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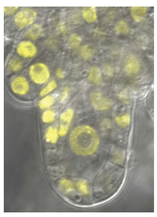

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RESEARCH PAPER

DNA methylation in promoter regions of genes involved in the reproductive and metabolic function of children born to women with PCOS

Bárbara Echiburú [a], Fermín Milagro [b,c], Nicolás Crisosto[a,d], Francisco Pérez-Bravo [e], Cristian Flores[a], Ana Arpón [b], Francisca Salas-Pérez[b], Sergio E. Recabarren[f], Teresa Sir-Petermann[a], and Manuel Maliqueo [a]

[a]Endocrinology and Metabolism Laboratory, West Division, School of Medicine, University of Chile, Santiago, Chile; [b]Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain; [c]Centro De Investigación Biomédica En Red Fisiopatología De La Obesidad Y Nutrición (Ciberobn), Instituto De Salud Carlos III, Madrid, Spain; [d]Unit of Endocrinology, Clínica Las, Santiago, Chile; [e]Laboratory of Nutritional Genomics, Department of Nutrition, Faculty of Medicine, University of Chile, Santiago, Chile; [f]Laboratory of Animal Physiology and Endocrinology, Department of Animal Science, Faculty of Veterinary Sciences, University of Concepcion, Chillán, Chile

ABSTRACT

Clinical and experimental evidences indicate that epigenetic modifications induced by the prenatal environment are related to metabolic and reproductive derangements in polycystic ovary syndrome (PCOS). Alterations in the leptin and adiponectin systems, androgen signalling and antimüllerian hormone (AMH) levels have been observed in PCOS women and in their offspring. Using a targeted Next-Generation Sequencing (NGS), we studied DNA methylation in promoter regions of the leptin (*LEP*), leptin receptor (*LEPR*), adiponectin (*ADIPOQ*), adiponectin receptor 1 and 2 (*ADIPOR1* and *ADIPOR2*), *AMH* and androgen receptor (*AR*) genes in 24 sons and daughters of women with PCOS (12 treated with metformin during pregnancy) and 24 children born to non-PCOS women during early infancy (2–3 months of age). Genomic DNA was extracted from whole blood, bisulphite converted and sequenced by NGS. Girls showed differences between groups in 1 CpG site of *LEPR*, 2 of *LEP*, 1 of *ADIPOR2* and 2 of *AR*. Boys showed differences in 5 CpG sites of *LEP*, 3 of *AMH* and 9 of *AR*. Maternal metformin treatment prevented some of these changes in *LEP*, *ADIPOR2* and partially in *AR* in girls, and in *LEP* and *AMH* in boys. Maternal BMI at early pregnancy was inversely correlated with the methylation levels of the ChrX-67544981 site in the whole group of girls ($r = -0.530$, $p = 0.008$) and with the global Z-score in all boys ($r = -0.539$, $p = 0.007$). These data indicate that the intrauterine PCOS environment predisposes the offspring to acquire certain sex-dependent DNA methylation patterns in the promoter regions of metabolic and reproductive genes.

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Introduction

Polycystic ovary syndrome (PCOS) is a highly prevalent and heterogenic disorder in women of reproductive age, characterized by hyperandrogenism and chronic anovulation, and closely associated with insulin resistance and obesity [1]. We and other authors have demonstrated that androgen excess and metabolic disturbances persist in PCOS women during pregnancy, predisposing to complications such as gestational diabetes (GDM) and pregnancy-induced hypertension. Moreover, this adverse maternal environment has also been associated with deleterious consequences for the foetus such as alterations in birth weight and development of metabolic and cardiovascular diseases during adult life [2,3]. Some of these disturbances are sex specific indicating that the maternal environment affects the male and female foetuses differently.

In previous studies we have observed higher leptin concentrations in cord blood of PCOS newborns compared to controls [4], a marker that has been associated with disturbances in BMI and insulin levels in PCOS women [5]. Daughters of PCOS women have higher antimüllerian hormone (AMH) concentrations since early infancy to puberty, evidencing an increased follicular mass [6,7] and an increased ovarian volume and hyperandrogenism during early and late puberty, respectively [8]. Regarding metabolic alterations, hypoadiponectinemia is present before the onset of

CONTACT Manuel Maliqueo mmaliqueo@uchile.cl Lab. Of Endocrinology, Dept. Of Medicine W. Division, School of Medicine, Carlos Schachtebeck (Ex Las Palmeras) 299, Interior Quinta Normal, Casilla 33052, Correo 33, Santiago 8320000, Chile
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puberty and hyperinsulinemia, reflecting insulin resistance, since prepuberty and later on [8,9]. On the other hand, sons of PCOS mothers show higher AMH concentrations from early infancy to prepuberty, suggesting an increase in the Sertoli cell number or function [10]. Moreover, these boys usually exhibit higher body weight since early infancy and insulin resistance when they get older [11]. Therefore, it seems that altered levels of leptin, adiponectin and AMH along with an abnormal androgen action are common features that appear since early infancy in both daughters and sons born to PCOS mothers. It is known that the androgen receptor modulates androgen action and several studies have supported that a higher activity in the AR is a determining factor in PCOS development [12–14].

During the last years, metformin has been used to treat the maternal complications associated with GDM and PCOS. Moreover, we have previously demonstrated that metformin can improve the altered endocrine-metabolic environment of PCOS mothers reducing AMH levels in their daughters, which might be associated with a decrease in their follicular mass [15]. Nevertheless, its long-term effects in the offspring could be debatable and need more research to be established.

It is now known that multiple mechanisms contribute to foetal programming in PCOS including genetic, epigenetic and environmental factors [16–19]. Epigenetics is a molecular phenomenon that regulates gene expression without changes in the DNA sequence modulating tissue-specific gene expression, genomic imprinting and X-chromosome inactivation [20]. Epigenetic modifications consist mainly of DNA methylation, histone modifications, chromatin reconstruction, and expression of non-coding RNA [21]. DNA methylation is the most stable and best understood epigenetic mechanism [22,23], which consists in the addition of methyl (-CH3) groups to the 5-carbon of cytosine mainly in CpG-dinucleotides (CpGs) regulating transcriptional expression of specific genes [24]. It has also been proposed that exposure to androgen excess during prenatal life may induce epigenetic changes inducing long-term modifications in the offspring [25]. Moreover, in PCOS women, specific genes have been demonstrated to be associated with aberrant DNA methylation in tissues and pathways associated to PCOS dysfunction [26–31]. In this context, it has been proposed that differences in the androgen receptor DNA methylation pattern could be associated to hyperandrogenism [32]. Recent studies suggest that adipokines are involved in the foetal metabolic health programming through epigenetic adaptations [33]. Finally, recent results showed a decreased methylation level of the AMH gene associated with an increase in AMH follicular levels in PCOS women [34]. Then, alterations observed in children born to PCOS women could be attributed to these modifications. Until now, only one study has approached this issue showing a differential DNA methylation pattern in umbilical cord blood from children born to PCOS women [35].

