep

Adipose tissue is the primary target of FGF21 action (Véniant et al., 2012) where it upregulates the activity of PPARG (Dutchak et al., 2012), the master regulator of adipogenesis, and results in increased adiponectin expression (Lin et al., 2013). As we

have previously shown that both PPARG and *ADIPOQ* were significantly downregulated in SAT of adolescent PA sheep (Siemienowicz et al., 2021) we examined the expression of FGFR1 and its KLB co-receptor, which regulate FGF21 action, in adipose tissue.

In adolescence, in SAT there was a reduction of *KLB* with similar levels of FGFR1 (Fig. 2A; $P<0.05$) while there was no difference in the expression of *KLB* and *FGFR1* in VAT (Fig. 2B). Conversely, in adulthood there was no differences in *KLB* and *FGFR1* in SAT (Fig. 2C) however, both *KLB* and *FGFR1* were increased in the VAT of PA sheep when compared to controls (Fig. 2D; $P<0.05$). In addition, apart from *PPARG* in adult VAT, there was a positive correlation between *KLB* and *PPARG* expression (Fig. 2E; $P<0.01$-0.0001) and *ADIPOQ* expression (Fig. 2E; $P<0.05$-0.0001) in both VAT and SAT, in adolescence (11M) and adulthood (30M) (Fig. 2E).

3.3 Reduction in *FGF21* and *PPARGC1A* expression is androgen and sex specific

Maternal androgen injection during gestation increases fetal androgen concentrations as well as estrogen concentrations as a result of placental aromatisation (Rae et al., 2013). To further investigate the direct role of prenatal androgens in the 'programming' of these metabolic alterations, we assessed hepatic *FGF21* expression in animals directly injected with steroid hormones during fetal life. Adolescent female sheep directly injected with testosterone in fetal life have a closely comparable metabolic profile to sheep exposed to increased androgens *in utero* through maternal injections (Hogg et al., 2011; Ramaswamy et al., 2016; Siemienowicz et al., 2021). Expression of *FGF21* was reduced in adolescent prenatally androgenised females when assessed through RNAseq (Fig. 3A; $P<0.05$) and qRT-PCR (Fig. 3B; $P<0.05$), and there was a positive correlation between RNAseq and qRT-PCR results (Fig. 5C; $P<0.0001$), extending confidence in parallels between both models and technical assays. Comparable to maternal injection model, adolescent females

directly treated with testosterone *in utero* had decreased hepatic expression of *PPARGC1A* (Fig. 3D; P<0.01). Hepatic expression of *FGF21* (Fig. 3E) and *PPARGC1A* (Fig. 3F) was no different in adolescent females exposed to prenatal estrogens *in utero*, suggesting direct androgenic programming. There was and no difference in *FGF21* (Fig. 3. G) and *PPARGC1A* (Fig. 3H) adolescent males directly exposed to elevated levels of androgens in fetal life, suggesting sex-specificity of this prenatal in utero androgen excess model.

3.4 Adolescent PA sheep have decreased hepatic lipid oxidation and increased hepatic lipid content and inflammation

As FGF21 can improve lipid profiles and reduce hepatic fat content we investigated the liver in detail in the PA female animals during adolescence using the FI model. In these sheep there was a trend for increased circulating free fatty acids (Fig. 4A; P=0.07). We assessed fatty acid oxidation in the liver in different cellular compartments. In the mitochondrial compartment prenatally androgenized sheep had decreased expression of hepatic *CPT1B* (Fig. 4B; P<0.05) with a trend towards reduced expression of *SLC25A20* (Fig. 4B; P=0.07) and *CPT2* (Fig 4B; P=0.06) that are rate-limiting factors, with regards to getting fatty acids into the mitochondria for beta oxidation (Fig. 4B). There was no difference in the expression of genes associated with mitochondrial beta oxidation (Fig.4C).

With regards to beta oxidation in the peroxisomes, there was decreased expression of *ABCD3* (Fig. 4D; P<0.05) and *ACAA1* (Fig. 4D; P<0.05), genes involved in the initial peroxisomal beta oxidation of larger fatty acids. The endoplasmic reticulum is responsible for omega oxidation and prenatally androgenized sheep had decreased expression of *CYP4F11* (Fig. 4E; P<0.05) and a trend towards decreased *CYP4F3* (Fig.4E; P=0.058) and *CYP4A11* (Fig. 4E; P=0.06), which are key genes involved in omega oxidation. Overall there was a consistent trend for reduced fatty acid oxidation and this is associated with

increased hepatic triglyceride content (Fig. 4F). There was a positive correlation between hepatic *PPARGC1A* expression and genes involved in lipid oxidation (Table 1; P<0.05-0.0001).

Dysregulated immune response play a central role in the development and progression of NAFLD (Gao and Tsukamoto, 2016; Oates et al., 2019). Adolescent PA sheep had increased expression of molecular markers of classically activated, pro-inflammatory (M1) macrophages, *CD68*, *ADGRE1*, *TLR2* and *TLR4* (Fig. 5A; P<0.05-0.01), a trend for increased *CD86* (Fig. 5A; P=0.054) and *IL1R* (Fig. 5A; P=0.07). In addition, there was increased expression of proinflammatory cytokines *IL1B* and *IL18* (Fig. 5B; P<0.05), and chemokines *CXCL9*, *CXCL10* and *CCL5* (Fig. 5C; P<0.05). Overall the PA female adolescent ewes with reduced FGF21 show reduced fatty acid usage in the liver as well as increased liver fat and increased liver inflammation.

