REPRODUCTION
REVIEW

DNA methylation in the pathogenesis of polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is the leading endocrine and metabolic disorder in premenopausal women characterized by hyperandrogenism and abnormal development of ovarian follicles. To date, the PCOS etiology remains unclear and has been related to insulin resistance, obesity, type 2 diabetes mellitus, cardiovascular disease and infertility, among other morbidities. Substantial evidence illustrates the impact of genetic, intrauterine and environmental factors on the PCOS etiology. Lately, epigenetic factors have garnered considerable attention in the pathogenesis of PCOS considering that changes in the content of DNA methylation, histone acetylation and noncoding RNAs have been reported in various tissues of women with this disease. DNA methylation is changed in the peripheral and umbilical cord blood, as well as in ovarian and adipose tissue of women with PCOS, suggesting the involvement of this epigenetic modification in the pathogenesis of the disease. Perhaps, these defects in DNA methylation promote the deregulation of genes involved in inflammation, hormone synthesis and signaling and glucose and lipid metabolism. Research on the role of DNA methylation in the pathogenesis of PCOS is just beginning, and several issues await investigation. This review aims to provide an overview of current research focused on DNA methylation and PCOS, as well as discuss the perspectives regarding this topic.

Reproduction (2019) **158** R27–R40

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine and metabolic dysfunction characterized by hyperandrogenism and incomplete development of ovarian follicles that result in an anovulatory state (Dumesic & Richards 2013, Trikudanathan 2015, Patel 2018). Alteration of the hypothalamic–pituitary–ovarian (HPO) axis is one major dysfunction observed in PCOS; this modification is because of an increase in the pulse frequency release of the gonadotropin-releasing hormone (GnRH) from the hypothalamus, which, in turn, favors the synthesis and secretion of luteinizing hormone (LH) instead of follicle-stimulating hormone (FSH) by the pituitary gland. An elevation in serum levels of LH results in a high LH/FSH ratio (higher than 2:1) that promotes androgen production in the ovarian theca cells (Trikudanathan 2015).

Remarkably, exposure to elevated androgens early in life, such as in women with congenital adrenal hyperplasia and prenatally androgenized animals, correlates with a decline in the negative feedback effect of estradiol and progesterone in the GnRH-mediated release of LH, which is a typical characteristic in women with PCOS (Blank *et al.* 2006). A study recently proposed that prenatal exposure to androgens results in aberrant fetal programming in GnRH neurons that impairs their response to the steroid hormones negative feedback in mice (Silva *et al.* 2018).

Substantial evidence demonstrates that fetal programming with prenatal testosterone treatment results in a phenotype similar to PCOS during adulthood in models of sheep and monkeys (Padmanabhan & Veiga-Lopez 2013, Rae *et al.* 2013, Abbott *et al.* 2016, Ramaswamy *et al.* 2016). In addition, prenatal exposure to elevated levels of anti-Müllerian hormone (elevated in pregnant women with PCOS) in pregnant mice programs female offspring to develop a PCOS phenotype during adulthood; however, this functional correlation has not been established in humans (Tata *et al.* 2018).

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-18-0449
Online version via https://rep.bioscientifica.com

Anovulation in women with PCOS is accompanied by menstrual dysfunction such as oligomenorrhea (menstrual periods occurring >35 days apart) or amenorrhea (absence of menstruation for, at least, 3 months), hyperandrogenism (evidenced by hirsutism, acne and alopecia), infertility and metabolic dysfunctions (Trikudanathan 2015, McCartney & Marshall 2016). Recently, the updated diagnosis criteria and the international guidelines for the assessment and management of women with PCOS have been published (Neven *et al.* 2018, Teede *et al.* 2018).

Although PCOS is one of the leading endocrine and metabolic disorders in premenopausal women (range: 6–20% worldwide), its etiology remains unclear to date (Teede *et al.* 2010, Goodarzi *et al.* 2011, Barber *et al.* 2015, Escobar-Morreale 2018*a*). PCOS is a chronic disease that directly affects the quality of life of reproductive age women as it is related to insulin resistance (60–70% of cases), obesity, type 2 diabetes mellitus, cardiovascular disease and mood disorders (primarily depression), among others morbidities and is the leading cause of anovulatory infertility (Brassard *et al.* 2008, Teede *et al.* 2010, Sirmans & Pate 2013, Reyes-Muñoz *et al.* 2016). The ovary is not the only affected tissue in the disease, as women with PCOS also exhibit adipose tissue dysfunction (Cortón *et al.* 2007, Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2008, Martínez-García *et al.* 2013, Montes-Nieto *et al.* 2013), insulin resistance in the skeletal muscle (Silva Dantas *et al.* 2013), elevated serum levels of inflammatory markers (Orio *et al.* 2005, Diamanti-Kandarakis *et al.* 2006*a*, Escobar-Morreale *et al.* 2011) and endometrial alterations (Piltonen 2016). Several studies have demonstrated the impact of genetic and environmental factors on the PCOS etiology (Abbott *et al.* 2005, Diamanti-Kandarakis *et al.* 2006*b*, Azziz *et al.* 2016, Mykhalchenko *et al.* 2017). Lately, epigenetic factors have garnered considerable attention in the study of the PCOS pathogenesis, considering that intrauterine and environmental insults, as well as lifestyle factors, might predispose to the disease development (Escobar-Morreale 2018*b*, Patel 2018, Smyka *et al.* 2018, Tata *et al.* 2018). Some studies have revealed alterations in the content of miRNAs and long noncoding RNAs in tissues affected by PCOS (Sørensen *et al.* 2016, Concha *et al.* 2017, Liu *et al.* 2017, Sagvekar *et al.* 2018). In addition, other studies have indicated that modifications in histone acetylation could be involved in the acquisition of the PCOS phenotype (Nelson-DeGrave *et al.* 2004, Wood *et al.* 2005, Qu *et al.* 2012). In particular, DNA methylation is altered in the peripheral and umbilical cord blood, as well as in ovarian, adipose tissue and skeletal muscle of women with PCOS (Table 1), suggesting the involvement of DNA methylation in the PCOS pathogenesis as evidenced in the functions probably altered by this epigenetic modification (Fig. 1) and because of its participation in the regulation of gene expression and chromatin structure (Andersen & Tost 2018). However, further investigation is warranted to establish the functional role of DNA methylation in patients with PCOS because of a lack of cause and effect studies. Hence, this review aims to provide an overview of current research focused on DNA methylation and PCOS, as well as discuss the perspectives regarding this topic.