In the present study we hypothesized that, depending on their sex, children born to PCOS women could have a particular methylation pattern in the promoter region of key reproductive and metabolic genes, which may be modulated by the intrauterine environment. Therefore, our aim was to evaluate, in genomic DNA from whole blood, the methylation pattern of promoter regions of reproductive and metabolic genes in the offspring of PCOS women during early infancy (2–3 months of age) and compare it with controls and with children form PCOS women treated with metformin during pregnancy. Based on previous observations, we focused our analysis on the promoter regions of leptin (*LEP*), leptin receptor (*LEPR*), adiponectin (*ADIPOQ*), adiponectin receptor 1 and 2 (*ADIPOR1, ADIPOR2*), antimüllerian hormone (*AMH*), and the androgen receptor (*AR)* genes.

Subjects and methods

Subjects

Twenty-four Chilean infants born to PCOS women (PCOS) and 24 born to non-PCOS women (control) were included in the study. The PCOS daughters and sons groups included 12 female and 12 male infants (2–3 months old), born to PCOS mothers. The control daughters and sons groups included 12 female and 12 male infants born to mothers with regular menses and without hyperandrogenism. None of the subjects included in the study were genetically related with

each other. In the PCOS group, 12 women were treated with metformin during the whole period of pregnancy (PCOS+M). PCOS and control infants were born from spontaneous singleton pregnancies. All infants were studied during early infancy (2–3 months of age). Most of these infants were included in previous studies carried out by our group [10,11,15]. Inclusion criteria for PCOS mothers and control mothers were similar to those previously reported [8,15].

The protocol was approved by the Institutional Review Board of the Faculty of Medicine University of Chile (Approval of Research Project Nº032-2015). All parents signed an informed consent before entering the study.

Pregnant women study protocol

PCOS mothers were recruited from patients attending the Unit of Endocrinology and Reproductive Medicine at the University of Chile who had desired fertility. Diagnosis of PCOS was made according to the diagnostic criteria for PCOS of the National Institutes of Health (NIH) consensus [36]. As part of their initial evaluation, all the patients underwent a lifestyle assessment and were placed on a diet and exercise treatment programme as previously described [15]. In addition, most of them received 1,500–2,000 mg metformin in standard formulation based on their weight, medication tolerance, and insulin levels. PCOS patients were instructed to stop metformin treatment upon a positive pregnancy test and those that required it continued with the medication during the whole pregnancy.

No medications to induce ovulation, such as clomiphene citrate or exogenous gonadotropins, were used. We excluded patients with hyperprolactinaemia, androgen-secreting neoplasm, Cushing syndrome, late-onset 21-hydroxylase deficiency, or thyroid disease.

As a control group, we selected pregnant women of similar age and socioeconomic level as the PCOS patients. These pregnant women had a history of regular 28- to 32-day menstrual cycles, absence of hirsutism and other clinical manifestations of hyperandrogenism, infertility, pregnancy complications, galactorrhoea, and thyroid dysfunction. All were healthy and were not receiving any drug therapy. These women were recruited from the antenatal care unit of San Juan de Dios hospital (Santiago, Chile) from the 12th week of gestation.

In all pregnant women, duration of gestation, initial and final body mass index (BMI), and weight gain during pregnancy were recorded. During gestational weeks 22–28, all women were classified as having gestational diabetes mellitus or pregnancy-induced hypertension in accordance with the World Health Organization criteria (fasting glucose values >105 mg/dL; 2-hour glucose postload >140 mg/dL), pregnancy-induced hypertension (blood pressure ≥140/90 mm Hg without proteinuria at a gestational age > 20 weeks on two or more occasions) or preeclampsia (blood pressure ≥ 140/90 mm Hg with proteinuria > 0.3 g/24 h after 20 weeks' gestation).

Offspring study protocol

All infants were examined twice, once during the first 3 days of life and again at 2–3 months of age. On both occasions the physical examination included weight and length, following the scheme described in previous studies in which most of these children participated [7,10,11,15]. Gestational age and the type of feeding was registered (exclusive breastfeeding, formula or mixed). In all infants, a blood sample was taken and stored at −80°C for DNA analysis.

Methylation analysis

DNA isolation. Genomic DNA was extracted from peripheral blood leukocytes in all infants using the E.Z.N.A.® Blood DNA Midi Kit (Omega Bio-tek, Inc.Qiagen, Hilden, Germany) following instructions provided by the manufacturer. The concentration and purity of DNA was determined using a Nanodrop spectrophotometer (Tecan Infinite 200 PRO).

Assay design, sample preparation, and multiplex targeted amplification. We selected the promoter regions of seven genes that, according to our previous observations and the literature, could be involved in the metabolic and endocrine changes found in the offspring of PCOS women, including leptin (*LEP*), leptin receptor (*LEPR*), adiponectin

(*ADIPOQ*), adiponectin receptor 1 (*ADIPOR1*), adiponectin receptor 2 (*ADIPOR2*), anti-Müllerian hormone (*AMH*), and androgen receptor (*AR*) (Supplementary Table 1). Genomic DNA was bisulphite modified and then sequenced by the Zymo Research Corporation (Irvine, CA, USA). Assays were designed targeting CpG sites in the specified regions of interest (ROI) using primers created with Rosefinch, Zymo Research's proprietary sodium bisulphite converted DNA-specific primer design tool (Supplementary Table 2). DNA samples were bisulphite converted using the EZ DNA Methylation-Lightning ™ Kit (D5030, Zymo Research) according to the manufacturer's instructions. The following processes included a targeted sequencing for DNA methylation analysis at multiple loci using a multiplex PCR strategy in combination with Next-Generation Sequencing (NGS) (MiSeq, Illumina, Inc., San Diego, CA). Multiplex amplification of all samples using ROI specific primer pairs and the Fluidigm Access Array™ System was performed according to the manufacturer's instructions. The resulting amplicons were pooled for harvesting and subsequent barcoding according to the Fluidigm instrument's guidelines. After barcoding, samples were purified using ZR-96 DNA Clean & Concentrator™ (D4023, Zymo Research), and then prepared for massively parallel sequencing using a MiSeq V2 300bp Reagent Kit and paired-end sequencing protocol according to the manufacturer's guidelines.

Targeted sequence alignments and data analysis. Sequence reads were identified using standard Illumina base-calling software and then analysed using a Zymo Research proprietary analysis pipeline, which is written in Python. Low quality nucleotides and adapter sequences were trimmed off during analysis QC. Sequence reads were aligned back to the reference genome using Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/), an aligner optimized for bisulphite sequence data and methylation calling [37]. Paired-end alignment was used as default thus requiring both read 1 and read 2 to be aligned within a certain distance; otherwise both read 1 and read 2 were discarded. Index files were constructed using the bismark_genome_preparation command and the entire reference genome (GRCh38/hg38). The non-directional parameter was applied while running Bismark. All other parameters were set to default. Nucleotides in primers were trimmed off from amplicons during methylation calling.