4. Discussion

Prenatally androgenized sheep had decreased hepatic expression and circulating concentrations of FGF21 in adolescence (11M) and during the transition from adolescence to adulthood (22M). FGF21 is a primarily hepatic hormone, which regulates glucose metabolism, insulin sensitivity, lipid homeostasis and energy balance (Lewis et al., 2019). FGF21 knockout (FGF21-KO) mice are hyperinsulinemic. These animals exhibit increased pancreatic beta cell proliferation (So et al., 2015), increased hepatic fat content (Badman et al., 2009; Tanaka et al., 2015), decreased expression of hepatic PGC1α (encoded by *PPARGC1A*) involved in fatty acid β-oxidation (Badman et al., 2009), increased hepatic macrophage infiltration and pro-inflammatory cytokines (Liu et al., 2016). As a result, they display delayed weight gain with mild obesity after 24 weeks on standard diet (Badman et al., 2009). Taken together with our data showing decreased expression of FGF21 and altered associated receptor and metabolic systems in prenatally androgenized sheep, we conclude that lowered FGF21 in adolescence contributes to the perturbed metabolic phenotype in PCOS.

Our adolescent sheep, from both models employed in the current study (indirect and direct exposure to increased androgens *in utero*), have hyperinsulinemia and increased pancreatic beta cell content (Rae et al., 2013; Ramaswamy et al., 2016), fatty liver (Hogg et al., 2011), and decreased energy expenditure with increased body weight in adulthood (Siemienowicz et al., 2020). We have now confirmed increased hepatic triglyceride content in adolescent sheep directly treated with androgens *in utero*, and further demonstrated decreased hepatic *PPARGC1A* expression, reduced fatty acid oxidation capacity and increased hepatic expression of inflammatory markers in adolescent PA sheep. This series of parallels between models of FGF21 manipulation, and prenatal androgen exposure,

direct us to conclude that FGF21 reduction during adolescence is a critical component underpinning the metabolic profile which develops in adulthood in such PA models.

Adipose tissue is the primary target of FGF21 action (Véniant et al., 2012), in which it preferentially binds to FGFR1 linked to KLB co-receptor (Yang et al., 2012), a key component of FGF21 signalling (Ding et al., 2012). Consequently, beneficial effects of FGF21 treatment as regards decreasing fat mass, restoring insulin sensitivity and reducing blood lipids are compromised in mice with adipocyte-selective ablation of FGFR1 (Adams et al., 2012b) or KLB (Adams et al., 2012a). FGF21 functions in a feed-forward loop in adipose tissue, regulating PPARG activity, considered to be the 'master regulator' of adipogenesis (Dutchak et al., 2012). Evidentially, FGF21 deficient mice have defects in PPARG signalling and decreased body fat (Dutchak et al., 2012), with selective SAT volume reduction, but no changes in VAT (H. Li et al., 2018).

FGF21 treatment promotes SAT expansion, through adipocyte hyperplasia, and reverses insulin resistance in FGF21-KO mice (H. Li et al., 2018). Hepatic overexpression of FGF21 in obese mice reverses adipocyte hypertrophy and inflammation (Jimenez et al., 2018). SAT is considered a healthy fat depot and is thought to be protective while increased VAT volume correlated with pathologic inflammation and insulin resistance (Booth et al., 2014). In humans, serum FGF21 concentration and *KLB* expression in SAT positively correlate with the SAT volume and maintenance of insulin sensitivity (H. Li et al., 2018). Collectively this indicates that FGF21 acts as selective regulator of the SAT storage capacity, and SAT is an important component as regards positive effects of FGF21 on insulin sensitivity. FGF21-KO mice have decreased expression of *KLB, PPARG*, *CEBPA, INSR, IRS1, and SLC2A4* in adipose tissue, particularly in SAT (Badman et al., 2009; Dutchak et al., 2012;

H. Li et al., 2018) and when fed high-fat diet, they have elevated circulating FFA, increased hepatic fat accumulation and enlarged adipocytes (Dutchak et al., 2012). These metabolic phenotypes parallel our ovine model of PCOS, with adolescent PA sheep having decreased FGF21 concentration, decreased expression of *KLB*, adipogenesis markers (*PPARG, CEBPA* and *CEBPB*) and reduced insulin signalling potential in SAT, but not VAT, while adult PA sheep present with obesity, elevated circulating FA and adipocyte hypertrophy and reduced adipogenesis in SAT, but not VAT (Siemienowicz et al., 2021). This data provides a compelling case for targeting SAT expansion in adolescence through FGF21 treatment, representing a novel therapeutic strategy to combat metabolic problems associated with PCOS.