DNA methylation in PCOS

DNA methylation is referred as an enzymatic reaction consisting of the addition of a methyl group generally at the carbon in the 5′ position of the pyrimidine ring of a cytosine followed by a guanine, called CpG dinucleotides (Illingworth *et al.* 2008). In addition, DNA methylation is involved in essential processes such as transcriptional regulation, X chromosome inactivation, imprinting, gametogenesis, development and differentiation, among others (Senner 2011). Alterations in DNA methylation are related to the pathogenesis of several diseases such as type 2 diabetes mellitus, neurodegenerative diseases, cardiovascular disease and cancer (Berson *et al.* 2018, Nebbioso *et al.* 2018, Rosa-Garrido *et al.* 2018, Zhou *et al.* 2018).

The interest in the role of DNA methylation in the PCOS pathogenesis began, at least, 10 years ago; however, a growing body of evidence reveals that women with PCOS have an altered epigenetic program due, in part, to this covalent modification (Fig. 1 and Table 1). In addition, alterations in DNA methylation have been noted in the peripheral and umbilical cord blood, suggesting a correlation between the PCOS phenotype and epigenetic changes in cells from systemic and fetal circulation. Remarkably, changes in DNA methylation have also been reported in tissues affected in the disease, including the ovary, adipose tissue and skeletal muscle (Table 1). Table 1 summarizes the studies that report DNA methylation changes related to PCOS, which includes observed changes in the gene expression, ontology terms obtained with the g:Profiler software (Reimand *et al.* 2016), and correlations reported between DNA methylation and clinical or functional findings observed in PCOS. Moreover, recent studies have highlighted the plasticity of DNA methylation observed across age and through therapeutic intervention in animal models and women with PCOS, which, in turn, has been related to phenotypical changes in the tissues affected by the disease (Xu *et al.* 2011, Cui *et al.* 2018, Kokosar *et al.* 2018). The fact that DNA methylation, gene expression and the corresponding phenotype can be modified opens the possibility of therapeutic interventions in PCOS.

Table 1 Differentially methylated genes reported in tissues from patients with PCOS.

Tissue	Genes and their associated changes in DNA methylation in patients with PCOS as compared with healthy women*	Gene expression changes associated with differential DNA methylation	Gene ontology and human phenotype ontology terms associated with differentially methylated genes#	Clinical or functional findings associated with DNA methylation changes	References
Peripheral blood	No change: *SRD5A1*, *CYP11A1*, *RPS4X* and *KCNJ11* Increased: *LY6G6F*, *KCTD21*, *ADCY9*, *RABL2B*, *ZNF611*, *VASH1*, *FST*, *LMNA* and *PPARGC1A* Decreased: *L-1*, *TMSB15B*, *RPF1*, *DNA2*, *EPHA8*, *LHCGR* and *EPHX1* Unspecified: *JAML*, *KBTBD12*, *SLC29A1*, *GPR176*, *MYOZ2*, *PIGT*, *C2CD4B*, *PCDHA7*, *HMGA1* and *PCDH18*	Not reported	Response to gonadotropin and cellular response to gonadotropin stimulus	Increased prolactin and estradiol levels in serum, increased free androgen index, insulin resistance, increased triglyceride levels in plasma and risk for metabolic syndrome	Sang *et al.* (2013, 2014), Shen *et al.* (2013), Ting *et al.* (2013), Wang *et al.* (2014*a*), Li *et al.* (2017), Sagvekar *et al.* (2017), Zhao *et al.* (2017)
Umbilical cord blood	Increased: *PRKN*, *PAX6*, *B4GALT7*, *MEST*, *CACNA2D2*, *RGMA* and *PRDM10* Decreased: *ESR1*, *APP*, *RBPMS LHCGR*, *CASP10*, *SPHK1*, *PCSK6*, *ARHGAP45* and *MIB2*	Not reported	Regulation of cell communication, spontaneous synaptic transmission and cell surface receptor signaling pathway, regulation of I-κB kinase/ NF-κB signaling, regulation of signaling and growth	Not reported	Lambertini *et al.* (2017)
Whole ovarian tissue	Increased: *FBN1*, *NAV2*, *PRDM1*, *RNF213*, *SSBP2*, *TNIK*, *ZFAND3*, *ZNF503*, *SLC2A8*, *NRIP1*, *IGF2BP2*, *CYP19A1*, *AMHR2*, *SNURF*, *SUMO3*, *PNMA6A*, *ADRA1D* and *SCML1* Decreased: *C2CD6*, *NR0B1*, *INSR*, *AMH*, *SPANXD*, *TUBA3E*, *FAM47B*, *MAB21L1* and *RBM3*	Increased: *FBN1*, *NAV2*, *RNF213*, *TNIK* and *ZFAND3* Decreased: *PRDM1*, *SSBP2*, *ZNF503* and *CYP19A1* Increased: *C2CD6* and *NR0B1*	Development of primary sexual characteristics (female gonad development), reproductive system development and Developmental process involved in reproduction (female sex differentiation)	Reduced aromatase activity	Yu *et al.* (2013), Wang *et al.* (2014*b*), Yu *et al.* (2015)
Granulosa cells	No change: *HDAC3*, *SERPINE1*, *SPP1*, *ANGPTL4*, *CYP17A1*, *PEX3*, *DIRAS3*, *PTX3* and *SLC12A8* Increased: *MATN4*, *DLGAP2*, *CDH13*, *GAREM2*, *GSC*, *ANKRD34C*, *ATP8B2* and *PPARG* Decreased: *L-1*, *LHCGR*, *SMG6*, *CCR5*, *LHB*, *NTN1*, *ARFGAP1*, *MDGA1*, *NCOR1*, *YAP1*, *CD9*, *NR4A1*, *EDN2*, *BNIP3* and *LIF*	Not reported Decreased: *PPARG* Increased: *LHCGR*, *NCOR1*, *YAP1*, *CD9*, *NR4A1*, *EDN2*, *LIF* and *BNIP3*	Tube development, hormone-mediated signaling pathway, response to organic substance and cellular response to low-density lipoprotein particle stimulus	Hyperandrogenism (evidenced by the increase in the free androgen index from follicular fluid)	Qu *et al.* (2012), Wang *et al.* (2014*a*), Xu *et al.* (2016), Jiang *et al.* (2017), Sagvekar *et al.* (2017)

