Molecular and Cellular Endocrinology 595 (2025) 112416

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Insights into the effects of sex and tissue location on the evolution of adipocyte dysfunction in an ovine model of polycystic ovary syndrome (PCOS)

Giovanni Levate [a], Yuan Wang [a], Riada McCredie [a], Megan Fenwick [a], Michael T. Rae [b], W. Colin Duncan [a,*], Katarzyna J. Siemienowicz [b]

[a] Centre for Reproductive Health, Institute for Regeneration and Repair, The University of Edinburgh, Edinburgh, UK
[b] Centre for Biomedicine and Global Health, Edinburgh Napier University, Edinburgh, UK

ARTICLE INFO

Keywords:
Androgen
Bone marrow
Adipose tissue
Inflammation
Sex differences
Sheep

ABSTRACT

Adipose tissue dysfunction is one of the features of Polycystic Ovary Syndrome (PCOS) with dysregulated adipogenesis, altered functional pathways and increased inflammation. It is increasingly clear that there are also male correlates of the hormonal and metabolic features of PCOS. We hypothesised that the effects of adipose tissue dysfunction are not sex-specific but rather fat depot-specific and independent of obesity. We used a clinically realistic ovine model of PCOS where pregnant sheep are injected with 100 mg of testosterone propionate twice weekly from day 62 to day 102 of gestation. We studied control and prenatally androgenised (PA) female and male offspring during adolescence and weight-matched control and PA female sheep during adulthood. We examined subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT) and in adult female sheep bone marrow adipose tissue (BMAT). Adipogenesis related gene expression in SAT was similar in adolescent female and male controls and the reduction in adipogenesis related gene expression by PA in female adipose tissue was not observed in males. Differences in expression of genes associated with adipose tissue function in adolescence in SAT driven by PA were found in both sexes. In adulthood, the changes seen in adolescent females were absent or reversed but there was an increase in inflammatory markers that was weight independent. In addition, BMAT showed increased inflammatory markers. Adipose dysfunction evolves with time and is focussed on SAT rather than VAT and is generally sex-specific although there are also effects of prenatal androgenisation on male SAT. In female adults, the inflammation seen in SAT is also present in BMAT and the development of blood cells in an inflammatory environment may have systemic implications.

1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders affecting women of reproductive age (Fauser et al., 2012). Its aetiology is complex and multifactorial, with reproductive features such as hyperandrogenism and anovulation, as well as metabolic associations like obesity, insulin resistance, liver steatosis and white adipose tissue (WAT) dysfunction (Ehrmann, 2005; Moran et al., 2015; Goodman et al., 2015; Hoeger et al., 2021).

WAT is the most abundant type of fat in humans and is the main storage and metabolic site for dietary lipids (Sun et al., 2011). The two main kinds of WAT, subcutaneous and visceral, differ at the anatomical, cellular and molecular level (Pou et al., 2007; Kahn et al., 2022). Subcutaneous adipose tissue (SAT) is primarily located in the femoral-gluteal and abdominal wall areas, and is made up of smaller, insulin-sensitive adipocytes (Després, 2012). Visceral adipose tissue (VAT) is commonly located around organs in the abdominal area and consists of larger, insulin-resistant adipocytes (Després, 2012). Visceral adipocytes are more metabolically active than subcutaneous adipocytes, showing increased lipolytic rate and triglyceride release (Amer, 1995). In addition, adipocytes are also present in the bone marrow, and notably bone marrow adipose tissue (BMAT), as well as having a metabolic role, is also linked to immune function (Cawthorn et al., 2014; Horowitz et al., 2017; Sebo et al., 2019).

* Corresponding author. Centre for Reproductive Health Institute for Regeneration and Repair Edinburgh BioQuarter, 4Little France Drive, Edinburgh, EH16 4UU, UK.
E-mail address: W.C.Duncan@ed.ac.uk (W.C. Duncan).

https://doi.org/10.1016/j.mce.2024.112416
Received 4 July 2024; Received in revised form 11 November 2024; Accepted 13 November 2024
Available online 16 November 2024

SAT dysfunction is very common in women affected by PCOS (Echiburú et al., 2018; Divoux et al., 2022). Such dysfunction is characterised by enlarged adipocytes which quickly reach their storage capacity, resulting in "lipotoxicity" when lipids overspill from SAT into the general circulation, accumulating in peripheral organs, such as the liver, and blood vessels as well as driving increased storage in visceral adipose tissue (VAT) (Spritzer et al., 2015; Pellegrinelli et al., 2016; Longo et al., 2019). Further to storing and metabolising lipids and thus participating in whole-body homeostasis, adipocytes also secrete hormones, inflammatory cytokines and adipokines (Galic et al., 2010). Adipocyte dysfunction is associated with inflammation within adipose tissue, which is commonly seen in PCOS (Spritzer et al., 2015; Longo et al., 2019).

Although PCOS is typically considered a female reproductive disorder, it has recently been identified that male descendants of women affected by PCOS similarly develop metabolic, cardiovascular, and hormonal abnormalities (Recabarren et al., 2008; Lenarcik et al., 2011; de Wilde et al., 2018; Yilmaz et al., 2018) and this warrants further research.

Our group has previously shown that androgen exposure of ewes during pregnancy leads to clinically realistic ovarian, hormonal and metabolic features similar to those present in PCOS (Hogg et al., 2011, 2012; Rae et al., 2013). Male offspring from such pregnancies display similar metabolic disturbances to those seen in sons born to women with PCOS (Siemienowicz et al., 2019, 2022). We have also previously reported that SAT of female adolescent prenatally androgenised (PA) sheep with a PCOS-phenotype shows impaired adipogenesis, while adult PCOS-sheep are insulin-resistant, overweight and their subcutaneous adipocytes are hypertrophic and more inflamed (Siemienowicz et al., 2021a).

We hypothesised that: 1) prenatal androgenisation causes a similar adipose tissue phenotype in females and males; 2) the hypertrophy of subcutaneous adipocytes in female adult PCOS-sheep is weight independent; 3) adipose tissue inflammation in PCOS-sheep is age and fat depot-specific. In this study, we assess sex-differences in adolescent adipose tissue function and examine its evolution into adulthood. We investigate adipocyte size and function in different fat depots in weight-matched adult female control and PCOS-sheep.

2. Materials and methods

2.1. Animals and treatments

Experiments were conducted under a project license from the UK Home Office after institutional ethical review. Animal husbandry, experimental protocols and tissue collection were performed as previously described (Hogg et al., 2011, 2012; Rae et al., 2013). 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.

Pregnant ewes were randomised to either intramuscular 100 mg testosterone propionate (TP), in 1 ml vegetable oil, or 1 ml vegetable oil control (C) twice weekly from day (D)62 to D102 pregnancy (total gestation 147 days). Pregnancies were carried to term and lambs were weaned at 3 months and fed hay or grass *ad libitum* until sacrifice at 11 months, [female adolescent, weight-matched (C = 5; TP = 9)], 6 months [male adolescent (C = 7, TP = 7)] or 30 months [female adult, weight-matched (C = 4; TP = 4)]. For BMAT one weight-matched control tissue was missing so three additional non-weight matched controls were used (C = 6, TP = 4). As males undergo puberty earlier than females, developmental stage was used rather than age to define adolescence. Due to practical and economic constraints only female offspring were reared through to adulthood.

Animals were sacrificed as described previously (Hogg et al., 2011), adipose tissue was carefully removed and fixed in Bouins solution for 24 h, transferred to 70% ethanol and processed into paraffin wax and/or snap frozen immediately upon removal and stored at −80C. Visceral adipose tissue was collected from the omentum, subcutaneous adipose tissue from the groin and bone marrow adipose tissue from the femur shaft.

2.2. Adipocyte morphometric analysis

For adipocyte morphometric analysis, three 5 μm sections were cut per adipose tissue sample, a minimum of 100 μm apart, and mounted on positively charged slides (Superfrost, Thermoscientific, UK). Sections were then dewaxed, rehydrated and stained with haematoxylin and eosin following standard protocol. Two randomly selected fields per section were captured at × 4 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 ImageJ Software in a blinded manner.