Adiponectin, an insulin sensitizing, anti-inflammatory and hepatoprotective factor synthesized by adipocytes, is a critical downstream effector of FGF21 (Lin et al., 2013). FGF21 induces adiponectin gene expression and secretion from adipocytes through a PPARG dependent mechanism (Lin et al., 2013). The effects of FGF21 treatment on regulating insulin sensitivity, alleviation of dyslipidaemia, NAFLD and NASH are dependent on the presence of adiponectin (Bao et al., 2018; Holland et al., 2013; Lin et al., 2013). We recently demonstrated that adolescent PA sheep have decreased adiponectin levels paralleled by decreased *ADIPOQ* expression in SAT (Siemienowicz et al., 2021), which is mirrored in adolescent and adult women with PCOS (Cankaya et al., 2014; Escobar-Morreale et al., 2006; Maliqueo et al., 2012). FGF21-KO mice have low levels of circulating adiponectin, while treatment with recombinant FGF21 increases serum adiponectin in those animals (Lin et al., 2013). This link between FGF21 and adiponectin is further emphasized by clinical trials, where administration of an FGF21 analogue to patients with NAFLD or type 2 diabetes and non-human primates resulted in increased circulating adiponectin

levels in dose-dependent manner (Gaich et al., 2013; Sanyal et al., 2019; Talukdar et al., 2016). Furthermore, in age 6-18 humans FGF21 concentration is positively correlated with adiponectin concentration, and an overall healthier metabolic profile, whereas children with diminished FGF21 had highest proportion of insulin resistance and metabolic syndrome (G. Li et al., 2017).

In the pediatric population, FGF21 deficiency is considered to play a role in the pathogenesis of insulin resistance, components of metabolic syndrome, fatty liver and low levels of adiponectin, independent of BMI (Alisi et al., 2013; G. Li et al., 2017). Interestingly, males have lower levels of FGF21 than females during puberty (Bisgaard et al., 2014; G. Li et al., 2017) and adulthood (Hanssen et al., 2015). Therefore, it is possible that sex hormones might have a role in regulation of FGF21 expression. There are no studies investigating FGF21 levels in adolescent girls with PCOS. Adult women with PCOS were reported to have comparable FGF21 levels with BMI-matched controls (Gorar et al., 2010; Sahin et al., 2014), again, matching our observations, in that there was no difference in FGF21 levels between adult controls and PCOS-like sheep.

The metabolic consequences of PCOS can be extremely serious. NAFLD describes a spectrum of liver pathologies, from simple hepatic steatosis, characterized by more than 5% fat infiltration to non-alcoholic steatohepatitis (NASH), a combination of hepatocellular injury, inflammation, and an increased risk of liver fibrosis (Fazel et al., 2016). PCOS sufferers are at increased risk of developing NAFLD and are likely to have more severe forms of NAFLD (Sarkar et al., 2020). The estimated prevalence of NAFLD in women with PCOS varies between 34 to 70%, compared to 25 to 30% in the general population (Paschou et al., 2020); during adolescence, there is more than double the incidence of

NAFLD as when compared with non-PCOS girls (Ayonrinde et al., 2016). FGF21 deficiency promotes the development of steatosis, hepatic inflammation, hepatocyte damage, and fibrosis, whereas FGF21 treatment ameliorates NASH by attenuating these processes (Zarei et al., 2020). Likewise, genetic polymorphism that reduce PGC1α expression correlates with the development of NAFLD in children and adults (Lin et al., 2013;Yoneda et al. 2008), while in NAFLD patients expression of PGC1α is decreased (Westerbacka et al., 2007). In the paediatric population hepatic FGF21 is inversely correlated with non-alcoholic fatty liver progression (Alisi et al., 2013). In adult population however the opposite is true, with higher levels of FGF21 in patients with NAFLD and NASH, positively corelating with the disease progression (Barb et al., 2019; Dushay et al., 2010), suggesting FGF21 resistance (Fisher et al., 2010). FGF21-null mice are more prone to developing NASH, have decreased PGC1α expression, reduced hepatic FA activation and beta-oxidation (Fisher et al., 2014; Liu et al., 2016; Potthoff et al., 2009).

Pharmacological administration of FGF21 analogues reduces hepatic fat content, inflammation and fibrosis in mice and humans (Coskun et al., 2008; Sanyal et al., 2019), by inducing PGC1α and its downstream genes, *CPT1A, CPT1B,* and promoting hepatic FA oxidation (Fisher et al., 2014; Keinicke et al., 2020). PGC1α regulates energy homeostasis and mitochondrial number and function (Piccini et al. 2018). PGC1α overexpression results in increased fatty acid oxidation and decreased haptic triglyceride content (Morris et al., 2012) while PGC1α deficienty results in decreased lipid oxidation and hepatic steatosis (Estall et al., 2009; Leone et al. 2005). Decreased expression of genes involved in rate limiting mitochondrial transport of FA for beta oxidation, peroxisomal beta oxidation and omega oxidation combined with increased hepatic triglycerides in adolescent female PA sheep may therefore be a consequence of decreased expression of *FGF21* and

PPARGC1A, further supported by our observation of positive correlation between hepatic *PPARGC1A* expression and genes involved in lipid oxidation.