(Continued)

Table 1 Continued.

Tissue	Genes and their associated changes in DNA methylation in patients with PCOS as compared with healthy women*	Gene expression changes associated with differential DNA methylation	Gene ontology and human phenotype ontology terms associated with differentially methylated genes#	Clinical or functional findings associated with DNA methylation changes	References
Subcutaneous adipose tissue	Increased: *ZZEF1*, *TPT1*, *STUB1*, *DMAP1*, *RAB5B*, *PPARG*, *SVEP1*, *SAV1*, *RORA*, *RAB6A* and *CNST*	Increased: *STUB1*, *RAB5B*, *SAV1* and *RAB6A* Decreased: *ZZEF1*, *TPT1*, *DMAP1*, *PPARG*, *SVEP1*, *RORA* and *CNST*	Oligomenorrhea	Increased testosterone levels in circulation	Kokosar *et al.* (2016)
	Decreased: *PUM1*, *DIP2C*, *SNX8*, *SRGAP3*, *ZFHX3*, *OR52W1* and *BBX*	Increased: *OR52W1* Decreased: *PUM1*, *DIP2C*, *SNX8*, *SRGAP3*, *ZFHX3* and *BBX*			
Skeletal muscle	Increased: *CST3*, *SPRTN*, *COL1A1*, *SCMH1*, *VAT1*, *CSPP1*, *ERP29*, *ADK* and *KLF10*	Increased: *SPRTN*, *SCMH1*, *CSPP1*, *ADK* and *KLF10* Decreased: *CST3*, *COL1A1*, *VAT1* and *ERP29*	Rhythmic process (regulation of circadian rhythms)	Not reported	Nilsson *et al.* (2018)
	Decreased: *HEATR3*, *HJV*, *MAP2K6* and *FOXO3*	Increased: *HEATR3*, *HJV*, *MAP2K6* and *FOXO3*			

*The top 10 genes with the most significant changes in DNA methylation were included when considering genome-wide DNA methylation studies, as well as those related with the pathogenesis of the disease. Only studies assessing DNA methylation levels in specific loci were included in the list of genes without changes in DNA methylation; #obtained by functional enrichment analysis of the differentially methylated genes included in this table using the web server g:Profiler and considering a corrected *P* value <0.05 (Reimand *et al.* 2016).

DNA methylation in the peripheral and cord blood from women with PCOS

The first study reporting DNA methylation levels in women with PCOS was reported in 2010; the study obtained global levels of methylated DNA from the peripheral blood that were assessed by ELISA and found no significant differences between PCOS women and controls (Xu *et al.* 2010). These findings raised the question of whether changes in DNA methylation were specific to the genomic region and tissue in women with PCOS; however, technical limitations, such as small sample size, could not be eliminated to elucidate these results. Conversely, a decline in the DNA methylation levels of a CpG located at the 5′-untranslated region of long interspersed nucleotide element-1 (L-1) correlated with a global hypomethylated state of the genome in the peripheral blood of women with PCOS compared with controls, as L-1 is a well-known marker for global DNA methylation (Sagvekar *et al.* 2017).

After that, studies based on next-generation sequencing were conducted to assess genome-wide DNA methylation levels in the peripheral blood of women with PCOS, and numerous differentially methylated genes were found when compared with women without the disease. In particular, a total of 40 differentially methylated genes were found between PCOS women and controls using the genome-wide methylated DNA immunoprecipitation (meDIP) analysis, and most detected genes correlated with cancer, immune response, transcription regulation and metabolism (Shen *et al.* 2013). In addition, Shen *et al.* (2013) detected 79 differentially methylated genes when comparing PCOS women with insulin resistance and those without insulin resistance, suggesting that insulin resistance affects the phenotype of women with PCOS, perhaps, via the *CEBPB* gene, as altered DNA methylation of this gene correlated with a deregulation of several genes involved in the modulation of the inflammatory response and metabolism. Remarkably, *CEBPB* encodes a transcription factor that participates in the ovarian follicle development and insulin signaling, both altered in women with PCOS (Yamamoto *et al.* 2002, Huang *et al.* 2007). Another study reported 52 differentially methylated CpGs between women with PCOS and controls using the bisulfite-based genome-wide DNA methylation analysis (Li *et al.* 2017). In this study, different genes with altered DNA methylation were identified in comparison with Shen *et al.* (2013),