2.3. Quantitative (q)RT-PCR

RNA was extracted from adipose tissue using the Lipid RNeasy Mini Kit (Qiagen Ltd.) following the manufacturer's instructions. RNA concentration and purity was assessed using a NanoDrop One spectrometer (ThermoFisher Scientific, UK). Complimentary DNA (cDNA) was synthesised using Nanoscript cDNA Reverse Transcription Kit (Novacyt, UK). Housekeeping genes for ovine visceral and subcutaneous adipose tissue have previously been identified using Genorm (Siemienowicz et al., 2019), and 18S was the most stable housekeeping gene and was therefore utilised for bone marrow adipose tissue.

Primers (Table 1) were designed and synthesised as described previously (Siemienowicz et al., 2020). Quantitative RT-PCR was performed on 96-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 QuantStudio 5 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 2-ΔΔCt method (Livak and Schmittgen, 2001).

Table 1
Primers. Details of the primers used for RT-PCR.

Gene	Forward Primer	Reverse Primer
18S	CAACTTTCGATGGTAGTCG	CCTTCCTTGGATGTGGTA
ACTB	ATCGAGGACAGGATGCAGAA	CCAATCCACACGGAGTACTTG
ADIPOQ	AGAGATGGCACCCCTGGT	GACCTTCGATCCCAGTGATT
AGPAT2	CAGCATCATCAGTTGGTTCG	GTACATGATCAGGCCCACAG
ATGL	GACGGTGGCATCTCAGACAA	GTAGGGTACCATCATGGCCG
CCL2	GACCCCAACCTGAAATGGGT	GCAGTTAGGGGAAGCCAGAA
CEBPA	GTGGACAAGAACAGCAACGA	CGCAGTGTGTCCAGTTCG
CEBPB	GACAAGCACAGCGACGAGTA	AGCTGCTCCACCTTCTTCTG
CEBPD	CGAGTACCGGCAGCGAC	GTCGCGCAGTCCGGC
CD68	CAGCCCAGGATTCACCAGTT	CCGAGAATGTCCACTGTGCT
CYP17A1	GGACACAACTCATCTCGCCA	CGCACCTCGATCTTCACCTT
CYP19A1	GGTGTCCGAAGTTGTGCCTA	CATGAGGGTCAACAC
DGAT1	CAGACAAGGACGGAGACGTAG	CACACACCAATTCAGGATGC
FABP4	CAAACTGGGCGTGGAATTCG	ACCAGCTTGTCACCATCTCG
HIF1	GCAAGACTTTTCTCA	TTGGTCAGATGGTCA
HSL	ACCGAGACAGGCCTCAGTGTG	GAATCGGCCACCGGTAAAGAG
IL6	AAATGACACCACCCCAAGCA	CTCCAGAAGACCAGCAGTGG
LEP	ATCTCACACACGCAGTCCGT	CCAGCAGGTGGAGAAGGTC
PEA-15	GACCAACAACATCACCCTTGA	TCTCCAGGAAGCTAAACCAGG
PLIN1	CATCTCTACCCGCCTTCGAA	TGCTTGCAATGGGCACACT
PPARG	TGCAGTGGGGATGTCTCATA	CAGCGGGAAGGACTTTATGT
SLC2A4	CCAGCATCTTTGAGTCAGCA	CAGAAGCAGAGCCACAGTCA
TNF	GGTGCCTCAGCCTCTTCT	GAACCAGAGGCCTGTTGAAG
VEGF	TCTTCAAGCCATCCTGTGTG	TGCATTCACATTTGTTGTGC

2.4. Statistical analysis

For comparing means of two treatment groups with equal variances Student's *t*-test was used, otherwise a Mann-Whitney test was used, in both cases accepting $P < 0.05$ as significant. For more than two comparisons, ANOVA was used with Tukey post hoc test or a Kruskal Wallis test with Dunn's multiple comparisons when variances were not equal. Statistical analysis was performed using GraphPad Prism 10.2 software (GraphPad Prism Software, San Diego, CA, USA). Asterisks were used to indicate level of significance based on the following criteria: $*P < 0.05$, $**P < 0.01$.

3. Results

3.1. Is altered adipogenesis induced by prenatal androgenisation in adolescence sex specific?

There was no difference in SAT adipogenesis markers between control adolescent females and males, and prenatal androgen exposure had no effect on expression of adipogenesis markers in male SAT (Fig. 1A). However, adolescent females exposed to increased prenatal androgens have reduced transcript abundance of markers of adipogenesis (*PPARG*, *CEBPA*, *CEBPB*) in SAT compared to contemporaneous weight-matched vehicle controls (Fig. 1A). There was no difference in the expression of *PPARG*, *CEBPA*, *CEBPB* and *CEBPD* in VAT between control adolescent males and females (Fig. 1B), and prenatal androgenisation had no effect on the expression of these genes in adolescent male or female VAT when

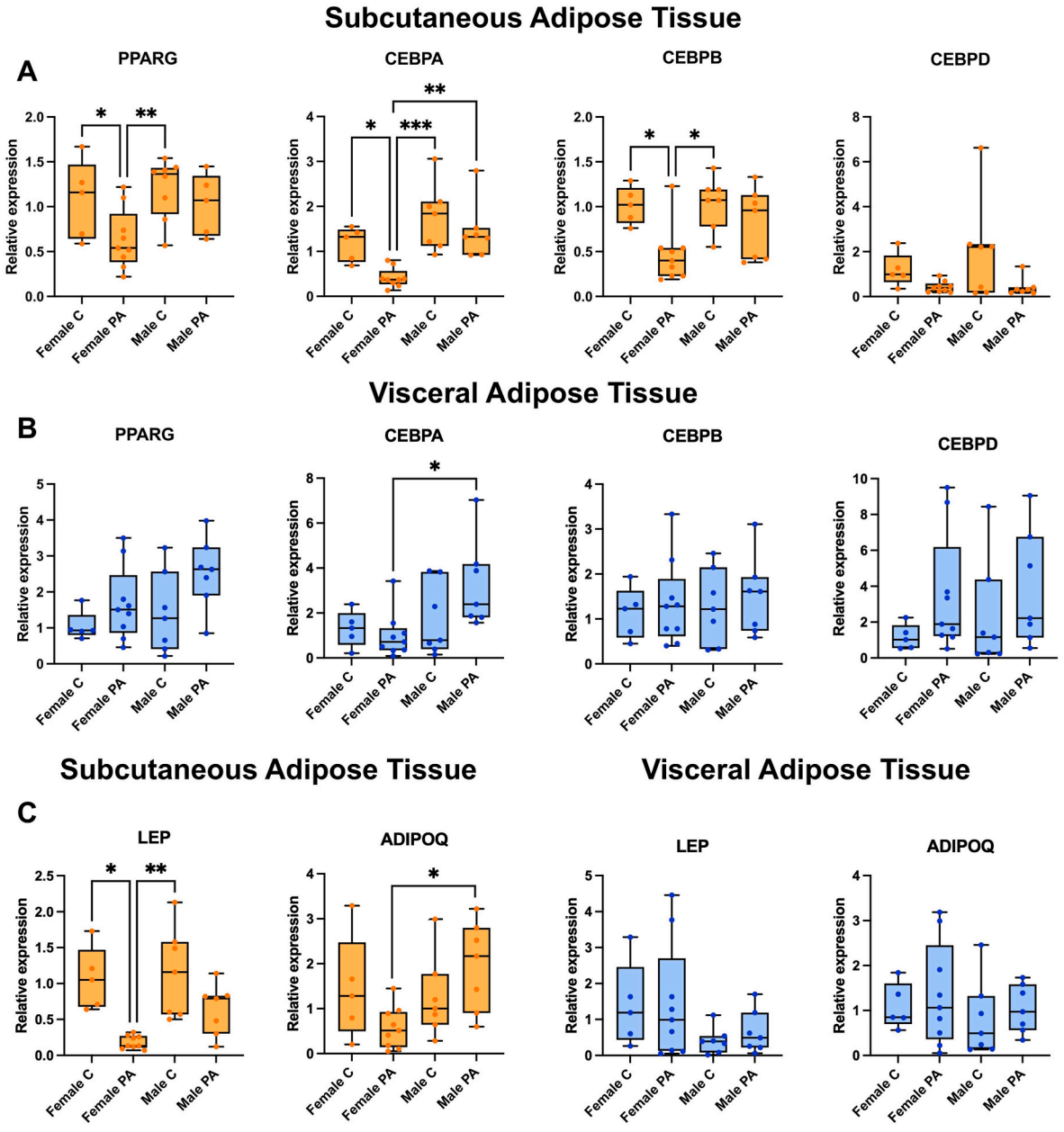

Fig. 1. Adipogenesis gene expression. Comparison of the expression of the adipogenesis genes *PPARG*, *CEBPA*, *CEBPB* and *CEBPD* between control (C) and prenatally androgenised (PA) female and male adolescent sheep in **A)** subcutaneous adipose tissue and **B)** visceral adipose tissue. Expression of the mature adipocyte markers *LEP* and *ADIPOQ*, and the effect of prenatal androgenisation, in female and male adolescent sheep in **C)** subcutaneous and visceral adipose tissue. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

compared to controls (Fig. 1B).