In addition to its metabolic function, PGC1α protects against inflammation, decreasesing expression of pro-inflammatory cytokines and stimulating expression of anti-inflammatory factors (Leveille et al., 2020). In animal models reduced levels of PGC1α potentiate progression of NAFLD to NASH and increase pro-inflammatory environment in liver tissue (Besse-Patin et al., 2017) while FGF21 deficiency results in increased hepatic macrophage infiltration, augmented inflammation with elevated expression of pro-inflammatory and pro-fibrotic cytokines (Liu et al., 2016; Zheng et al., 2020), whilst gene therapy increasing hepatic FGF21 synthesis inhibits macrophage infiltration, inflammation and fibrosis (Jimenez et al., 2018). Pharmacological administration of FGF21 in animal models of hepatic injury, alcoholic and non-alcoholic steatosis decreases hepatic expression of molecular markers of pro-inflammatory macrophages, *CD68*, F4/80 (encoded by *ADGRE1*), and pro-inflammatory cytokines, including *IL1B* and *TNF* (Bao et al., 2018; Cui et al., 2020; Lee et al., 2016). We have observed herein that adolescent PA sheep had increased mRNA expression of markers of pro-inflammatory macrophages, *CD68*, *ADGRE1* (coding for F4/80), *TLR2* and *TLR4*, pro-inflammatory cytokines *IL1B* and *IL18* and chemokines *CXCL9*, *CXCL10* and *CCL5*. Again, our data appears in agreement with studies on FGF21 and PGC1α deficiency animal models.

In conclusion, based on evidence presented using realistic clinical model of PCOS, targeting FGF21 expression during adolescence may be a potential therapeutic option to prevent onset of adipocyte and liver dysfunction, and thus sidestep the subsequent serious health relevant consequences associated with PCOS.

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Declaration of interest

The authors have no conflicts of interest to declare.

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Figure legends

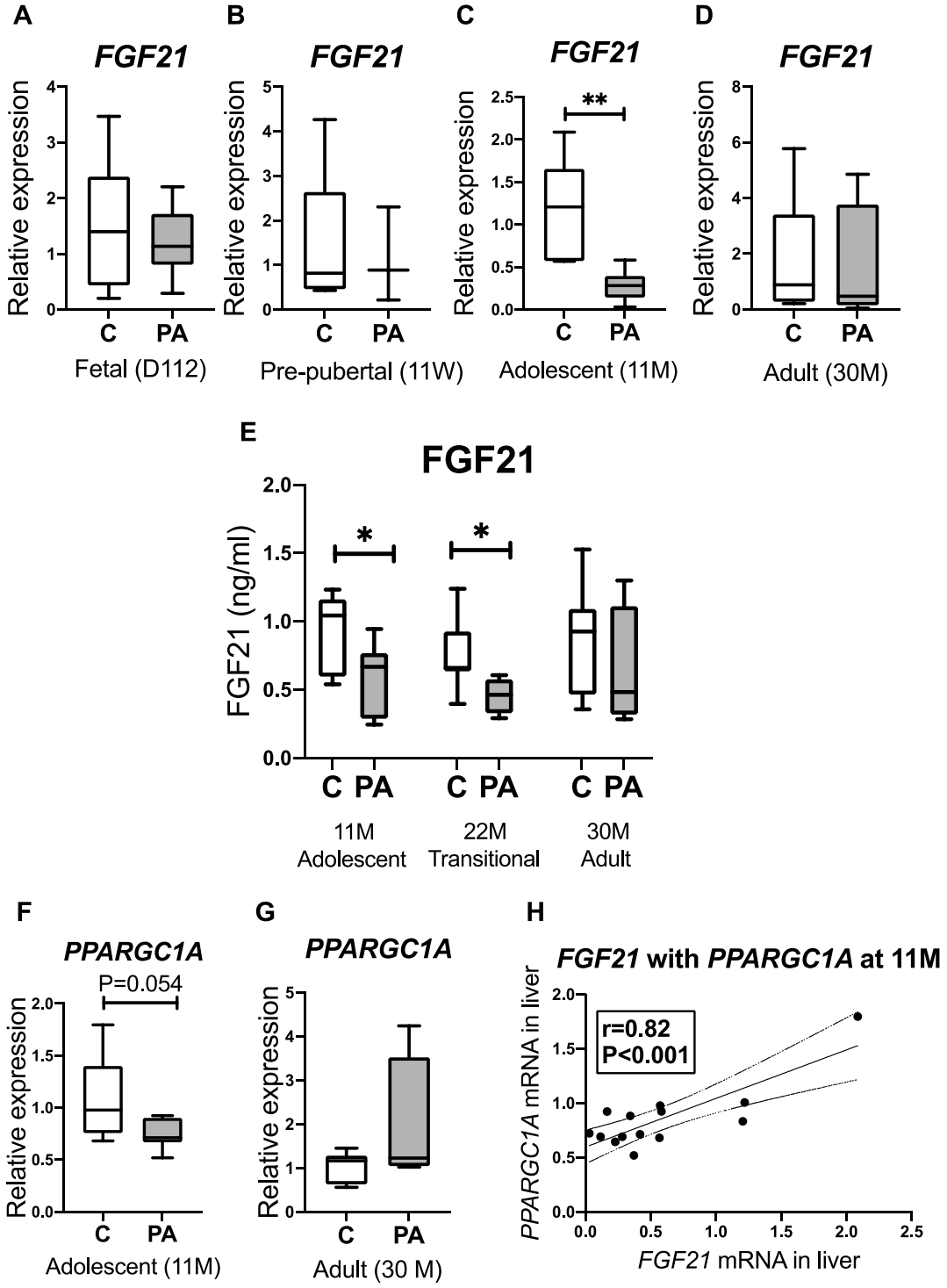

Figure 1. FGF21 and *PPARGC1A* expression in controls (C) and prenatally androgenised sheep (PA) from maternal injection cohort. There was no difference in expression of *FGF21* in (**A**) fetal, (**B**) pre-pubertal and (**D**) adult life. (**C**) Hepatic *FGF21* was reduced in adolescent PA sheep. (**E**) The changes in the hepatic *FGF21* expression were mirrored by