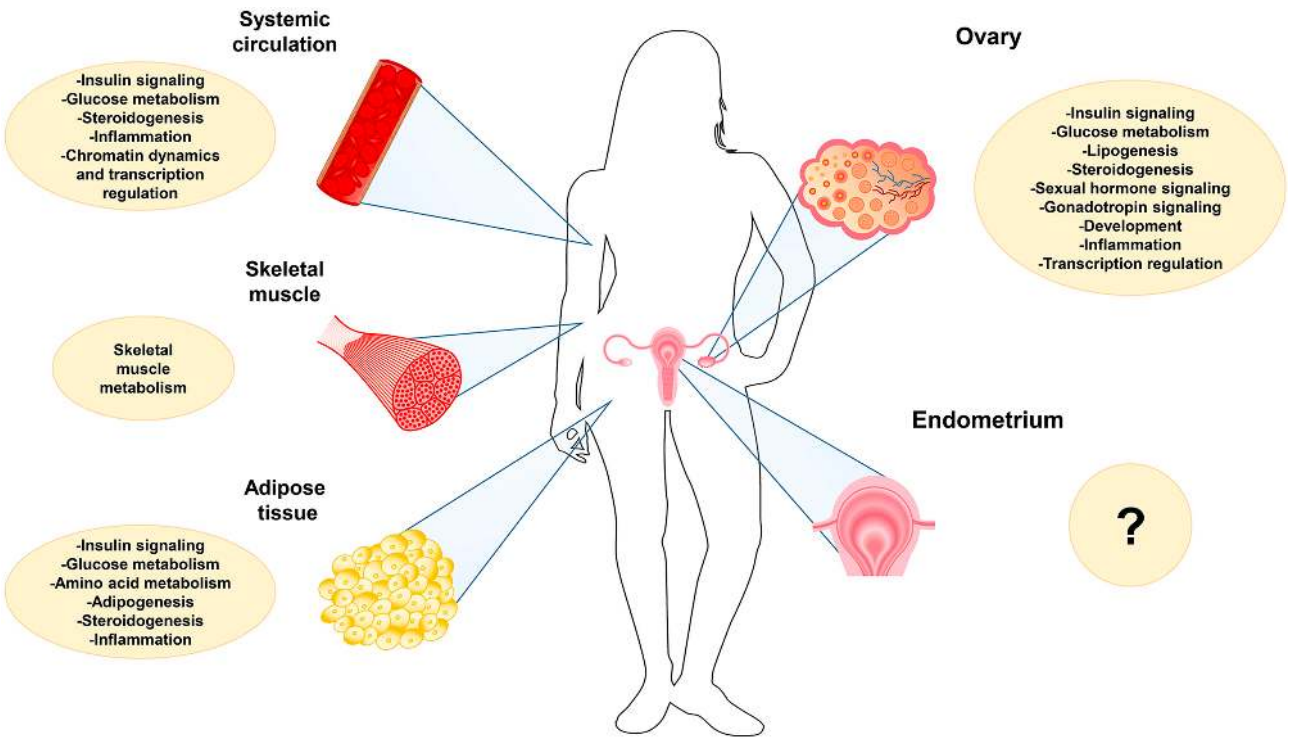

Figure 1 Functions probably related to DNA methylation in different tissues of patients with PCOS.

perhaps, because of differences in the technique used (the meDIP assay assesses relative levels of DNA methylation in genome fragments <200 bp, whereas the techniques based on the conversion with bisulfite, detect changes in DNA methylation in a specific cytosine), sample size, ethnicity and the heterogeneous phenotype of PCOS women; however, genes identified with differential methylation also correlated with inflammation and metabolism (Li *et al.* 2017). Remarkably, Li *et al.* (2017) found a correlation between levels of DNA methylation in specific CpG sites and altered clinical variables in women with PCOS, such as serum levels of estradiol and prolactin, perhaps, because of the HPO axis dysfunction.

Together with the genome-wide DNA methylation studies conducted in the peripheral blood of patients with PCOS, studies focusing on the analysis of the DNA methylation content in specific genes have been conducted. In particular, an elevation of DNA methylation has been observed in the promoter of *FST* (which encodes follistatin), *LMNA* (encodes Lamin A/C) and *PPARGC1A* (encodes the peroxisome proliferator-activated receptor gamma coactivator 1-α), while reduced levels of this epigenetic mark have been reported in the promoter of the *LHCGR* gene (encodes the LH receptor) and *EPHX1* gene (encodes epoxide hydrolase 1) in women with PCOS compared with controls (Sang *et al.* 2013, 2014, Ting *et al.* 2013, Wang *et al.* 2014*a*, Zhao *et al.* 2017). These alterations in DNA methylation correlated with various molecular pathways and physiological processes that are dysregulated in PCOS such as follicular development (Eldar-Geva *et al.* 2001), infertility (Hai *et al.* 2015), steroidogenesis (Korhonen *et al.* 2003), glucose metabolism and insulin signaling (Vandenbeek *et al.* 2018). In addition, DNA methylation defects in *PPARGC1A* have been related to insulin resistance and high serum androgen levels in women with PCOS, as well as with a decline in the mitochondrial DNA content, a well-known marker of metabolic disease when reduced in the peripheral blood (Zhao *et al.* 2017). Furthermore, Lamin A/C, which plays a pivotal role in chromatin dynamics and transcriptional programming during development, should affect the transcriptional program of several genes; thus, future studies should address this interesting issue (Solovei *et al.* 2013).