There were no differences in adolescent SAT *LEP* and *ADIPOQ* expression between control adolescent males and females and the reduction in *LEP* and trend to reduction *ADIPOQ* seen here after prenatal androgenisation in female adolescent SAT was not observed in males (Fig. 1C). Prenatal androgenisation had no effect on the *LEP* and *ADIPOQ* in adolescent male or female VAT (Fig. 1C). The prenatal androgenic reduction in adolescent adipogenesis is both SAT and sex-specific, and the effect of female prenatal androgenisation is not to make adipogenesis more male-like.

3.2. Effects of sex and prenatal androgenisation on adipocyte lipid metabolism

The impact of altered adipogenesis on the evolution of adipocyte lipid metabolism was assessed in both sexes.

In adolescence, there were no differences between control male and female SAT *HSL*, *ATGL*, *AGPAT2, DGAT1, FABP4* and *PLIN1* transcript abundance (Fig. 2A). In adolescent female SAT prenatal androgenisation significantly reduced *HSL*, *ATGL*, *AGPAT2* compared to vehicle controls (Fig. 2A). In adolescent males, *FABP4* was reduced after prenatal androgenisation (Fig. 2A). In adolescent VAT there were no differences between males and females and no effect of prenatal androgenisation on *HSL*, *ATGL*, *AGPAT2, DGAT1, FABP4* and *PLIN1* transcript abundance (Fig. 2B).

In mature adult females the transcript abundance of *HSL* and *DGAT1* were significantly increased in SAT with a trend towards increased *PLIN1* after prenatal androgenisation. In contrast *ATGL* in SAT tended to be reduced ($P = 0.056$) after prenatal androgenisation compared to contemporaneous weight-matched vehicle controls (Fig. 2C). There were no differences in VAT *HSL*, *ATGL*, *AGPAT2*, *DGAT1, FABP4* and *PLIN1* in mature weight matched female adults (Fig. 2D).

Prenatal androgenisation has effects on lipid metabolism that is limited to SAT, evolves with age and is not limited to females.

3.3. The effect of sex and prenatal androgenisation on adipocyte metabolic and endocrine function

We next assessed the impact of prenatal androgenisation on candidate metabolic and endocrine transcripts.

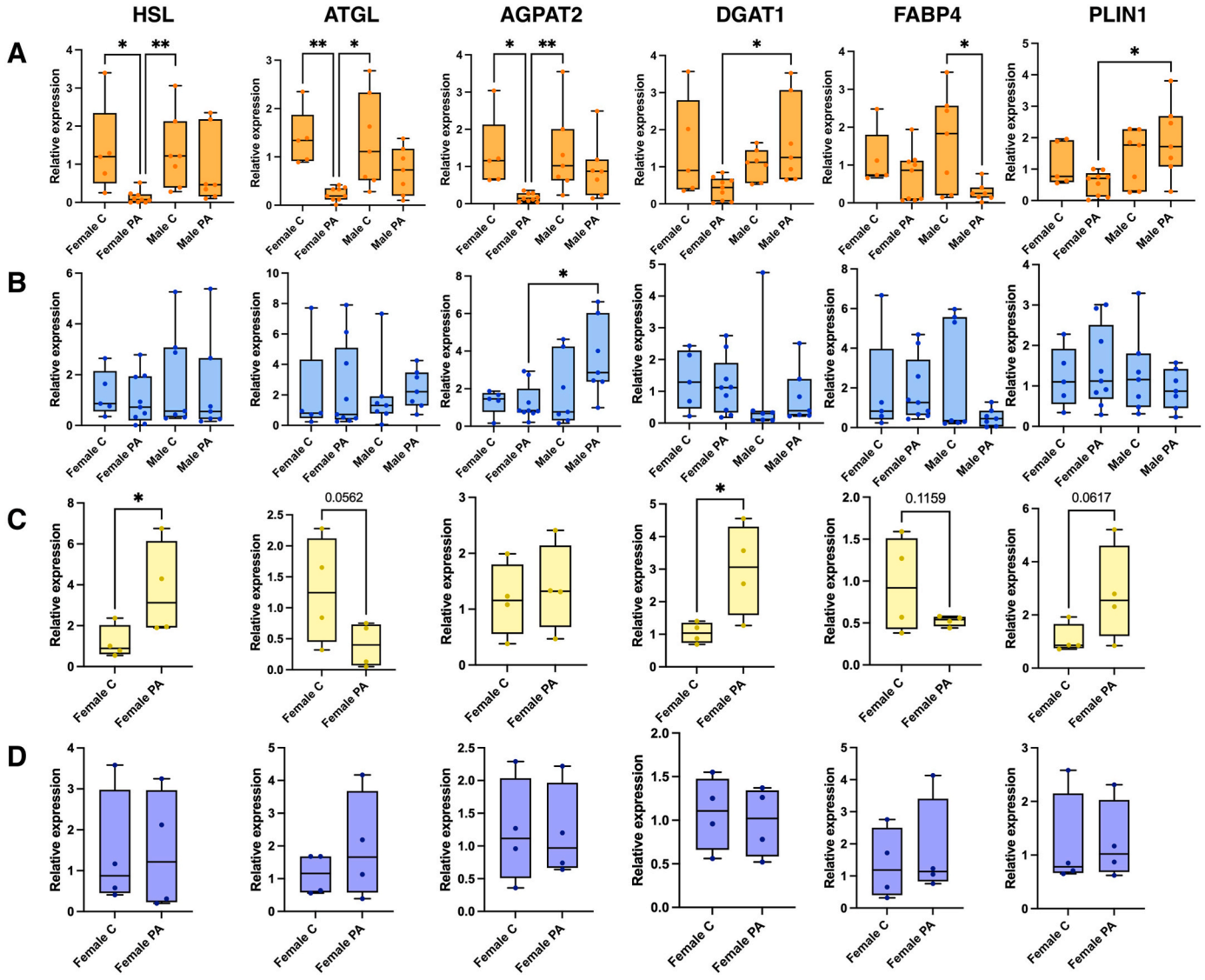

Fig. 2. Lipid metabolism gene expression. Comparison of the expression of the lipid metabolism genes *HSL*, *ATGL*, *AGPAT2, DGAT1, FABP4* and *PLIN1* between control (C) and prenatally androgenised (PA) female and male adolescent sheep in **A)** subcutaneous adipose tissue and **B)** visceral adipose tissue. The effect of prenatal androgenisation of the expression of *HSL*, *ATGL*, *AGPAT2, DGAT1, FABP4* and *PLIN1* in **C)** subcutaneous adipose tissue and **D)** visceral adipose tissue of mature adult female sheep. *$P < 0.05$, **$P < 0.01$.

In adolescence, there was no difference between control female and male *HIF* and *VEGF* expression In SAT. In females, prenatal androgenisation increased *HIF1* in SAT with a similar trend in *VEGF* (Fig. 3A). The effect of prenatal androgenisation on adolescent male SAT showed a similar pattern to females for *HIF1* and *VEGF* but it was not significantly different (Fig. 3A). Likewise in both female and male VAT the pattern in *HIF1* and *VEGF* expression was similar to SAT but there were no significant differences (Fig. 3B).