Data analysis. The methylation level (β-value) of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Differential methylation was assessed per CpG site including samples with at least 10 reads. Moreover, Z-score value to every CpG site of each gene promoter region was calculated according the method proposed by Hertzberg [38]. For the calculation of the z-score of each promoter region, the sum of each individual z-score value was considered. Finally, we calculated a global z-score value, with the sum of each site that was statistically different between the PCOS and control group.

Transcription factors analysis. In order to identify potential transcription factor binding sites in a promoter sequence, computational identification was performed using TFSITESCAN tools and database (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl). Only transcription factors binding on or closely near the CpGs were further studied.

Statistical evaluation

Data are expressed as median and range for anthropometric and biochemical variables, and as mean and standard deviation for DNA methylation. Normal distribution was assessed by the Kolmogorov-Smirnov test. The β-value and z-scores comparisons were performed according to the sex of the infants. Differences between groups were calculated through one-way ANOVA followed by Bonferroni post hoc test when data were normally distributed or Kruskal Wallis followed by Dunn test for skewed data. Categorical data were analysed using $\chi 2$ or Fisher's exact test. Spearman correlations were used to evaluate the relationship between the variables of interest. Statistical analysis was performed using SPSS 23.0 package. A p-value of less than 0.05 was considered to be statistically significant.

Results

Clinical maternal data

Clinical characteristics of pregnant control and PCOS women (treated and non-treated with metformin during pregnancy) are shown in Table 1. PCOS women treated with metformin were older than control women ($P = 0.010$). Moreover, pre-pregnancy body weight, BMI and the prevalence of overweight/obesity were significantly higher in those PCOS women that required metformin during pregnancy compared to control women ($P = 0.002$, $P < 0.001$ and $P = 0.004$, respectively). At the beginning of pregnancy, both PCOS women treated and non-treated with metformin showed higher BMI compared to control women ($P = 0.002$ and $P < 0.002$, respectively). At the third trimester, BMI was higher in PCOS women compared to control women ($P = 0.029$). In turn, gestational weight gain (GWG) was lower in PCOS treated with metformin compared to controls and non-treated PCOS ($P = 0.001$ and $P = 0.035$, respectively). No differences were observed in the prevalence of pregnancy-induced hypertension but gestational diabetes mellitus (GDM) was more prevalent in both groups of PCOS women compared to controls. The clinical characteristics of PCOS women at diagnosis are shown in supplementary table 3.

Infant clinical data

Clinical and anthropometric characteristics of infants born to control and PCOS women are shown in Table 2. At birth, daughters born to PCOS+M showed lower gestational age than controls ($P = 0.004$), whereas birth length tended to be lower in this group of girls compared to controls ($P = 0.074$). On the other hand, in sons, there were no differences between groups regarding clinical and anthropometric variables. The prevalence of small (SGA) and large (LGA) for gestational age was similar between groups in both daughters and sons.

At the time of study, there were no differences in age between groups both in daughters and sons. Z-score of weight and length were lower in daughters of PCOS women treated with metformin during pregnancy compared to girls born to control women ($P = 0.034$ and $P = 0.010$). In turn, sons of PCOS+M tended to be heavier and gained more weight from birth to study time ($P = 0.060$ and $P = 0.037$, respectively). By the time of the study, the prevalence of exclusive maternal breastfeeding was similar between groups both in daughters and sons.

Table 1. Clinical and biochemical characteristics of control and PCOS pregnant women with (PCOS+M) and without metformin (PCOS).

	Control (n = 24)	PCOS (n = 12)	PCOS+M (n = 12)	*P-values*
Pre-pregnancy				
Age (years)	23.5 (20.2–30.7)	29.5 (26.0–31.0)	30.5 (30.0–35.2) [b]	**0.013**
Weight (kg)	59.5 (52.8–66.0)	69.0 (56.0–80.0)	72.0 (68.0–76.8)[b]	**0.002**
BMI (Kg/m^2)	22.9 (21.1–26.0)	26.1 (23.7–29.1)	29.3 (27.1–30.9) [b]	**<0.001**
Overweight/obesity (%)	29.2	58.3	83.3[b]	**0.007**
At beginning				
Weight (kg)	59.0 (52.5–66.0)	69.0 (57.0–80.0)	70.8 (68.0–77.0)[b]	**0.002**
BMI (Kg/m^2)	23.7 (21.1–25.7)	27.6 (24.4–29.9)[a]	28.8 (27.1–30.9) [b]	**<0.001**
At third trimester				
Weight (Kg)	71.0 (65.8–77.8)	78.0 (69.0–90.0)[a]	78.8 (70.9–81.5)	0.096
BMI	29.1 (26.7 – 31.3)	30.2 (28.5 – 36.1)[a]	31.9 (28.2 – 32.4)	**0.026**
SBP (mm Hg)	120 (110–120)	110 (105–110)	120 (110–120)	0.087
DBP (mm Hg)	70 (60–77)	62 (60–70)	70 (70 – 70)	0.448
GWG (Kg)	12.9 (8.2–16.5)	11.0 (6.5–15.8)	5.0 (2.8–7.9)[b, c]	**0.001**
PIH (%) (n)	0	8.3 (1/12)	0 (0/12)	0.216
GDM (%) (n)	0	16.7 (2/12)[a]	66.7 (8/12) [b,c]	**<0.001**

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; GWG: gestational weight gain; PIH: pregnancy induced-hypertension; GDM: gestational diabetes mellitus. Data are expressed as median and interquartile range for continuous variables and percentage for categorical variables. For continuous variables differences were calculated by one way ANOVA test followed by Bonferroni test or Kruskal Wallis followed by Dunn tests according to the normal distribution of data. Categorical variables were analysed by chi-square test. [a]$P < 0.05$ between control and PCOS; [b]$P < 0.05$ between control and PCOS+M; [c]$P < 0.05$ between PCOS and PCOS+M.

Table 2. Clinical characteristics of control and PCOS infants (daughters and sons) born to control and PCOS mothers treated (PCOS+M) and non-treated (PCOS) with metformin during pregnancy.