848 circulating FGF21, with reduced levels in adolescence and in the early adulthood, that
849 normalised in adulthood at 30 months of age. FGF21 induces PPARGC1A expression. (**F**)
850 Adolescent PA sheep showed a strong trend for decreased *PPARGC1A.* (**G**) There was no
851 difference in the expression of *PPARGC1A* in adulthood*.* (**H**) There was a correlation
852 between hepatic *FGF21* and *PPARGC1A* expression in the adolescent liver. Box plot
853 whiskers are lowest and highest observed values, box is the upper and lower quartile, with
854 median represented by line in box. Unpaired, two-tailed Student's t test was used for
855 comparing means of two treatment groups with equal variances accepting $P<0.05$ as
856 significant. Correlation was assessed by calculation of Pearson product-moment co-
857 efficient. (*P<0.05; ** P<0.01).
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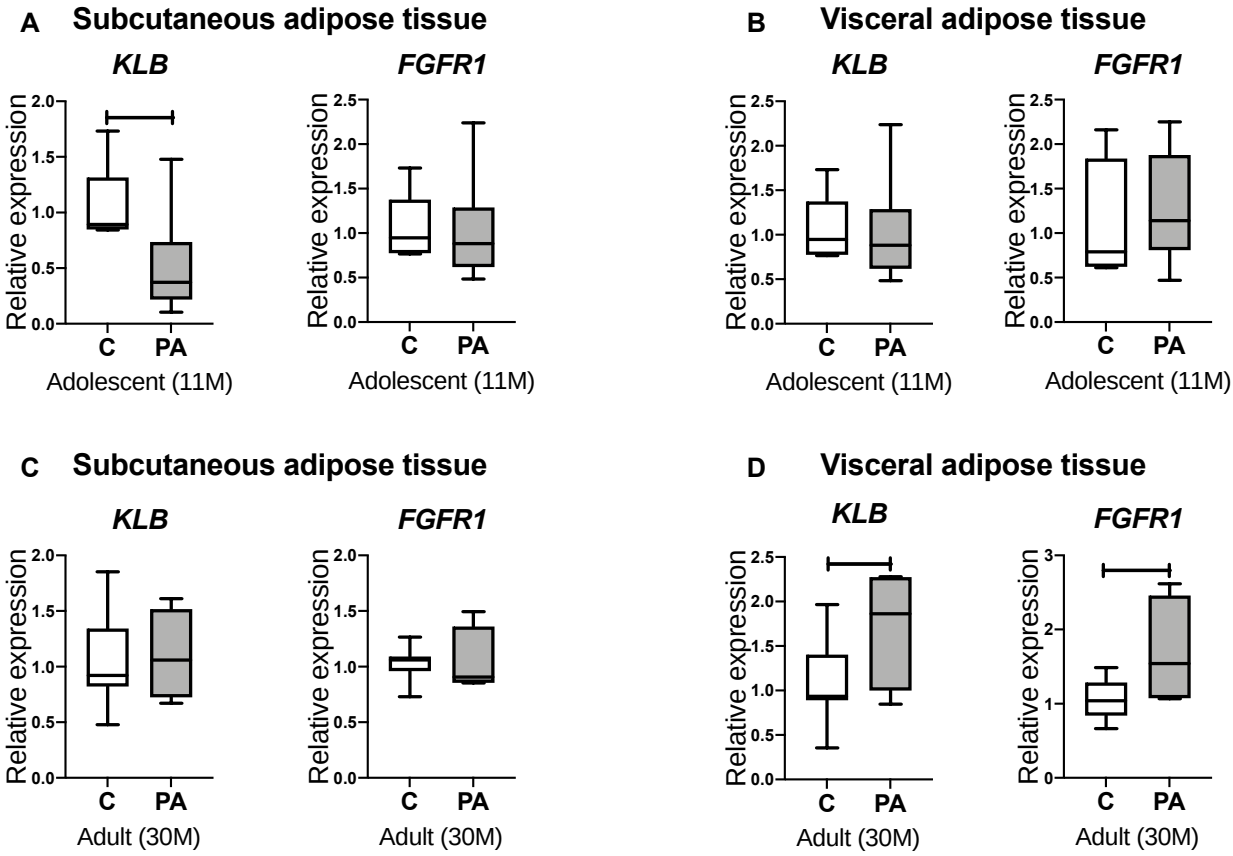

E

Correlation	Tissue	Animals Age	Pearson r	P value
KLB with PPARG	SAT	Adolescent (11M)	0.93	<0.0001
	VAT	Adolescent (11M)	0.90	<0.0001
	SAT	Adult (30M)	0.75	0.0012
	VAT	Adult (30M)	0.21	n.s.
KLB with ADIPOQ	SAT	Adolescent (11M)	0.80	0.0006
	VAT	Adolescent (11M)	0.65	0.013
	SAT	Adult (30M)	0.55	0.033
	VAT	Adult (30M)	0.92	<0.0001

Figure 2. FGF21 signalling in adipose tissue in controls (C) and prenatally androgenised sheep (PA) from maternal injection cohort (androgens reached the fetuses via transplacental transfer from the mother). **(A)** In adolescence, PA sheep had reduced expression of in *KLB* in SAT, with no difference in the expression of *FGFR1*. (**B**) There was no difference in the expression of *KLB* and *FGFR1* in VAT. (**C**) In adulthood, there was no differences in *KLB* and *FGFR1* in SAT, but (**D**) both *KLB* and *FGFR1* were increased in the

VAT of PA sheep. (**E**) There was a positive correlation between *KLB* and *PPARG* expression and *ADIPOQ* expression in both VAT and SAT, in adolescence (11 months) and adulthood (30 months), with exception of *PPARG* in adult 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 co-efficient. (*P<0.05).