In adolescence, adipose tissue *CYP17A1, CYP19A1* and *SLC2A4* expression in SAT was the same in control female and males (Fig. 3A). Prenatal androgenisation had no effect in expression of *CYP17A1*, *CYP19A1* and *SLC2A4* in female adolescent SAT while it reduced in *CYP19A1* in male adolescent SAT (Fig. 3A). Prenatal androgenisation caused an increase in female adolescent VAT *SLC2A4* with a non-significant trend (P = 0.06) to its increase in male adolescent VAT (Fig. 3B).

In mature female adults there was no effect of prenatal androgenisation on *HIF1* or *VEGF* in SAT or VAT when compared to weight-matched contemporaneous controls (Fig. 3C and D). There was no effect of prenatal androgenisation on *CYP17A1* in SAT or VAT in mature adult females (Fig. 3C and D). In mature female adults however *CYP19A1* was reduced by prenatal androgenisation in SAT (Fig. 3C)

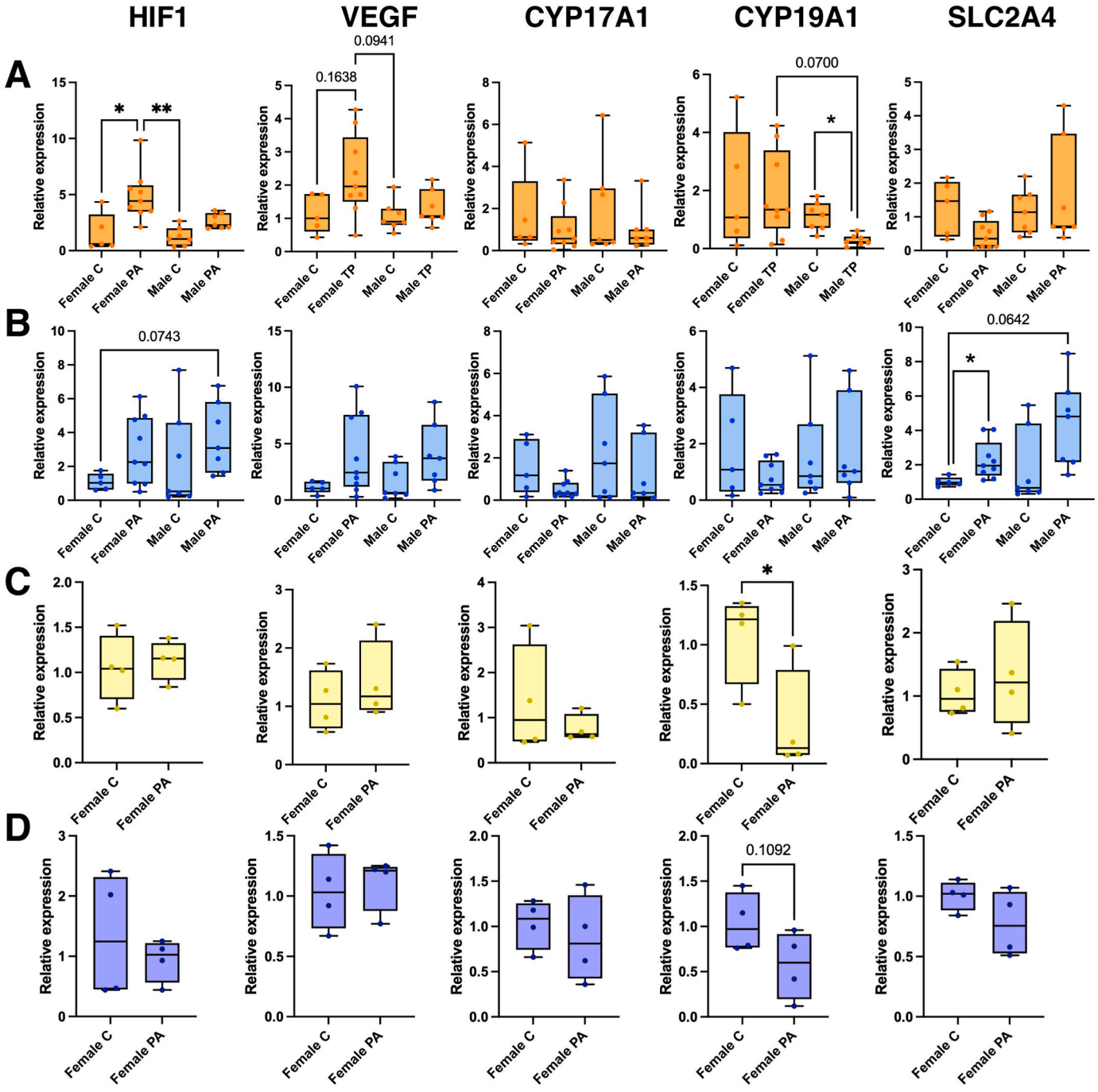

Fig. 3. Metabolism and endocrine gene expression. Comparison of the expression of the metabolism and steroidogenic genes *HIF1*, *VEGF*, *CYP17A1, CYP19A1,* and *SLC2A4* between control (C) and prenatally androgenised (PA) male and female adolescent sheep in **A)** subcutaneous adipose tissue and **B)** visceral adipose tissue. The effect of prenatal androgenisation of the expression of *HIF1*, *VEGF*, *CYP17A1, CYP19A1,* and *SLC2A4* in **C)** subcutaneous adipose tissue and **D)** visceral adipose tissue of mature adult female sheep. *P < 0.05, **P < 0.01.

with no significant reduction in VAT (Fig. 3D). In mature female adults there was no effect of prenatal androgenisation on *SLC2A4* in SAT or VAT when compared to weight-matched contemporaneous controls (Fig. 3C and D).

Adipocyte metabolism and endocrinology can be altered by prenatal androgenisation with effects on males and females and in different depots that changes with age.

3.4. Adipose tissue structure in mature adults

To determine if the effects of prenatal androgenisation on adipose tissue structure in mature adults was independent from weight we examined adipose tissue in weight-matched prenatally androgenised sheep and contemporaneous controls. In SAT there was a reduction in the number of adipocytes ($P < 0.05$) with a trend to an increase in mean adipocyte size ($P = 0.05$) in mature female adults after prenatal androgenisation when compared to contemporaneous weight-matched vehicle controls (Fig. 4A). There were no differences in the number of adipocytes and mean adipocyte size in VAT (Fig. 4B).

The structural change in SAT in mature adult females was independent of weight.

3.5. Is PEA15 involved in the regulation of fat cell size after prenatal androgenisation?

We looked at the adipocyte marker *PEA15* that is associated with increasing adipocyte size. In adolescence prenatal androgenisation had no effects on female or male adipose tissue PEA15 expression in SAT or VAT. In adult females prenatal androgenisation had no impact on adipocyte *PEA15* expression in SAT or VAT (Fig. 5). PEA15 does not seem to be involved in the weight independent increase in adult female adipocyte size as a result of prenatal androgenisation.

3.6. The evolution of adipose tissue inflammation

We examined whether the inflammation seen in mature female adult SAT after the development of obesity as a consequence of prenatal androgenisation was present in adolescence in females and males.

In adolescence, prenatal androgenisation had no effects on *TNF*, *IL6*, *CCL2* and *CD68* expression in SAT in females and males when compared to vehicle controls (Fig. 6A). In VAT there was no effect of prenatal androgenisation on *TNF*, *IL6* and *CD68* expression in adolescent females and males (Fig. 6B). However, in VAT, prenatal androgenisation increased *CCL2* in adolescent males with a similar non-statistically significant pattern in adolescent females (Fig. 6B).

In mature female adults matched for weight we showed inflammation after prenatal androgenisation with increased *TNF* and *IL6* in SAT (Fig. 6C) with no differences in VAT (Fig. 6D). In bone marrow adipocytes of mature female adults prenatal androgenisation increased *TNF, IL6* and *CD68* transcript abundance (Fig. 6E).