	Daughters				Sons			
	Control (n = 12)	PCOS (n = 6)	PCOS+M (n = 6)	P-values	Control (n = 12)	PCOS (n = 6)	PCOS+M (n = 6)	P-values
At birth								
Gestational age (wk)	39.0 (39.0–40.0)	39.0 (38.0–40.0)	38.0 (36.0–38.0)[b]	**0.005**	39.5 (38.2–40.0)	39.5 (38.7–41.0)	39.0 (37.7–39.5)	0.474
Weight (g)	3545 (3257–3750)	3275 (2690–3905)	3285 (2895–3515)	0.346	3575 (3402–4010)	3588 (2965–3875)	3417 (3015–3692)	0.534
Z-score weight	0.76 (0.15–1.06)	0.04 (−1.26–1.87)	0.34 (−0.25–0.93)	0.601	1.11 (0.55–1.93)	1.03 (−0.73–1.58)	0.57 (−0.25–1.34)	0.631
Length (cm)	50.0 (49.0–51.0)	47.2 (44.7–50.4)	47.7 (45.9–49.2)	**0.046**	50.0 (49.2–51.7)	50.0 (49.2–51.0)	49.5 (48.2–51.4)	0.659
Z-score length	0.22 (−0.27–0.68)	−0.39 (−1.38–0.60)	−0.12 (−0.83–0.17)	0.381	0.22 (0.06–0.85)	0.30 -(0.25–0.54)	0.13 -(0.70–0.88)	0.775
SGA (%) (n)	0 (0/12)	16.7 (1/6)	0 (0/6)	0.209	8.3 (1/12)	16.7 (1/6)	0 (0/6)	0.579
LGA (%) (n)	16.7 (2/12)	33.3 (2/6)	0 (0/6)	0.301	33.3 (4/12)	33.3 (2/6)	16.7 (1/6)	0.739
At study								
Age (years)	0.17 (0.17–0.23)	0.25 (0.17–0.25)	0.25 (0.23–0.25)[b]	**0.006**	0.17 (0.17–0.25)	0.21 (0.17–0.27)	0.25 (0.17–0.33)	0.077
Weight (g)	6115 (5327–6650)	6335 (5425–6605)	5175 (4747–6337)	0.336	5600 (5400–8160)	6400 (6050–7540)	6375 (5587–7012)	**0.040**
Z-score weight	0.94 (0.18–1.48)	0.73 (−0.04–1.28)	−1.02 (−1.52–0.42)[b]	**0.032**	−0.29 (−0.95–0.48)	0.49 (−0.04–1.79)	0.68 (−0.79–2.06)	0.080
Length (cm)	58.0 (57.0–60.7)	57.5 (55.5–59.0)	57.5 (55.2–60.1)	0.419	59.0 (58.0–60.9)	60.0 (59.0–62.5)	57.5 (57.0–61.0)	0.267
Z-score length	−0.13 (−0.59–1.45)	−0.73 (−1.56 – −0.08)	−1.30 (−2.17 – −0.20)[b]	**0.008**	−0.24 (−0.71–0.62)	0.03 (−0.57–1.43)	−0.33 (−1.71–1.36)	0.789
Weight gain (g)	2735 (1820–3212)	2620 (2390–3170)	2485 (1480–3327)	0.886	2145 (1652–2732)	2967 (2270–4147)	3615 (2881–4510)[b]	**0.020**
EMB (%) (n)	80 (8/10)	83.3 (5/6)	50.0 (3/6)	0.338	77.8 (7/9)	25.0 (1/4)	66.7 (4/6)	0.186

Results are expressed as median and interquartile range. SGA: small for gestational age; LGA: large for gestational age and EMB: exclusive maternal breastfeeding. Data are expressed as median and interquartile range for continuous variables and percentage for categorical variables. For continuous variables differences were calculated by one way ANOVA test followed by Bonferroni test or Kruskal Wallis followed by Dunn tests according to the normal distribution of data. Categorical variables were analysed by chi-square test. [a]$P < 0.05$ between control and PCOS; [b]$P < 0.05$ between control and PCOS+M; [c]$P < 0.05$ between PCOS and PCOS+M.

Methylation analysis in promoter region of candidate genes

In total, the methylation levels in 368 CpG sites distributed among the promoter regions of 7 genes (*LEP, ADIPOQ, AMH, LEPR, ADIPOR1, ADIPOR2* and *AR*) were analysed.

Daughters showed differences in 1 CpG site located in the promoter region of *LEPR*, 2 in *LEP*, 1 in *ADIPOR2* and 2 in *AR* (Figure 1(a–d)). In the Chr1-65419664 site of the *LEPR* promoter, the proportion of methylation was higher in infants born to PCOS and PCOS+M compared to those born to control women ($P = 0.016$ and $P = 0.037$, respectively) (Figure 1(a)). Moreover, the Chr7-128240906 and Chr7-128241078 sites in the promoter of *LEP* exhibited higher methylation levels in PCOS+M infants compared to PCOS ($P = 0.007$ and $P = 0.033$, respectively). A trend to higher methylation in the Chr7-128240906 site was observed in control infants compared to PCOS ($P = 0.072$) (Figure 1(b)). In the Chr12-1690290 site of the *ADIPOR2* promoter, increased methylation levels were observed in infants born to PCOS mothers compared to controls and PCOS+M. ($P = 0.022$ and $P = 0.019$, respectively) (Figure 1(c)). Moreover, the ChrX-67543969 and ChrX-67544981 sites of the *AR* promoter were less methylated in PCOS compared to controls ($P = 0.005$ and $P = 0.049$, respectively) (Figure 1(d)).

In sons, 5 CpG sites in the promoter region of *LEP*, 3 in *AMH*, and 9 in *AR* showed differences in methylation levels between groups (Figure 2(a–c)). In the promoter region of *LEP* (Figure 2(a)), the Chr7-128240873 site had lower methylation levels in PCOS compared to controls ($P = 0.037$), whereas in Chr7-128241155 methylation was lower in PCOS than in PCOS+M ($P = 0.034$). On the other hand, methylation in Chr7-128241074 was higher in PCOS compared to controls and PCOS+M ($P = 0.008$ and $P = 0.012$, respectively). In turn, methylation levels at the Chr7-128241028 site were lower ($P = 0.028$), whereas in Chr7-128241387 were higher in PCOS +M compared to controls ($P = 0.028$). In the promoter region of *AMH* (Figure 2(b)), methylation levels in Chr19-2248956 and Chr19-2249331 were lower in PCOS than in controls ($P = 0.030$ and $P = 0.010$, respectively) and the last site was also lower in PCOS compared to PCOS+M ($P = 0.010$). At Chr19-2249336, sons born to PCOS+M mothers had higher methylation levels than controls ($P = 0.046$). Finally, the *AR* promoter showed higher methylation levels in PCOS+M sons compared to controls and PCOS at the ChrX-67543762 site ($P < 0.0001$ and $P < 0.0001$, respectively) and lower methylation levels at ChrX-67544032 compared to controls and PCOS ($P < 0.0001$ and $P = 0.048$, respectively). In the other CpG sites, lower methylation levels were observed in sons born to PCOS+M compared to controls (ChrX-67543849 ($P = 0.006$), ChrX-67543889 ($P = 0.043$), ChrX-67543895 ($P = 0.015$), ChrX-67543899 ($P = 0.017$), ChrX-67544040 ($P = 0.006$), ChrX-67544221 ($P = 0.041$) and ChrX-67545002 ($P = 0.022$)) (Figure 2(c)).

Methylation Z-score

The Z-scores for the promoter regions of *LEP, LEPR, ADIPOQ, ADIPOR1, ADIPOR2 AMH and AR* were comparable between the groups in both daughters and sons. The same was observed for the global Z-score in daughters (Figure 3(a)). However, the global Z-score was higher in control sons compared to PCOS and PCOS+M ($P = 0.019$ and $P = 0.019$, respectively) (Figure 3(b)).