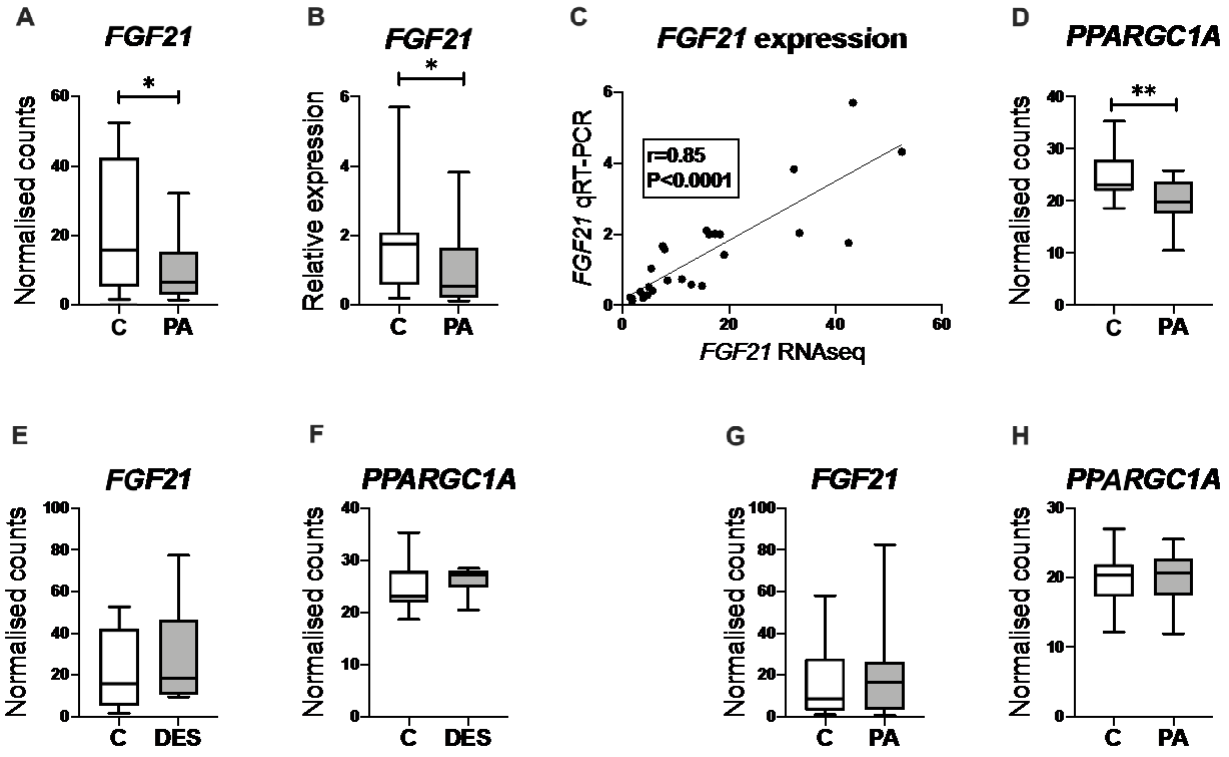

Figure 3. Hepatic *FGF21* and *PPARGC1A* expression in controls (C) and prenatally androgenised sheep (PA) from fetal injection cohort (fetuses directly injected with androgen during fetal life (day 62 and 82)). Adolescent female PA sheep had reduced hepatic expression of *FGF21* when assessed through (**A**) RNAseq and (**B**) qRT-PCR, and (**C**) there

884 was a positive correlation between RNAseq and qRT-PCR results. (**D**) Adolescent PA
885 females had decreased hepatic expression of *PPARGC1A*. (**E**) There was no difference in
886 the hepatic expression of *FGF21* and (**F**) *PPARGC1A* in adolescent females exposed to
887 prenatal estrogens (DES). (**G**) There was and no difference in *FGF21* and (**H**) *PPARGC1A*
888 adolescent PA males. Box plot whiskers are lowest and highest observed values, box is the
889 upper and lower quartile, with median represented by line in box. Unpaired, two-tailed
890 Student's t test was used for comparing means of two treatment groups with equal
891 variances accepting $P<0.05$ as significant. Correlation was assessed by calculation of
892 Pearson product-moment co-efficient. (*P<0.05; ** P<0.01).