Adipose tissue inflammation does not appear to be sex-specific but it is depot-specific and increases with age.

4. Discussion

In this study, we demonstrate for the first time that: 1) exposure to increased prenatal androgens leads to sex-specific effects in the subcutaneous adipose tissue of adolescent sheep and PA female sheep adipose tissue is altered in a way that is not more like a male; 2) The altered size and dysfunction of adipocytes in adult female PA sheep is independent of obesity; 3) The expansion in adipocyte size in adult subcutaneous adipose tissue in adult female PA sheep is not linked to alterations in PEA15, an emerging protein linked to adipose tissue expansion in high calorie diets; 4) The increased inflammatory signal in adipose tissue is fat depot specific and bone marrow adipose tissue in adult female PA sheep has an inflammatory phenotype.

Prenatal androgenisation resulted in significant decreases in the expression of adipogenesis (*PPARG*, *CEBPA* and *CEBPB*) in female but not male SAT of PA sheep. These effects were also tissue-specific and limited only to SAT and not VAT. What is remarkable is that the normal exposure to androgens in male development had no effect on adipogenesis as there were no differences between male and female control adolescent sheep.

The impaired adipogenesis observed in the female adolescent cohort can be attributed to the significant decrease in CCATT enhancer-binding protein (C/EBP)-β, the main regulator of cell proliferation and DNA replication in differentiating adipocytes (Guo et al., 2015; Siersbæk et al., 2011). Binding to the DNA, it stimulates activation of further transcription factors C/EBP-α and peroxisome proliferator-activated receptor (PPAR)-γ. Low C/EBP-B expression detected in female

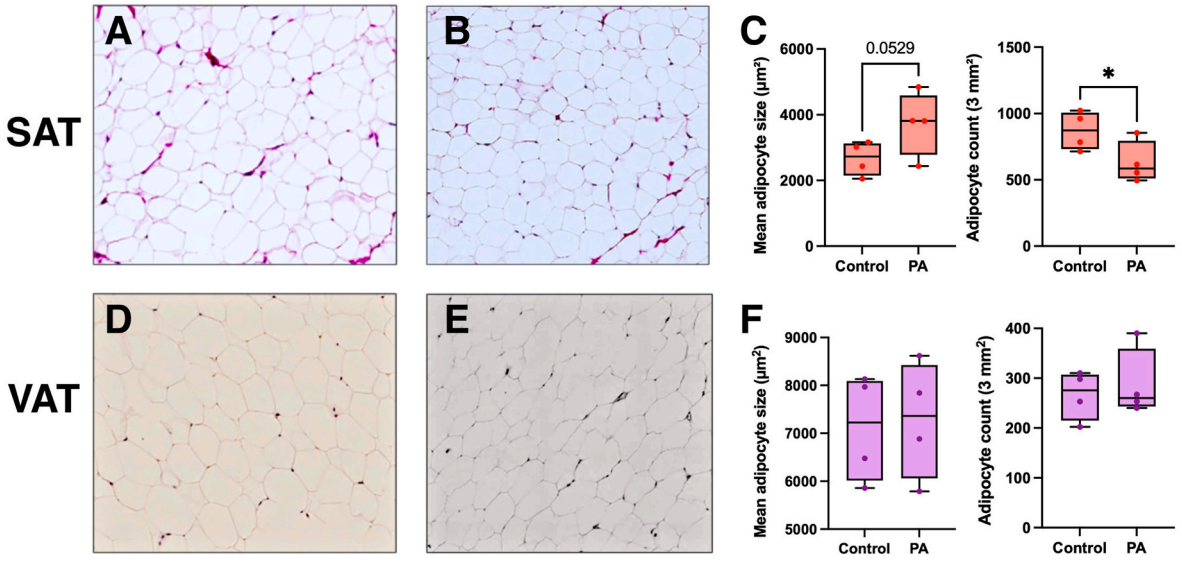

Fig. 4. Adipose tissue morphology. Morphology of adipose tissue in subcutaneous adipose tissue (SAT) in **A)** Control (C) adult female sheep and **B)** prenatally androgenised (PA) adult female sheep. **C)** Mean adipocyte size and adipocyte count in C and PA adult female sheep. Morphology of adipose tissue in visceral adipose tissue (VAT) in **D)** Control adult female sheep and **E)** prenatally androgenised (PA) adult female sheep. **F)** Mean adipocyte size and adipocyte count in C and PA adult female sheep. Scale bar 100 µm *$P < 0.05$.

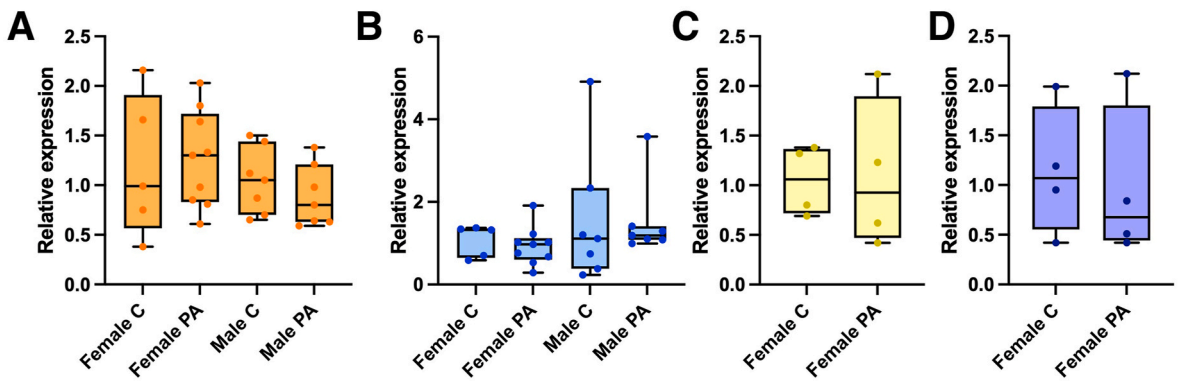

Fig. 5. *PEA15* gene expression. Comparison of the expression of *PEA15* between control (C) and prenatally androgenised (PA) female and male adolescent sheep in **A)** subcutaneous adipose tissue and **B)** visceral adipose tissue. The effect of prenatal androgenisation of the expression of *PEA15* in **C)** subcutaneous adipose tissue and **D)** visceral adipose tissue of mature adult female sheep.

adolescent PA sheep thus prevents recruitment of PPAR-G and C/EBP-A, also significantly decreased compared to control sheep. C/EBP-α and PPAR-ɣ prevent cell division and instead favour differentiation and lipid accumulation in normal conditions (Ghaben and Scherer, 2019). The molecular regulation of this is not yet fully established. We have reported that this process may be endocrinologically regulated through a reduction in hepatic FGF21 expression (Siemienowicz et al., 2021b). Recently is has been suggested that the cell surface glycoprotein CD44 may also have a role in co-ordinating the adipogenesis response (Weng et al., 2024).

The consequence of reduced adipogenesis is that the resulting adipocytes should therefore be less mature or less differentiated than the control. This is shown by a reduction in the expression of leptin in females but not males. We have previously reported a reduction in leptin and adiponectin in adolescent female PA sheep (Siemienowicz et al., 2021a). In this study the trend of reduced adiponectin did not reach significance when multiple comparison statistical analysis was used, with males included in the analysis. Although, like female PA sheep (Siemienowicz et al., 2021a), PA male sheep have dyslipidaemia, metabolic dysfunction (Siemienowicz et al., 2022) and insulin resistance (Siemienowicz et al., 2019), unlike females this does not appear to be associated with impaired adipogenesis.