Correlation analysis

In daughters, the methylation levels of the chrX-67544981 site (*AR*) were significantly and inversely correlated with maternal BMI at early pregnancy (Figure 4(a)). On the other hand, in sons, the methylation levels in chr7-128241028 (*LEP*) and chrX-67543762 (*AR*) were significantly and positively correlated with the Z-Score of length and with the postnatal weight gain between birth and time of study, respectively (Figure 4(b–c)). Moreover, in sons, the global Z-Score of methylation was negatively associated with maternal BMI at early pregnancy and with postnatal weight gain (Figure 4(d–e)).

Predicted transcription factor (TF) binding sites

In daughters, the differently methylated site, Chr7-128240906, in the *LEP* promoter has putative binding sites for the transcription factors SP1

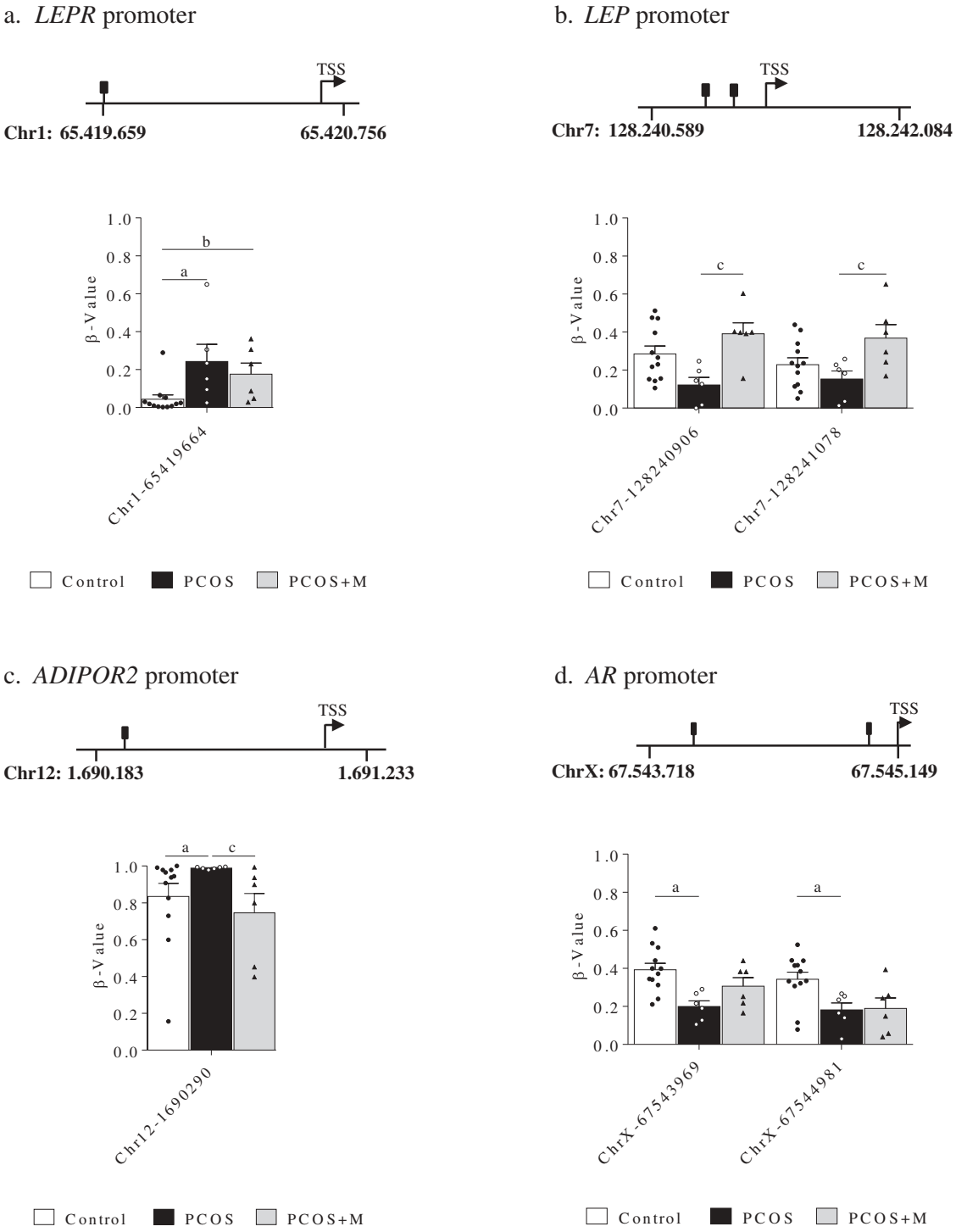

Figure 1. Methylation levels (β-value) in the promoter regions of the leptin receptor (*LEPR*) (a), leptin (*LEP*) (b), adiponectin receptor 2 (*ADIPOR2*) (c) and androgen receptor (*AR*) genes (d) in daughters of control (control, n = 12), PCOS women (PCOS, n = 6) and PCOS women treated with metformin during pregnancy (PCOS+M, n = 6). Data are shown as median ± SEM. Dots indicate the cases in each group. Differences were calculated by one-way ANOVA followed by Bonferroni test or Kruskal-Wallis test followed by Dunn test. [a]$P < 0.05$ between control and PCOS; [b]$P < 0.05$ between control and PCOS+M; [c]$P < 0.05$ between PCOS and PCOS+M.

(specificity protein 1) and GKLF (gut-enriched Krüppel:like factor). Moreover, the Chr1-65419662 site of the *LEPR* promoter binds SOX9 (SRY-Box 9 Protein), and the Chr12-1690290 site of the *ADIPOR2* promoter binds E4 F1 (E4F transcription factor 1). In turn, the *AR* promoter has different methylation sites associated with EGR1 (early growth response 1), SET (Suppressor of