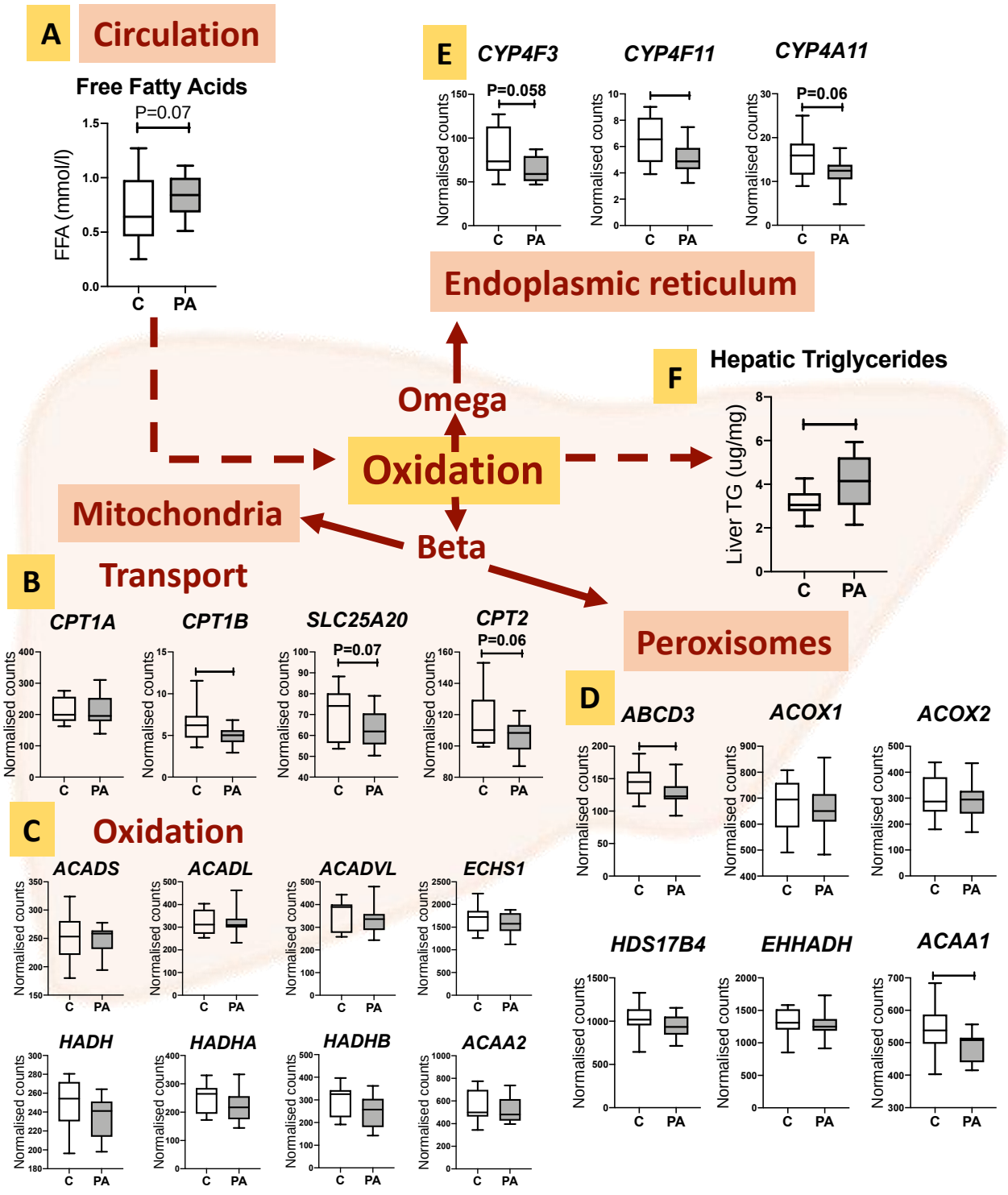

Figure 4. FFAs, hepatic oxidation and liver triglycerides in controls (C) and prenatally androgenised sheep (PA) from fetal injection cohort. (**A**) Adolescent PA sheep had a trend for increased circulating FFAs. (**B**) Adolescent PA sheep had decreased expression of hepatic *CPT1B,* with a trend towards reduced expression of *SLC25A20* and *CPT2*, genes

involved in rate limiting mitochondrial transport of FFAs for beta oxidation. (**C**) There was no difference in the expression of genes associated with mitochondrial beta oxidation. (**D**) There was decreased expression of genes involved in the peroxisomal beta oxidation, *ABCD3* and *ACAA1,* in adolescent PA sheep. (**E**) Adolescent PA sheep had decreased expression of *CYP4F11* and a trend towards decreased *CYP4F3* and *CYP4A11* (Fig. 4E; P=0.06), key genes involved in omega oxidation. (**F**) Decreased oxidative potential in adolescent PA sheep resulted in increased hepatic triglyceride content. 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).