Decreased adipogenesis also means these adipocytes could be less likely to efficiently store and metabolise lipids. This is supported by the observation of impaired lipid metabolism capability, both in terms of lipolysis as evidenced by reduced *HSL* and *ATGL* expression, as well as lipogenesis, as evidenced by decreased *AGPAT2* expression, in PA adolescent female sheep compared to control sheep. Decreased *AGPAT2* expression is a marker of decreased adipogenesis (Gale et al., 2006; Cautivo et al., 2016). After a meal, free fatty acids (FFAs) and glycerol are converted to triglycerides (TGs) during 'lipogenesis' by AGPAT2 and DGAT. TGs are consequently stored in lipid droplets, cytosolic structures which can occupy up to 90% of the cell volume (Ahmadian et al., 2009). Low *AGPAT2* expression in female PA sheep thus could prevent TGs synthesis, accumulation and storage in adipocytes in adolescence. At the same time, significantly decreased expression of *ATGL* and *HSL*, which account for more than 90% of total adipocyte lipolysis (Schweiger et al., 2006) underlines the inability of freeing FFAs and glycerol in the general circulation following prolonged fasting or physical exercise. Decreased expression of ATGL and HSL in SAT is associated with insulin resistance (Jocken et al., 2007), which PA female sheep demonstrate (Siemienowicz et al., 2020) and decreased PLIN1 is associated with decreased adipogenesis (Lyu et al., 2015).

Although there was no effect on these pathways in male PA SAT, analysis of the expression of the maturation marker fatty acid-binding protein (FABP)-4, which did not change in female sheep, was significantly decreased in male sheep during adolescence. This suggests that there are functional differences in male adolescent PA SAT that are different from those in female PA adolescents. Once more these changes were only seen in SAT and were not present in VAT. There was, however, some evidence of VAT dysfunction in adolescence with PA being associated with an increase in GLUT4 (SLC2A4) expression. This is evidence of potential VAT dysfunction. Interestingly in adult obese women with PCOS VAT GLUT4 was seen to be reduced (Chen et al., 2014). Although it wasn't significantly reduced in PA adult VAT it tended to be lower and the reduction in female adolescent SAT lost significance when multiple comparison statistics including males was used. This suggests a dynamic effect on glucose uptake in VAT that changes with maturity. In adulthood there were differences in lipid metabolism markers in SAT in female PA sheep but of note they tended to be in the different direction when compared to adolescence. The SAT hypertrophy in adult PA females is in agreement with increased *PLIN1* and *DGAT1* as both are markers of lipid droplet accumulation (Grahn et al., 2013; Harris et al., 2011). This suggests augmented lipid metabolism in adulthood that could further contribute to SAT dysfunction.

The endocrine and steroid metabolic characteristics of fat in both males and females are well known (Galic et al., 2010; Le Magueresse-Battistoni, 2020). Prenatal androgenisation might have a long-lasting effect. In adolescence aromatase (CYP19A1), which converts androgens into estrogens, was reduced in PA male SAT. This was not seen in adolescent female PA sheep but aromatase was reduced in the SAT of adult PA sheep. This has been reported in women with PCOS (Wang et al., 2012), suggesting an additional mechanism to augment androgens. However, this change in women is inconclusive as SAT *CYP19* was increased in those women with PCOS and higher testosterone and blood pressure (Lecke et al., 2011). This suggests that in some circumstances increased androgens may promote SAT CYP19 expression in PCOS. It is unclear why this was not the case in the PA adult sheep, although it should be noted that the tissue was collected at sacrifice in the non-breeding season to remove the impact of ovarian hormones in control and PA differences. Overall, the local effects, and metabolism, of androgens and estrogens in adipose tissue fat in PCOS are unclear and require further study.

Although there were no differences as a result of prenatal androgenisation in inflammatory markers in SAT in adolescence this was not the case in adult PA female SAT. We have previously reported increased inflammation in PA sheep when compared to contemporaneous controls (Siemienowicz et al., 2021a). However, the PA sheep weighed more than the control sheep. As obesity is associated with adipose tissue inflammation (Ellulu et al., 2017), here we looked only at weight-matched control and PA adult female sheep. Analysis of weight-matched sheep confirms that the adipocyte hypertrophy and inflammation is weight independent. In adult females PA is associated with a hypertrophic and pro-inflammatory phenotype in SAT but not VAT, as evidenced by

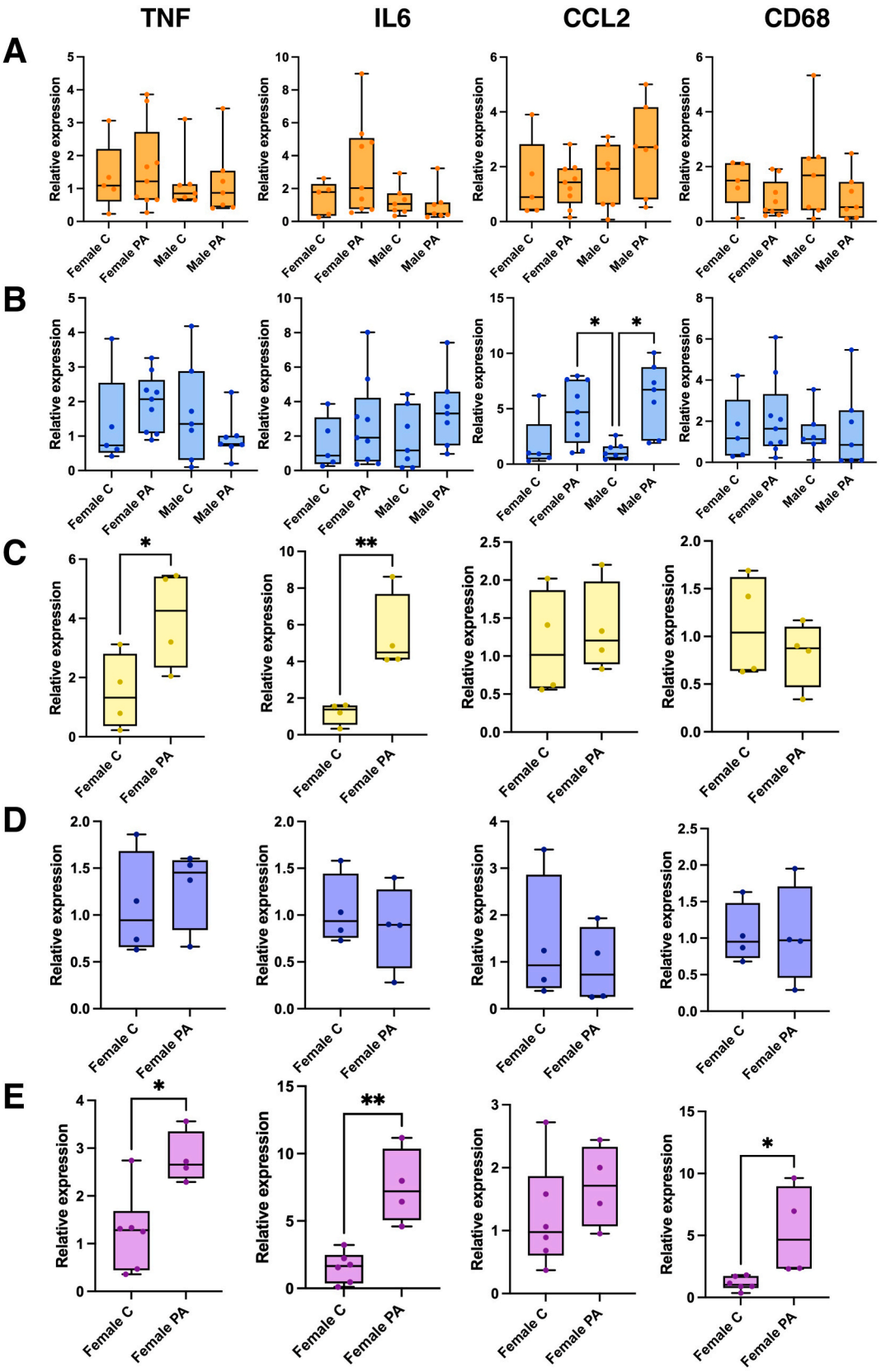

Fig. 6. Inflammation gene expression. Comparison of the expression of the inflammation genes *TNF*, *IL6*, *CCL2* and *CD68* between control (C) and prenatally androgenised (PA) female and male adolescent sheep in **A)** subcutaneous adipose tissue and **B)** visceral adipose tissue. The effect of prenatal androgenisation of the expression of *TNF*, *IL6*, *CCL2* and *CD68* in **C)** subcutaneous adipose tissue, **D)** visceral adipose tissue and **E)** Bone marrow adipose tissue of mature adult female sheep. *P $<$ 0.05, **P $<$ 0.01.

significantly larger white adipocytes and increased expression of *TNF* and *IL6* markers.