Intrauterine programming has been proposed as one of the plausible mechanisms that cause PCOS, as evidenced by the high heritability of the disease and animal models demonstrating that prenatal exposure to high levels of androgens or AMH results in the acquisition of the PCOS phenotype in the offspring (Filippou & Homburg 2017, Tata *et al.* 2018). To date, several studies have been conducted in prenatal androgenized sheep and monkeys in which a PCOS phenotype was observed during adulthood, which, in turn, has been related to DNA methylation changes in the case of monkeys (Manikkam *et al.* 2006, Abbott *et al.* 2008, 2017, Veiga-Lopez *et al.* 2008, 2012, Smith *et al.* 2009, Ortega *et al.* 2010, Xu *et al.* 2011, Padmanabhan *et al.* 2014, Puttabyatappa *et al.* 2018). Nevertheless, little

evidence exists about epigenetic alterations that could be related to intrauterine programming of the offspring of patients with PCOS. In particular, a genome-wide DNA methylation study examined the umbilical cord blood from women with PCOS; 614 hypermethylated and 1066 hypomethylated CpGs were reported (associated with 323 hypermethylated genes and 595 hypomethylated genes, respectively) compared with women without the disease (Lambertini *et al.* 2017). Remarkably, most affected genes correlated with glucose and lipid metabolism, hormone-related functions, regulation of inflammation and processes affected in PCOS women, suggesting that intrauterine programming of the PCOS phenotype could be mediated, at least, in part, by DNA methylation (Shen *et al.* 2013, Lambertini *et al.* 2017, Li *et al.* 2017). These findings should be confirmed in larger cohorts and it would be of great interest to follow-up the female progeny to ascertain if they develop PCOS symptoms during the reproductive age.

The limitation of studies assessing DNA methylation (and other epigenetic marks) in the peripheral blood is the fact that the total DNA is isolated from different cell types with their DNA methylation profile, whose content and epigenetic signature vary depending on diverse factors. Thus, the DNA methylation profile obtained is the average of the methylation levels in each cell type and should be deconvoluted to ascertain the epigenetic status of a specific cell type (Titus *et al.* 2017). In addition, the DNA methylation analysis of the peripheral blood does not necessarily reflect the status of specific organs or tissues affected by the disease. However, Li *et al.* (2017) reported that alterations in DNA methylation profiles of the peripheral blood from women with PCOS corroborate those obtained from the ovary tissue, indicating that certain alterations in DNA methylation should be inferred from the peripheral blood, which is of great significance, as this would avoid the invasive procedure of the biopsy. Remarkably, all studies examining the peripheral blood indicate that the PCOS phenotype correlates with an alteration in the DNA methylation content of genes involved in the inflammatory response, glucose and lipid metabolism and hormone signaling, which, in turn, is associated with the presence of a systemic dysfunction characterized by chronic inflammation accompanied by hyperandrogenism and insulin resistance (Shorakae *et al.* 2018). Besides, these alterations in the DNA methylation program could be acquired during fetal development as proposed by Lambertini *et al.* (2017).

DNA methylation in the ovarian tissue and granulosa cells from women with PCOS

Ovulation is one of the primary processes affected by PCOS as alterations in the HPO axis (that increase serum androgens) induce follicular atresia and follicular maturation arrest, which, in turn, cause oligo-ovulation or anovulation (Dumesic *et al.* 2015). In particular, ovarian follicular arrest correlates with a decline in FSH responsiveness of the ovarian tissue (Fauser & Van Heusden 1997). In turn, an increase in LH levels overstimulates theca cells to secrete excessive androgens that impair follicular growth and maturation, a situation that is augmented when combined with the gonadotrophic effect of insulin, resulting in the accumulation of atresic or immature ovarian follicles that cause polycystic ovarian morphology (Nestler *et al.* 1998). These molecular and morphological changes in the ovarian tissue could be attributed to epigenetic alterations as proposed in animal models of PCOS. Predominantly, studies have reported that prenatal testosterone treatment in sheep results in an increase in the occurrence of persistent and growing follicles (contributing to the morphology of a PCO), luteal and antral follicles defects, a decline in primordial follicles and an altered ovarian protein content of AMH, ovarian steroidogenic enzymes, matrix metalloproteases and proteins related to insulin pathway during adulthood (Manikkam *et al.* 2006, Veiga-Lopez *et al.* 2008, 2012, Smith *et al.* 2009, Ortega *et al.* 2010, Padmanabhan *et al.* 2014, Puttabyatappa *et al.* 2018). Remarkably, this fetal programming induced by prenatal testosterone correlates with defects in the expression of ovarian steroidogenic genes and miRNAs associated with insulin pathway in the ovaries of fetal ewes (Luense *et al.* 2011). Reportedly, a decline in DNA methylation levels of the promoter of *Gata6* and *Star* steroidogenic genes occurs in theca cells of prenatal androgenized rats (Salehi Jahromi *et al.* 2018).

DHEA treatment in prepubertal female mice results in a phenotype similar to PCOS. Remarkably, a decline in the total content of DNA methylation and the expression of *Dnmt1* has been observed in oocytes from female mice treated with DHEA (Eini *et al.* 2017). Using a mice model of hyperinsulinemia and hyperandrogenism combined with single-cell technology, DNA methylation changes in single oocytes reportedly associated with several molecular functions such as transcriptional regulation, Notch signaling and cell division (Li *et al.* 2018). In addition, prenatal exposure to androgens in rats produces a phenotype similar to PCOS that arises during adulthood; this PCOS-like phenotype correlates with alterations in the content of DNA methylation in the ovarian tissue, especially in the promoters of genes involved in the development of reproductive organs and hormone-related processes. These findings indicate that ovarian alterations observed in PCOS can be programmed during development (Zhang *et al.* 2014).