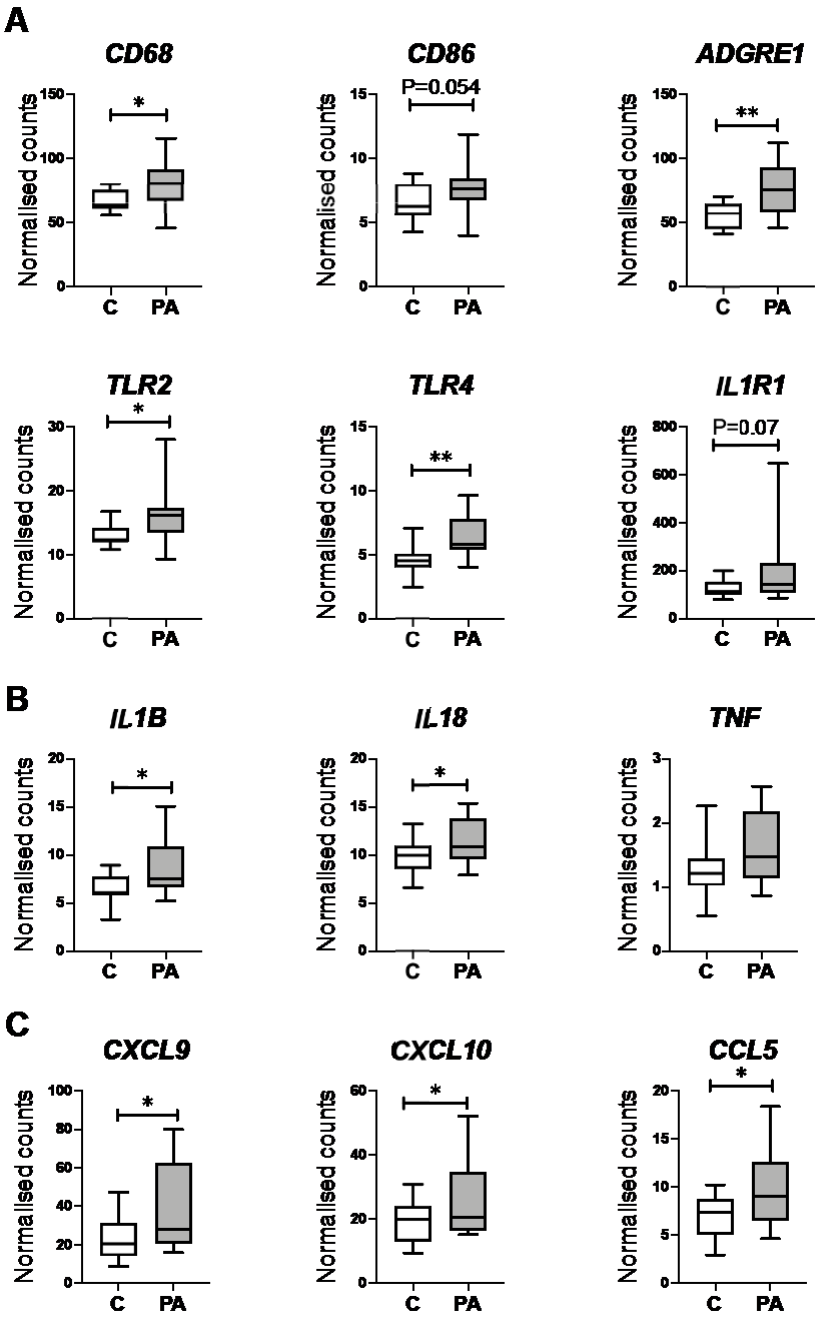

Figure 5. Molecular markers of pro-inflammatory macrophages, cytokines and chemokines in liver of controls (C) and prenatally androgenised sheep (PA) from fetal injection cohort. (**A**) Adolescent PA sheep had increased expression of molecular markers of classically activated, pro-inflammatory (M1) macrophages, *CD68*, *ADGRE1*, *TLR2* and *TLR4* and a trend for increased *CD86* and *IL1R*. (**B**) There was increased expression of proinflammatory cytokines *IL1B* and *IL18* and (**C**) chemokines *CXCL9*, *CXCL10* and *CCL5* in PA female adolescent ewes. 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

Correlation with hepatic *PPARGC1A* expression		
Gene	**Pearson r**	**P value**
CPT1B	0.56	0.002
CPT2	0.49	0.011
ACADL	0.41	0.004
HADH	0.45	0.021
HADHA	0.40	0.042
HADHB	0.39	0.045
ABCD3	0.74	<0.0001
ACOX1	0.45	0.021
ACOX2	0.46	0.017
CYP4F3	0.41	0.040
CYP4A11	0.54	0.004

Table 1

There was a positive correlation between hepatic expression of *PPARGC1A* and genes involved in lipid oxidation in adolescent control and PA female sheep from fetal injection cohort. Correlation was assessed by calculation of Pearson product-moment co-efficient.

Supplementary Table 1

Gene	Forward Primer	Reverse Primer
18S	CAACTTTCGATGGTAGTCG	CCTTCCTTGGATGTGGTA
ACTB	ATCGAGGACAGGATGCAGAA	CCAATCCACACGGAGTACTTG
FGF21	TCCCGAAAGTCTCTTGGAGC	CGATCCATACAGCTTCCCATCT
FGFR1	TCAGAGACCCACCTTCAAGC	GAAGCTGGGGGAGTATTGGT
KLB	CAGAGGATACCACAGCCATCT	CCAGGCTGTGTAACCAAACA
MDH1	TTATCTCCGATGGCAACTCC	GGGAGACCTTCAACAACTTTCC
PPARGC1A	ATGAGTCAGGCCACTGCAGAC	CTCTGCGGTATTCTTCCCTCT
RPS26	CAAGGTAGTCAGGAATCGCTCT	TTACATGGGCTTTGGTGGAG

Supplementary Table 1. Primers for real-time RT-PCR analysis. Forward and reverse primers were designed using Primer3 Input version 0.4 online software (http://frodo.wi.mit.edu) with DNA sequences obtained at Ensembl Genome Browser. To confirm the validity of the gene product in the sheep, both conventional PCR and amplicon sequencing were performed. Primer specificity and efficacy for qRT-PCR was evaluated through the generation of standard curves with serial dilutions of cDNA; a standard curve slope of approximately -3.3 was accepted as efficient, and a melt-curve analysis was also performed.