In order to investigate the mechanisms involved in adipose tissue hypertrophy we examined PEA15 expression. The small, cytoplasmic adaptor PEA15 has recently been identified as a novel regulator of white adipocyte size in mice (Verschoor et al., 2021). In this study, the lack of PEA15 in the knock-out group led to significantly larger white adipocytes than in WT mice. Even more interestingly, PEA15 has also previously been shown to be overexpressed in obese PCOS women (Savastano et al., 2011). Therefore, *PEA15* expression was investigated in our ovine model. *PEA15* levels were unchanged in female PA adult sheep, both in SAT and VAT. Similarly, no changes were detected during adolescence in both male and female sheep, in either SAT or VAT. The different models used make it difficult to directly compare our results. Moreover, the changes in cell size detected by Verschoor and colleagues were evident only after high fat diet, commonly used in murine models to induce an obese state, and not in chow conditions. However, PA sheep were fed a normal diet suggesting that *PEA15* is not involved in the SAT hypertrophy observed in our female PA sheep and may be more involved in dietary-induced SAT hypertrophy.

Adult female PA sheep also present a pro-inflammatory phenotype, and this study reveals for the first time this is not isolated to SAT but also to BMAT. This highly specialised adipose tissue depot is known to impact bone health, haematopoiesis, and participating to the metabolic homeostasis (Horowitz et al., 2017; Cawthorn et al., 2014). *TNF*, *IL6* and *CD68* were all found to be significantly increased in BMAT of PA adult sheep compared to the control, indicating increased recruitment of macrophages from the circulation, contributing to the worsening of adipocyte dysfunction. This could have profound implications for both bone formation and haematopoiesis, as bone marrow adipocytes actively participate in these two processes through cell-cell interaction as well as the release of adipokines and growth factors (Sebo et al., 2019). More investigations are needed to examine whether this is the case.

In summary, we have shown PA female sheep are phenotypically different from males in adolescence. At this developmental stage, impaired lipid metabolism capability was registered, in addition to impaired adipogenesis. We then confirmed this leads to SAT dysfunction, hypertrophic changes to adipocytes and increased inflammation during adulthood in females. Critically, we show for the first time this inflammatory phenotype is common to both SAT and BMAT, with potentially critical consequences for bone marrow immune system activity.

CRediT authorship contribution statement

Giovanni Levate: Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Yuan Wang:** Writing – review & editing, Methodology, Formal analysis. **Riada McCredie:** Writing – review & editing, Methodology, Formal analysis. **Megan Fenwick:** Writing – review & editing, Investigation, Formal analysis. **Michael T. Rae:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **W. Colin Duncan:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Katarzyna J. Siemienowicz:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Funding

This work was funded by Medical Research Council (MRC) project grants (G0500717; G0801807) and supported by (MR/W015439/1; MR/P011535/1).

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors wish to acknowledge Joan Docherty, John Hogg, Marjorie Thomson, Peter Tennant and James Nixon and the staff at the Marshall Building, University of Edinburgh for their excellent animal husbandry.

Data availability

Data will be made available on request.

References

Ahmadian, M., Duncan, R.E., Sul, H.S., 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. Trends Endocrinol. Metabol. 20, 424–428.
Amer, P., 1995. Differences in lipolysis between human subcutaneous and omental adipose tissues. Ann. Med. 27, 435–438.
Cautivo, K.M., Lizama, C.O., Tapia, P.J., Agarwal, A.K., Garg, A., Horton, J.D., Cortes, V. A., 2016. ACPAT2 is essential for postnatal development and maintenance of white and brown adipose tissue. Mol. Metabol. 5, 491–505.
Cawthorn, W.P., Scheller, E.L., Learman, B.S., Prale, S.D., Simon, B.R., Mori, H., et al., 2014. Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. Cell Metabol. 20, 368–375.
Chen, L., Xu, W.M., Zhang, D., 2014. Association of abdominal obesity, insulin resistance, and oxidative stress in adipose tissue in women with polycystic ovary syndrome. Fertil. Steril. 102, 1167–1174.e4.
de Wilde, M.A., Eising, J.B., Gunning, M.N., Koster, M.P.H., Evelein, A.M.V., Dalmeijer, G.W., Uiterwaal, C.S.P.M., Eijkemans, M.J.C., van der Ent, C.K., Meijboom, F.J., et al., 2018. Cardiovascular and metabolic health of 74 children from women previously diagnosed with polycystic ovary syndrome in comparison with a population-based reference cohort. Reprod. Sci. 25, 1492–1500.
Després, J.P., 2012. Body fat distribution and risk of cardiovascular disease: an update. Circulation 126, 1301–1313.
Divoux, A., Erdos, E., Whytock, K., Osborne, T.F., Smith, S.R., 2022. Transcriptional and DNA methylation signatures of subcutaneous adipose tissue and adipose-derived stem cells in PCOS women. Cells 11, 848.
Echiburú, B., Pérez-Bravo, F., Galgani, J.E., Sandoval, D., Saldias, C., Crisoto, N., Maliqueo, M., Sir-Petermann, T., 2018. Enlarged adipocytes in subcutaneous adipose tissue associated to hyperandrogenism and visceral adipose tissue volume in women with polycystic ovary syndrome. Steroids 130, 15–21.
Ehrmann, D.A., 2005. Polycystic ovary syndrome. N. Engl. J. Med. 352, 1223–1236.
Ellulu, M.S., Patimah, I., Khaza'ai, H., Rahmat, A., Abed, Y., 2017. Obesity and inflammation: the linking mechanism and the complications. Arch. Med. Sci. 13, 851–863.
Fauser, B.C., Tarlatzis, B.C., Rebar, R.W., Legro, R.S., Balen, A.H., Lobo, R., Carmina, E., Chang, J., et al., 2012. Consensus on women's health aspects of polycystic ovary syndrome (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group. Fertil. Steril. 97, 28–38.e25.
Gale, S.E., Frolov, A., Han, X., Bickel, P.E., Cao, L., Bowcock, A., Schaffer, J.E., Ory, D.S., 2006. A regulatory role for 1-Acylglycerol-3-phosphate-O-acyltransferase 2 in adipose differentiation. J. Biol. Chem. 281, 11082–11089.
Galic, S., Oakhill, J.S., Steinberg, G.R., 2010. Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol. 316, 129–139.
Ghaben, A.L., Scherer, P.E., 2019. Adipogenesis and metabolic health. Nat. Rev. Mol. Cell Biol. 20, 242–258.
Goodman, NF, Cobin, RH, Futterweit, W, Gluek, JS, Legro, RS, Carmina, E, et al., 2015. AMERICAN association of clinical endocrinologists, AMERICAN college of endocrinology, and androgen excess and PCOS society disease state clinical review: guide to the best practices in the evaluation and treatment of polycystic ovary SYNDROME–PART 1. Endocr. Pract. 21, 1291–1300.
Grahn, T.H.M., Zhang, Y., Lee, M.-J., Sommer, A.G., Mosyoslavsky, G., Fried, S.K., Greenberg, A.S., Puri, V., 2013. FSP27 and PLIN1 intercation promotes the formation of large lipid droplets in human adipocytes. Biochem. Biophys. Res. Commun. 432, 296–301.
Guo, L., Li, X., Tang, Q.Q., 2015. Transcriptional regulation of adipocyte differentiation: a central role for CCAAT/enhancer-binding protein (C/EBP) β. J. Biol. Chem. 290, 755–761.
Harris, C.A., Haas, J.T., Streeper, R.S., Stone, S.J., Kumari, M., Yang, K., Han, X., Brownwell, N., Gross, R.W., Zecher, R., Farese, R.V., 2011. DGAT enzymes are required for triacylglycerol synthesis and lipid droplets in adipocytes. J. Lipid Res. 52, 657–667.
Hoeger, K.M., Dokras, A., Piltonen, T., 2021. Update on PCOS: consequences, challenges, and guiding treatment. J. Clin. Endocrinol. Metab. 106, e1071–e1083.
Hogg, K., Wood, C., McNeilly, A.S., Duncan, W.C., 2011. The in utero programming effect of increased maternal androgens and a direct fetal intervention on liver and metabolic function in adult sheep. PLoS One 6, e24877.