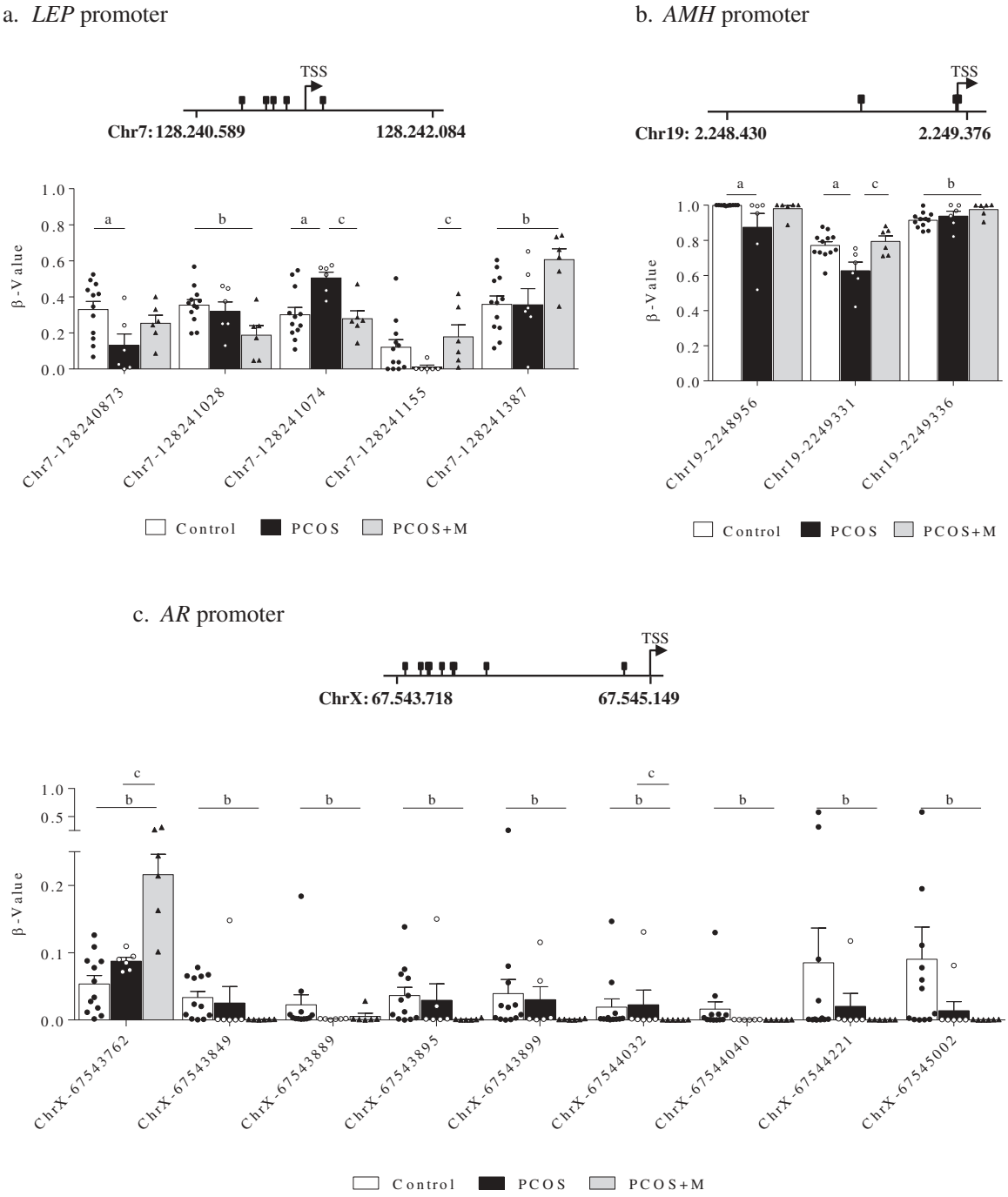

Figure 2. Methylation levels (β-value) in the promoter regions of the leptin (*LEP*) (a), antimüllerian hormone (*AMH*) (b) and androgen receptor (*AR*) genes (c) in sons of control (control, n = 12), PCOS women (PCOS, n = 6) and PCOS women treated with metformin during pregnancy (PCOS+M, n = 6). Data are shown as median ± SEM. Dots indicate the cases in each group. Differences were calculated by one-way ANOVA followed by Bonferroni test or Kruskal-Wallis test followed by Dunn test. [a]$P < 0.05$ between control and PCOS; [b]$P < 0.05$ between control and PCOS+M; [c]$P < 0.05$ between PCOS and PCOS+M.

variegation, Enhancer of Zeste, Trithorax) and MYND (myeloid:Nervy:DEAF1) in the ChrX-67543969 site, whereas the ChrX-67544580 site binds MED1 (mediator of RNA polymerase II transcription subunit 1).

In sons, CpG sites in the *LEP* promoter exhibit binding sites for AP2 (Activator protein 2) in Chr7-128241387 and GCF (GC factor) in Chr7-128241155. The Chr19-2249336 site of the *AMH* promoter can be regulated by CNRE (cAMP

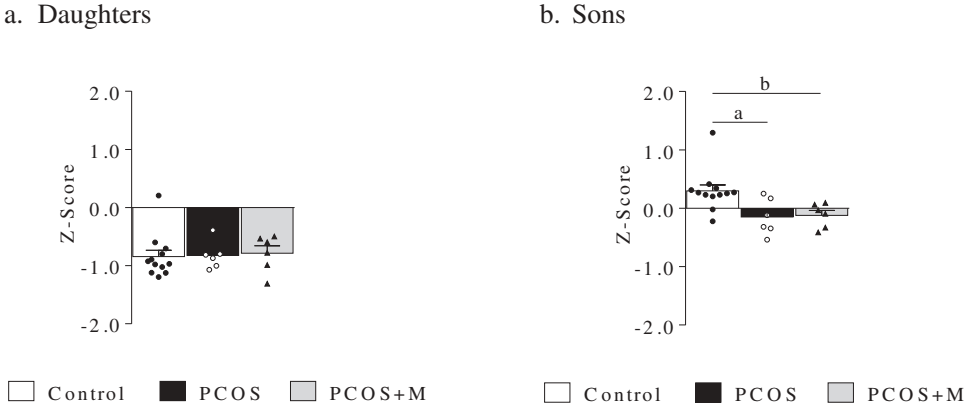

Figure 3. Z-Score of the promoter regions of *LEP, LEPR, ADIPOQ, ADIPOR1, ADIPOR2, AMH* and *AR* in daughters and sons of control (control, n = 12/12), PCOS women (PCOS, n = 6/6) and PCOS women treated with metformin during pregnancy (PCOS+M, n = 6/6). Data are shown as median ± SEM. Dots indicate the cases in each group. Differences were calculated by Kruskal-Wallis test followed by Dunn test. [a]$P < 0.05$ between control and PCOS; [b]$P < 0.05$ between control and PCOS+M.

negative response element). In the *AR* promoter, we found 4 sites that bind transcription factors: the ChrX-67543762 and ChrX-67543895 sites bind E2 F factor, the ChrX-67543849 site binds XRE (xenobiotic responsive element), and EBS1 (Ets binding site 1) and EGR1 are very close to this site. Finally, AP2 alpha and gamma, and CAF1 (chromatin assembly factor complex) are transcription factors associated to the ChrX-67545002 site.

Discussion

Using a targeted NGS approach, we found that the offspring of women with PCOS, at early infancy, show a sex-specific DNA methylation pattern in the promoter regions of genes associated with reproductive and metabolic features of PCOS. Interestingly, metformin treatment during pregnancy in PCOS women normalized the methylation levels in some of these CpG sites, suggesting that the intrauterine environment of PCOS women may confer a different methylation pattern to their offspring compared to children born to women without PCOS.