The study of DNA methylation in the ovarian tissue of women with PCOS has been conducted using techniques that analyze either the whole genome or a specific locus. Some genome-wide DNA methylation studies have been conducted in the ovarian tissue of women

with PCOS, which differ based on the specifically used technique (Wang *et al.* 2014*b*, Yu *et al.* 2015). In particular, variations in DNA methylation levels of genomic regions related to 342 genes were found in the ovarian tissue of women with PCOS compared with controls using meDIP coupled to microarray analysis (Yu *et al.* 2015). In this study, hypermethylated genomic regions in women with PCOS were preferably distributed on CpG island shores (genomic regions that are 1–2 kb from a CpG island) and promoters with high CpG content, whereas hypomethylated regions were found in gene bodies. Conversely, using the bisulfite-based genome-wide DNA methylation analysis, Wang *et al.* (2014*b*) reported that CpG islands and CpG island shores were hypomethylated in women with PCOS compared with controls. The differences between these two studies could be attributed to the small sample size analyzed in both studies, as well as to the heterogeneity of the disease. In addition, Wang *et al.* (2014*b*) reported a correlation between changes in DNA methylation and mRNA expression in 54 genes of 7929 differentially methylated CpGs, raising the question whether changes in DNA methylation observed in other regions of the genome correlate with the altered gene expression. In these studies, differentially methylated genes between the ovarian tissue from women with PCOS and controls correlated with hormone activity (including *CYP19A1*, *AMH* and *AMHR2* genes that are markedly related to the PCOS pathogenesis), transcriptional regulation, inflammation, glucose metabolism and insulin signaling (Wang *et al.* 2014*b*, Yu *et al.* 2015). Using methylation-specific PCR, an increase in the methylation levels of *CYP19A1* promoter was reported in the ovarian tissue of women with PCOS compared with controls, which correlated with a concomitant reduction in the mRNA and protein content, indicating a correlation between epigenetic alterations and an increase in androgen levels that results in the PCOS phenotype (Yu *et al.* 2013). *CYP19A1* encodes aromatase that converts androgens into estrogens and is expressed in differentiated preovulatory granulosa cells and luteal cells (Stocco 2012). The aromatase activity is decreased in patients with PCOS, perhaps, by an increase of androgens in circulation and follicular fluid (Chen *et al.* 2015, Yang *et al.* 2015). Reportedly, prenatal testosterone treatment downregulates the expression of aromatase in granulosa cells from adult sheep (Padmanabhan *et al.* 2014), indicating that the increase in DNA methylation in *CYP19A1* promoter observed in women with PCOS could be the result of the prenatal exposure to high levels of androgens.

Most studies focusing on DNA methylation and PCOS have been conducted in granulosa cells, which play a fundamental role in steroidogenesis and ovarian folliculogenesis and whose dysfunction correlates with the pathogenesis of the disease (Pellatt *et al.* 2007; Lan *et al.* 2015). Some studies have reported differences in the global content of DNA methylation in granulosa cells of women with PCOS compared with controls (Pruksananonda *et al.* 2016, Xu *et al.* 2016, Sagvekar *et al.* 2017, Pan *et al.* 2018). In particular, a global hypomethylated state of the genome in granulosa cells of women with PCOS (evidenced by a decline in the DNA methylation levels of L-1) correlated with the hormonal alterations of the disease (Sagvekar *et al.* 2017). A genome-wide study revealed that granulosa cells of PCOS women with obesity exhibit variations in the content of DNA methylation compared with PCOS women without obesity (5202 differentially methylated CpGs) and women without the disease (6936 differentially methylated CpGs), albeit the highest difference was observed between PCOS women without obesity and controls (12,245 differentially methylated CpGs), indicating that obesity affects not only the phenotype of women with PCOS but also their epigenetic program (Xu *et al.* 2016). However, no differences were observed in molecular functions related to the differentially methylated genes between the three groups, which correlated with the regulation of transcription and development. Although Xu *et al.* (2016) did not find differences in the global content of 5hmC, changes in specific genomic regions must be assessed to eliminate the participation of this epigenetic mark in the PCOS pathogenesis.

In addition, alterations in DNA methylation have been reported in several genes associated with the ovary function and morphology in granulosa cells of women with PCOS, which, in turn, correlates with the response to gonadotropins, insulin signaling and steroidogenesis (Qu *et al.* 2012, Wang *et al.* 2014*a*, Jiang *et al.* 2017, Pan *et al.* 2018).

Decreased levels of DNA methylation in the *LHCGR* gene promoter and a concomitant increase in the gene expression have been reported in granulosa cells of women with PCOS compared with controls (Wang *et al.* 2014*a*). In addition, *LHCGR* variants have been related to PCOS in genome-wide association studies (GWAS), and its overexpression has been associated with a hypersensitivity to LH impulses in ovarian follicles, which would contribute to the PCOS pathogenesis (Shi *et al.* 2012, Mutharasan *et al.* 2013). Another study analyzing granulosa cells from patients with PCOS reported the increased expression of *LHCGR*, along with an upregulated activity of this receptor (Kanamarlapudi *et al.* 2016). These studies indicate that the reduction in DNA methylation of the *LHCGR* gene promoter should correlate with the overexpression of an active form of LH receptor in granulosa cells that, in turn, results in a hyper-response to gonadotrophins in the ovaries of women with PCOS.