Hogg, K., Young, J.M., Oliver, E.M., Souza, C.J., McNeilly, A.S., Duncan, W.C., 2012. Enhanced thecal androgen production is prenatally programmed in an ovine model of polycystic ovary syndrome. Endocrinology 153, 450–461.
Horowitz, M.C., Berry, R., Holtrup, B., Sebo, Z., Nelson, T., Fretz, J.A., Lindskog, D., Kaplan, J.L., Ables, G., Rodeheffer, M.S., Rosen, C.J., 2017. Bone marrow adipocytes. Adipocyte 6, 193–204.
Jocken, J.W.E., Langin, D., Smit, E., Saris, W.H.M., Valle, C., Hull, G.B., Holm, C., Arner, P., Blaak, E.E., 2007. Adipose triglyceride lipase and Hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. J. Clin. Endocrinol. Metab. 92, 2292–2299.
Kahn, D., Macias, E., Zarini, S., Garfield, A., Berry, K.Z., MacLean, P., Gerszten, R.E., Libby, A., Solt, C., et al., 2022. Exploring visceral and subcutaneous adipose tissue secretomes in human obesity: implications for metabolic disease. Endocrinology 163, bqac140.
Le Magueresse-Battistoni, B., 2020. Adipose tissue and endocrine-disrupting chemicals: does sex matter? Int. J. Environ. Res. Publ. Health 17, 9403.
Lecke, S.B., Morsch, D.M., Spritzer, P.M., 2011. CYP19 gene expression in subcutaneous adipose tissue is associated with blood pressure in women with polycystic ovary syndrome. J Steroids 7676, 1383–1388.
Lenarcik, A., Bidzińska-Speichert, B., Tworowska-Bardzińska, U., Krępuła, K., 2011. Hormonal abnormalities in first-degree relatives of women with polycystic ovary syndrome (PCOS). Endokrynol. Pol. 62, 129–133.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Longo, M., Zatterale, F., Naderi, J., Parrillo, L., Formisano, P., Raciti, G.A., Beguinot, F., Miele, C., 2019. Adipose tissue dysfunction as determinant of obesity-associated metabolic complications. Int. J. Mol. Sci. 20, 2358.
Lyu, Y., Su, X., Deng, J., Liu, S., Zhao, X., Wei, S., Geng, B., Xu, X., 2015. Defective differentiation of adipose precursor cells from lipodystrophic mice lacking perlipin 1. PLoS One 10, e0117536.
Moran, L.J., Norman, R.J., Teede, H.J., 2015. Metabolic risk in PCOS: phenotype and adiposity impact. Trends Endocrinol. Metabol. 26, 136–143.
Pellegrinelli, V., Carobbio, S., Vidal-Puig, A., 2016. Adipose tissue plasticity: how fat depots respond differently to pathophysiological cues. Diabetologia 59, 1075–1088.
Pou, K.M., Massaro, J.M., Hoffmann, U., Vasan, R.S., Maurovich-Horvat, P., Larson, M. G., Keaney, JF Jr, Meigs, J.B., Lipinska, I., Kathiresan, S., Murabito, J.M., O'Donnell, C.J., Benjamin, E.J., Fox, C.S., 2007. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. Circulation 116, 1234–1241.
Rae, M., Grace, C., Hogg, K., Wilson, L.M., McHaffie, S.L., Ramaswamy, S., MacCallum, J., Connolly, F., McNeilly, A.S., Duncan, W.C., 2013. The pancreas is altered by in utero androgen exposure: implications for clinical conditions such as polycystic ovary syndrome (PCOS). PLoS One 8, e56263.
Recabarren, S.E., Smith, R., Rios, R., Maliqueo, M., Echiburú, B., Codner, E., Cassorla, F., Rojas, P., Sir-Petermann, T., 2008. Metabolic profile in sons of women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 93, 1820–1826.
Savastano, S., Valentino, R., Di Somma, C., Orio, F., Pivonello, C., Passaretti, F., Brancato, V., Formisano, P., et al., 2011. Serum 25- Hydroxyvitamin D levels, phosphoprotein enriched in diabetes gene product (PED/PEA-15) and leptin-to-adiponectin ratio in women with PCOS. Nutr. Metab. 8, 1743–7075.
Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R., Zimmermann, R., 2006. Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. J. Biol. Chem. 281, 40236–40241.
Sebo, Z.L., Rendina-Ruedy, E., Ables, G.P., Lindskog, D.M., Rodeheffer, M.S., Fazeli, P.K., Horowitz, M.C., 2019. Bone marrow adiposity: basic and clinical implications. Endocr. Rev. 40, 1187–1206.
Siemienowicz, K.J., Coukan, F., Franks, S., Rae, M.T., Duncan, W.C., 2021a. Aberrant subcutaneous adipogenesis precedes adult metabolic dysfunction in an ovine model of polycystic ovary syndrome (PCOS). Mol. Cell. Endocrinol. 519, 111042.
Siemienowicz, K., Furmanska, K., Filis, P., Talia, C., Thomas, J., Fowler, P.A., Rae, M.T., Duncan, W.C., 2021b. Pubertal FGF21 deficit is central in the metabolib pathophysiology of an ovine model of polycustic ovary syndrome. Mol. Cell. Endocrinol. 525, 111196.
Siemienowicz, K.J., Filis, P., Shaw, S., Douglas, A., Thomas, J., Mulroy, S., Howie, F., Duncan, W.C., Rae, M.T., 2019. Fetal androgen exposure is a determinant of adult male metabolic health. Sci. Rep. 9, 20195.
Siemienowicz, K.J., Filis, P., Thomas, J., Fowler, P.A., Duncan, W.C., Rae, M.T., 2022. Hepatic mitochondrial dysfunction and risk of liver disease in an ovine model of "PCOS males". Biomedicines 10, 1291.
Siemienowicz, K., Rae, M.T., Howells, F., Anderson, C., Nicol, L.M., Franks, S., Duncan, W.C., 2020. Insights into manipulating postprandial energy expenditure to manage weight gain in polycystic ovary syndrome. PCOS) iScience 23, 101164.
Siersbæk, R., Nielsen, R., John, S., Sung, M.H., Baek, S., Loft, A., Hager, G.L., Mandrup, S., 2011. Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. EMBO J. 30, 1459–1472.
Spritzer, P.M., Lecke, S.B., Satler, F., Morsch, D.M., 2015. Adipose tissue dysfunction, adipokines, and low-grade chronic inflammation in polycystic ovary syndrome. Reproduction 149, R219–R227.
Sun, K., Kusminski, C.M., Scherer, P.E., 2011. Adipose tissue remodeling and obesity. J. Clin. Invest. 121, 2094–2101.
Verschoor, P.J., Greig, F.H., Rochford, J.J., Levate, G., Delibegovic, M., Thomson Dleeson-Payne, A., Dekeryte, R., Banks, R., et al., 2021. Phosphoprotein enriched in astrocytes (PEA)-15 is a novel regulator of adipose tissue expansion. Sci. Rep. 11, 6949.
Wang, L., Li, S., Zhao, A., Tao, T., Mao, X., Zhang, P., Liu, W., 2012. The expression of sex steroid synthesis and inactivation enzymes in subcutaneous adipose tissue of PCOS patients. J. Steroid Biochem. Mol. Biol. 132, 120–126.
Weng, X., Jiang, H., Walker, D.J., Zhou, H., Lin, D., Wang, J., Kang, L., 2024. Deletion of CD44 promotrs adipogenesis by regulating PPARγ and cell cycle-related pathways. J. Endocrinol. 262, e240079.
Yilmaz, B., Vellanki, P., Ata, B., Yildiz, B.O., 2018. Metabolic syndrome, hypertension, and hyperlipidemia in mothers, fathers, sisters, and brothers of women with polycystic ovary syndrome: a systematic review and meta-analysis. Fertil. Steril. 109, 356–364.e32.