Both sons and daughters of women with PCOS showed differences in the methylation levels of specific sites in the promoter regions of *LEP* and *AR*. We also observed that daughters of these women had changes in the methylation levels of *LEPR* and *ADIPOR2*. Previous data indicate that deregulation of the expression and secretion of leptin in metabolic diseases is associated with modifications in its promoter methylation [39]. Interestingly, it has been described that DNA methylation of the *LEP* and *ADIPOR2* promoters in cord blood and placenta are associated with maternal and infant perinatal factors [40,41]. In this regard, in placenta of women with PCOS, a reduced gene expression of *LEP* and *LEPR* have been observed, whereas in cord blood, higher circulating levels of leptin have been reported in newborns of women with PCOS [4,42]. Similarly, prenatal androgenization in the sheep model, resembling PCOS pregnancy, produces an increase of the *ADIPOR2* gene expression in fat, muscle and liver of the female offspring at the peripubertal period [43]. It has been observed that the expression of adipokines and their receptors is modulated by the promoter methylation status [44–47], moreover, methylation of these genes has been associated with metabolic alterations and BMI [47,48]. In the same line, we have described that the circulating leptin-adiponectin ratio is associated with metabolic abnormalities in daughters of women with PCOS during the pubertal transition [49]. Therefore, it is possible to suggest that alterations in the leptin-adiponectin system that have been frequently observed at different ages in the offspring of women with PCOS, could be determined by epigenetic modifications that occur during early life.

Same as with the *LEP* promoter, *AR* exhibited a differential pattern of methylation according to sex, indicating that epigenetic modifications could affect the expression of the androgen receptor, and hence, androgen action. As stated before,

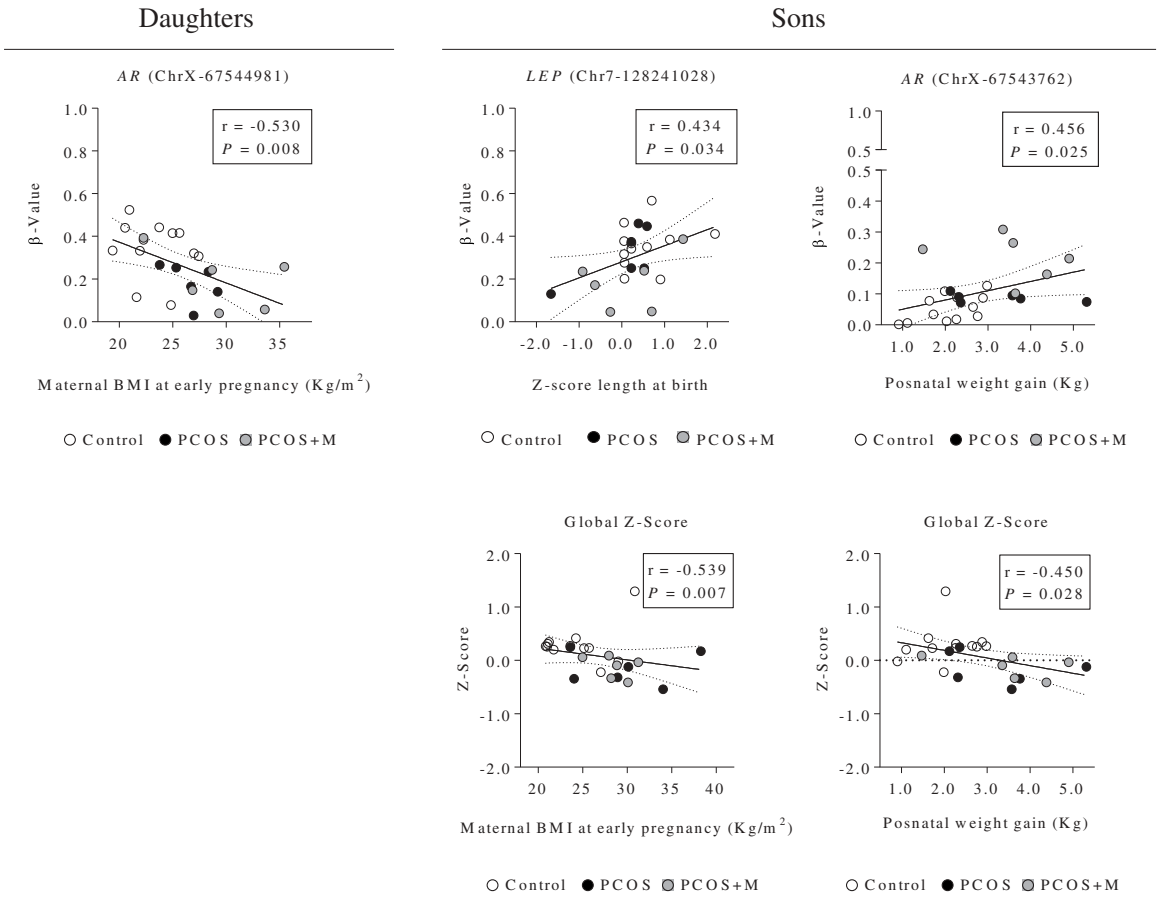

Figure 4. Correlation analysis between the methylation levels (β-value) of the CpG sites of the promoter regions of the *AR* (chromosome X, ChrX), and *LEP* (chromosome 7, Chr7) genes (and Z-Score of the analysed promoter regions), and the maternal characteristics and anthropometric parameters at birth and postnatal age in daughters and sons. Dots indicate the cases in each group. The associations between variables were calculated by Spearman's rank correlation analysis. $P < 0.05$ was considered as significant level.

hyperandrogenism is central in the pathophysiology of PCOS. Therefore, our observations in peripheral leukocytes from PCOS daughters agree with this asseveration because a hypomethylation in the *AR* promoter suggests an increased expression of the *AR*. On the other hand, DNA methylation is also an important epigenetic mechanism involved in the X chromosome inactivation (XCI), where the AR gene is [50]. It has been proposed that a non-random XCI, may contribute significantly to the expression of PCOS [14]. Nevertheless, while this analysis was not considered in the present study, in a previous report we did not find significant differences in the pattern of XCI between daughters of PCOS and control women at 2–3 months of life [51], as has been reported in adult PCOS women [32,52,53]. In addition, in that study we showed that shorter CAG repeats in the *AR*, which favours its activity, are associated with abnormalities in the lipid profile of young daughters of PCOS women [51]. Consistently, an elevated protein and gene expression of the androgen receptor have been found in the ovaries and liver of the female offspring of prenatally androgenized sheep, indicating that the mechanisms that regulate the sensitivity to androgen action are determined during foetal life [54,55]. Moreover, the AR promoter methylation has been linked with BMI and fat mass according to gender [56–58].

One of the most constant features that we have observed in the offspring of PCOS women is high AMH serum concentrations. In the present study only sons of PCOS women showed lower methylation levels in CpG sites of the *AMH* promoter, which is in accordance with the higher serum AMH concentrations described in them during infancy and childhood [59]. On the other hand, we did not observe differences in *AMH* DNA

methylation in girls, which could suggest that the increase in the follicular mass is responsible for this feature more than an increased gene expression. Thus, it seems that, opposite to what was observed in boys, apparently this gene is not modified by epigenetic regulation in girls.

It is currently accepted that an adverse *in utero* environment can influence the establishment of epigenetic marks during foetal development with consequences later in life [60,61]. In this regard, the negative correlation found between maternal BMI and the CpG methylation level in the *AR* promoter in girls and with the global z-score in boys, highlights the importance of the maternal metabolic condition for the acquisition of specific epigenetic marks in their offspring. On the other hand, the relationships found between the methylation levels of the *LEP* and *AR* sites and the global methylation z-score with postnatal anthropometric features in male newborns highlights the effect of these epigenetic mechanisms in postnatal life. Regarding these anthropometric parameters, only postnatal weight gain was different among sons, specifically sons of PCOS women who took metformin during pregnancy gained almost 1.5 kg more than sons born to control women, while the z-score for length at birth was comparable between groups.