Hyperandrogenism in women with PCOS has been related to a decline in DNA methylation levels of *NCOR1* promoter and with an elevation in those of *PPARG* promoter in granulosa cells, which

corresponded to a higher and lower gene expression, respectively, compared with PCOS women without hyperandrogenism (diagnosed according to the Rotterdam criteria) and women without the disease (Qu *et al.* 2012). *PPARG* encodes PPARG that regulates the ovarian function, whereas *NCOR1* encodes a nuclear corepressor of PPARG that is essential in reproductive functions and hormonal signaling (Gao *et al.* 2006, Toth *et al.* 2007). Thus, Qu *et al.* (2012) demonstrated the correlation between androgen excess and PCOS epigenetic alterations, as alterations in DNA methylation and gene expression of the *PPARG* and *NCOR1* genes in PCOS women with hyperandrogenism were replicated in *in vitro* and animal models. Remarkably, prenatal testosterone treatment induces the PPARG expression during fetal life in sheep ovaries, which correlates with the developmental programming of ovarian dysfunction observed in adult animals; however, no differences were reported in adult animals (Ortega *et al.* 2010). Further studies are warranted to investigate whether DNA methylation is involved in this fetal programming of increased *PPARG* expression mediated by the prenatal testosterone treatment. In addition, a study reported that PPARG agonists and insulin induce the expression of *PPARG*, *STAR*, *INSR* and *IRS-1* in human ovarian cells, indicating the interaction of insulin signaling and steroidogenesis (Seto-Young *et al.* 2007); this also highlights the therapeutic potential of PPARG agonists, as they enhance the ovarian insulin sensitivity and inhibit androgen production, which is altered in women with PCOS (Seto-Young *et al.* 2005). Hence, further studies should be assessed to investigate the effect of PPARG agonists on DNA methylation.

Yes-associated protein-1 (Yap1) regulates the proliferation of granulosa cells, and its hyperactivation by androgens inhibits ovulation in mice (Ji *et al.* 2017). Reportedly, several CpGs in the *YAP1* gene promoter are hypomethylated in granulosa cells of women with PCOS compared with controls (along with an elevation in protein and mRNA levels), perhaps, because of high androgen levels, suggesting that YAP1 hyperactivation might be mediated by DNA methylation (Jiang *et al.* 2017).

In granulosa cells, alterations in promoter DNA methylation and gene expression levels have been reported in several genes that participate in the synthesis of lipids and steroids (*CD9*, *BNIP3*, *EDN2*, *NR4A1* and *LIF*), indicating that epigenetic alterations could correlate with steroidogenesis and metabolic dysfunction observed in PCOS (Pan *et al.* 2018).

Further investigation with higher sample size and a more homogeneous population is warranted in the ovarian tissue of PCOS women to elucidate the regions in the genome that are differentially methylated in the disease. The use of single-cell technology coupled with next-generation sequencing should be applied to determine the alterations in the DNA methylation content of oocytes obtained from women with PCOS, which would exert a marked impact on assisted reproductive technology.

DNA methylation in the adipose tissue and skeletal muscle from women with PCOS

The presence of abdominal obesity and alterations in the adipose tissue in women with PCOS markedly correlates with insulin resistance, which, in turn, promotes the synthesis of androgens in the ovary that worsen metabolic dysfunctions (Delitala *et al.* 2017). Alterations in the morphology and function of the adipose tissue in women with PCOS have been described previously, including increased adipocyte size and reduced adiponectin expression and secretion (Mannerås-Holm *et al.* 2011). Reportedly, hyperinsulinemia promotes testosterone synthesis by inducing the activity of aldo-ketoreductase type 1C3 (that converts androstenedione to testosterone) in subcutaneous adipose tissue, contributing to elevated androgen levels in patients with PCOS (O'Reilly *et al.* 2015). Remarkably, prenatal androgen exposure in rhesus monkey induces modifications in DNA methylation levels in the visceral adipose tissue of infant and adult animals, establishing the impact of prenatal androgen exposure on intrauterine programming of the PCOS phenotype. Remarkably, a different set of genes were differentially methylated between infant and adult animals in this study, highlighting the plasticity of this epigenetic modification across age (Xu *et al.* 2011). These findings suggest that the DNA methylation profile observed in adults should not be the same presented in newborns destined to have adult PCOS, raising the question whether epigenetic alterations and the related increased adiposity are the consequence and not a fundamental feature of the disease.

Using a genome-wide approach, a study demonstrated that 440 CpGs are differentially methylated in the subcutaneous adipose tissue of women with PCOS compared with that of controls, and, notably, changes in DNA methylation in 33 CpG sites corroborated changes in the gene expression (Kokosar *et al.* 2016). In this study, authors inferred the functional role of the variations in DNA methylation at the expression level in the adipose tissue of women with PCOS, which enabled them to construct a complete scenario of the correlations between epigenetic changes and phenotype variables observed in the disease, including hyperandrogenemia, insulin resistance and adipocyte size. In particular, genes that exhibited alterations in DNA methylation and mRNA expression levels are involved in inflammation, adipogenesis, metabolism of sex hormones and glucose and amino acid metabolism, pathways commonly affected in PCOS and adipose tissue dysfunction (Delitala *et al.* 2017, Escobar-Morreale 2018*a*). In addition, Kokosar *et al.* (2016) reported differentially methylated

and expressed genes that were previously identified as candidate genes for PCOS and type 2 diabetes mellitus (*RAB5B*, *PPARG* and *SVEP1*), indicating their association with the disease at genetic and epigenetic levels.

Remarkably, low-frequency electrical stimulation acupuncture induces several changes in DNA methylation and mRNA levels in the adipose tissue of women with PCOS (Kokosar *et al.* 2018). In particular, a single bout of 45-min electroacupuncture promotes changes in the DNA methylation levels of 407 CpGs, which correlates with corresponding changes in the gene expression and glucose uptake in the whole body. This promising therapy induces muscle contractions by activating transcriptional and signaling programs similar to those activated during exercise, promoting the restoration of metabolic dysfunctions in women with PCOS (Stener-Victorin 2013, Benrick *et al.* 2017). These findings have been related to a partial restoration of DNA methylation content and gene expression levels previously associated with metabolic complications of PCOS, insulin resistance and alterations in adipogenesis and inflammation, highlighting the significance of lifestyle intervention in the management of PCOS (Kokosar *et al.* 2018). This study highlights the plasticity of DNA methylation, which can be modified by therapeutic intervention to reestablish the transcriptional program and restore the dysfunction observed in the adipose tissue of women with PCOS. Nevertheless, further studies are warranted to establish whether electroacupuncture therapy will maintain these epigenetic changes when applied regularly, as well as ascertain the duration of those changes. Furthermore, the participation of other epigenetic mechanisms must be investigated to decode the epigenetic program that arbitrates glucose uptake dependent on muscle contraction in women with PCOS.

In addition, further studies are needed to determine whether other therapies that focus on the metabolic improvement of women with PCOS (such as metformin, diet and physical activity) could modulate the content of DNA methylation in the adipose tissue of these women.

Insulin resistance in patients with PCOS correlates with defects in insulin signaling in the skeletal muscle, which, in turn, is considered a risk factor for type 2 diabetes mellitus (Dunaif *et al.* 2001, Li *et al.* 2002, Corbould *et al.* 2005, 2006, Skov *et al.* 2007). Only one recent study investigated DNA methylation in the skeletal muscle of women with PCOS and reported minor statistical differences in the levels of DNA methylation between women with PCOS and those without the disease. However, correlations between DNA methylation and gene expression were established in genes related to the regulation of circadian clock, skeletal muscle metabolism and skeletal muscle homeostasis (Nilsson *et al.* 2018). Nevertheless, more studies are needed to illustrate the functional role of DNA methylation in skeletal muscle insulin resistance. Besides, it is imperative to study other epigenetic mechanisms that could be involved in the disease.

Perspectives and conclusion

Research on the role of DNA methylation in the PCOS pathogenesis has just begun, and although significant progress has been made, several issues merit investigation.

Nevertheless, genome-wide DNA methylation studies indicate that differentially methylated genes in various tissues affected by PCOS are related to inflammation, hormone-related processes and glucose and lipid metabolism. However, more studies with a higher sample size and a more homogeneous population are warranted to attain more consistent results. Extensive evidence has revealed the alteration of DNA methylation in the peripheral blood of women with PCOS; however, no information exists about DNA methylation profiles in specific populations of leucocytes. The study of DNA methylation in specific cell populations from the peripheral blood will provide the molecular basis of chronic inflammation observed in women with PCOS. Remarkably, a higher number of genes with altered content of DNA methylation are detected in specific tissues affected by PCOS than that in the peripheral blood, highlighting the significance of the study of DNA methylation in specific tissues affected by PCOS. In fact, the endometrium is an established tissue affected by PCOS, as several functional markers are deregulated in women with the disease, and although transcriptomic and proteomic studies have been performed, genome-wide DNA methylation studies have not been addressed (Piltonen 2016, Amjadi *et al.* 2018; Fig. 1). In addition, the study of active DNA demethylation intermediates (such as 5-hydroxymethylcytosine) should assess the tissues affected by the disease via genome-wide or gene-specific studies.

A study recently reported that metformin regulates the DNA methylation process by controlling the activity of S-adenosylhomocysteine hydrolase, an enzyme that hydrolyzes S-adenosylhomocysteine, which inhibits the activity of DNMTs (Zhong *et al.* 2017). However, to date, no study has evaluated the impact of metformin on DNA methylation levels in women with PCOS, which is of great significance as metformin is one of the main lines of treatment against PCOS.

Finally, there is a paucity of knowledge about the functional role of DNA methylation changes reported in women with PCOS, as most changes do not have an apparent impact in the gene expression. Thus, more studies must address the significance of DNA methylation changes in the gene expression and chromatin structure in PCOS animal models. Prenatal androgenized animals and next-generation sequencing technologies could elucidate fetal programming of PCOS such as those

related to the onset of an epigenetic signature in the tissues affected by the disease. Furthermore, the advances in dCas9 technology will offer a more reliable tool to investigate the functional effects of specific changes in DNA methylation that result in a PCOS phenotype (Lei *et al.* 2017).

The alteration of DNA methylation in women with PCOS correlates with systemic and tissue-specific dysfunctions, which, in turn, are related to hyperandrogenism and insulin resistance; these defects in DNA methylation promote the deregulation of genes involved in inflammation, hormone-related processes and glucose and lipid metabolism. A growing body of evidence suggests that these alterations can be acquired during development, indicating a vital role in intrauterine programming. Nevertheless, more research is warranted to elucidate the role of DNA methylation in the PCOS pathogenesis; however, recent studies have provided crucial advances that raise the question about its participation in the development of this disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported by the Fondo Sectorial de Investigación en Salud y Seguridad Social (FOSISS) from the Consejo Nacional de Ciencia y Tecnología (CONACyT; grant number 272682) and the Instituto Nacional de Perinatología 'Isidro Espinosa de los Reyes' (INPer; grant number 569, 212250-3000-20209-04-16). Y I G V was supported by a master's scholarship from the CONACyT (scholarship number 711002).

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Received 24 August 2018
First decision 27 September 2018
Revised manuscript received 29 March 2019
Accepted 8 April 2019