Previously, we reported that metformin treatment ameliorates the endocrine and metabolic alterations in women with PCOS during pregnancy resulting in the improvement of ovarian PCOS markers in their female offspring [15]. Along with this, a recent study demonstrated that short-term metformin administration, at therapeutic doses, has a rapid effect on epigenetic regulation in human white blood cells producing both hypo and hypermethylation in the promoters of different genes [62]. In the present study, we observed that metformin treatment during pregnancy reversed the effect of PCOS on the methylation patterns of some CpG sites of the *LEP, ADIPOR2* and had a partial effect on the *AR* promoters in daughters, whereas in sons, it had an effect on the *LEP* and *AMH* promoters. Interestingly, in sons of women with PCOS, in the CpG sites of the *AR* gene, the methylation level was only modified in the metformin group, especially in ChrX-67543762 and ChrX-67544032, which seems to suggest that the PCOS effect was enhanced by metformin treatment. The impact of these findings is unclear as the long-term impact of intrauterine metformin exposure on childhood development is an unanswered question. Studies evaluating children of patients with gestational diabetes exposed to metformin vs. insulin show a neutral effect in body fat, visceral adipose tissue and intrahepatic fat [63]. We observed that sons of women with PCOS treated with metformin gained more weight from birth until 3 months old, which is consistent with previous observations that have shown an increase in BMI and in the prevalence of overweight or obesity in prepubertal PCOS sons exposed to metformin during pregnancy, (4–-7 years old) [64,65]. Nevertheless, it is important to note that in those PCOS women who were medicated with metformin during pregnancy, a large percentage developed GDM probably due to their higher pre-pregnancy metabolic risk, which makes difficult to dissect the effect of metformin from the effect of maternal GDM. In this regard, several studies have established an association between GDM and an altered epigenetic profile in the offspring, finding both increases and decreases of methylation levels depending on the genes studied [66,67]. The significance of these findings in terms of long-term cardiovascular risk is uncertain.

DNA methylation is involved in essential processes that regulate gene expression such as the binding of transcription factors to regulatory elements or direct transcriptional inhibition as in the X chromosome inactivation [68]. DNA methylation has been generally related to gene inactivation and to repression of transcription factor binding ability [69–74]. Therefore, the final effect depends on both mechanisms [24]. The consequences of the inactivation of a gene are relative, since it depends on whether the expression of such a gene is favourable or deleterious for a particular condition. Unfortunately, we were unable to perform gene expression analysis (RNA from blood leucocytes) since the original study was not designed for this purpose. The *LEP* promoter can potentially bind the transcription factors SP1 and GKLF (KLF4), which are important regulators of the leptin gene, adipogenesis and oxidative stress [75–78]. Particularly KLF4 also

promotes macrophage polarization towards an antiinflamatory phenotype (M2) [79]. A study with PCOS women showed activation of KLF-4 after treatment with electroacupunture, associated with epigenetic and transcriptional changes that elicit metabolic improvement [76]. On the other hand, SP1 may also function as a cellular glucose sensor and the effect of its regulation depends on the maturation of the adipocyte [75,78]. Many of these functions have to do with increased leptin transcription. In this context, if the leptin promoter is more methylated, this may indeed inhibit transcription factors binding, avoiding that they exert their action at the promoter level [77,78] and possibly reducing their positive metabolic effects. On the other hand, the transcription factors EGR, MED1, E2 F and XRE have been related to *AR* promoter regulation. In this regard, the overexpression of EGR-1 enhances AR translocation to the nucleus increasing its activity [80] and therefore, could contribute to the hyperandrogenic state; interestingly higher levels of this protein have been observed in obese women with PCOS [81]. In turn, MED1 and E2 F have been described as co-activators of the AR, participating in the regulation of the expression of cell cycle genes [82,83], which could be involved in the proliferation of ovarian granulosa cells, a phenomenon that has been observed in females born to prenatally androgenized sheep and in the offspring of women with PCOS [84,85]. Moreover, in humans, polymorphisms of the XRE gene have been associated with susceptibility to polycystic ovaries [86]. It is likely that lower DNA methylation of the AR promoter may favour DNA binding of EGR, MED1, E2 F and XRE increasing AR transcription, which could worsen hyperandrogenism and its consequences in the PCOS condition. Although, we cannot exclude that that the binding of some of these transcription factors, such as EGR1, to their target sequences may occur independently of the methylation status [87].

The differential methylation pattern observed in the offspring of PCOS women may reflect adaptive changes generated during pregnancy as a result of an altered intrauterine environment, they may be directly inherited from the mother, or result from a combination of both. Further modulation of these processes may occur during postnatal life through environmental exposure to other factors such as hormones, nutrients, lifestyle, etc. which may reverse or worsen these effects, making them susceptible for therapeutic interventions [88–90].

Although it would be ideal to evaluate the target tissue this is not always possible. Thus, peripheral blood leucocytes DNA is a good surrogate. In this regard, a genome-wide epigenetic study has reported high consistency between peripheral blood and ovarian tissue from PCOS women [29,91]. However, Sang et al did not found consistency between the methylation level of peripheral blood leukocytes and tissue regarding the follistatin promoter in endometrial tissue of PCOS women [92]. In turn, *LEP* and *ADIPOQ*, methylation in peripheral leukocytes is correlated with their expression in subcutaneous and visceral adipose tissue [93]. Therefore, whole blood could serve as a useful surrogate measure of the tissue status in terms of epigenetics in the PCOS scenario [94–96]. Another limitation of our study is the small simple size, mainly due to the age of the children studied, the careful selection of PCOS patients and the time required to follow the pregnancies. Finally, although it was not contemplated in this study, it would have been very interesting to have the mothers' DNA to compare the methylation pattern with that of their children, since these data would have enriched the interpretation of the results.

In summary, we observed that both daughters and sons born to women with PCOS have sex-dependent differences in the methylation levels of CpG sites in the promoter regions of metabolic and reproductive genes such as *LEP, LEPR, ADIPOR2, AMH* and *AR*. The intrauterine environment at least in part, mediates these modifications, as treatment with metformin during pregnancy is able to change them. Therefore, our data support the concept that the maternal environment in women with PCOS may induce epigenetic modifications in the DNA methylation profile both in their sons and daughters, which can program the expression of future reproductive and metabolic derangements.

Disclosure statement

Authors have no conflict of interests

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ORCID

Bárbara Echiburú http://orcid.org/0000-0001-6535-3104
Fermín Milagro http://orcid.org/0000-0002-3228-9916
Francisco Pérez-Bravo http://orcid.org/0000-0002-5660-5717
Ana Arpón http://orcid.org/0000-0002-9508-0431
Manuel Maliqueo http://orcid.org/0000-0003-3872-0217

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