LINE1 CpG-DNA Hypomethylation in Granulosa Cells and Blood Leukocytes Is Associated With PCOS and Related Traits

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Context: Altered global DNA methylation is indicative of epigenomic instability concerning chronic diseases. Investigating its incidence and association with polycystic ovary syndrome (PCOS) is essential to understand the etiopathogenesis of this disorder.

Objectives: We assessed global DNA methylation differences in peripheral blood leukocytes (PBLs) and cumulus granulosa cells (CGCs) of controls and women with PCOS; and their association with PCOS and its traits.

Design, Setting, Participants, Main Outcome Measure: This study included a total of 102 controls and women with PCOS. Forty-one women undergoing controlled ovarian hyperstimulation (COH) and 61 women not undergoing COH were recruited from *in vitro* fertilization (IVF) and infertility clinics. DNA methylation was measured by ELISA for 5′-methyl-cytosine content and bisulfite sequencing of 5′-untranslated region (5′-UTR) of long interspersed nucleotide element-1 (LINE1/L1).

Results: Total 5′-methyl-cytosine and L1 methylation levels in PBLs and CGCs were similar between controls and women with PCOS. Methylation assessed at CpG sites of L1 5′-UTR revealed a single CpG-site (CpG-4) to be consistently hypomethylated in PBLs of both PCOS groups and CGCs of stimulated PCOS group. In unstimulated women, hypomethylation at CpG-4 was strongly associated with PCOS susceptibility, whereas in stimulated group it showed strong associations with PCOS and its hormonal traits. Furthermore, CGCs demonstrated consistent global and CpG-DNA hypomethylation relative to PBLs, irrespective of normal or disease states.

Conclusion: Our study revealed strong association of single hypomethylated CpG-site with PCOS. Identification and characterization of more such methyl-CpG signatures in repetitive elements in larger study populations would provide valuable epigenetic insights into PCOS. ***(J Clin Endocrinol Metab* 102: 1396–1405, 2017)**

Polycystic ovary syndrome (PCOS), an endocrinopathy of unknown pathophysiologic origins, affects 6% to 15% of women in the reproductive age and is a major cause of anovulatory infertility (1). It is a multifactorial, heterogeneous condition that is clinically characterized by oligomenorrhea and/or amenorrhea, hyperandrogenemia

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Abbreviations: AMH, anti-Mullerian hormone; BS-PCR, bisulfite PCR; C-CC, cumulus granulosa cells of controls; CGC, cumulus granulosa cell; COH, controlled ovarian hyperstimulation; d-OPU, day of ovum pick-up; E_2, estradiol; FAI, free androgen index; FF, follicular fluid; HA, hyperandrogenic; IVF, *in vitro* fertilization; L1/LINE1, long interspersed nucleotide element-1; MII, matured metaphase II; NHA, nonhyperandrogenic; ORF, open reading frame; P-CC, cumulus granulosa cells of women with PCOS; P_4, progesterone; PBL, peripheral blood leukocyte; PCOS, polycystic ovary syndrome; ROF, rate of fertilization; SC, stimulated control; SP, stimulated women with polycystic ovary syndrome; TT, total testosterone; UC, unstimulated control; UP, unstimulated all-polycystic ovary syndrome; UTR, untranslated region.

and multicystic ovaries on ultrasound. These features typically manifest along with insulin resistance and compensatory hyperinsulinemia. There is increased GnRH pulsatility and LH hypersecretion, which in combination with elevated insulin, augments ovarian androgen synthesis. Additionally, insulin resistance increases susceptibility to metabolic defects such as glucose intolerance, type-2 diabetes mellitus, dyslipidemia, hypertension and cardiovascular disease (2, 3). Thus, hyperandrogenemia and hyperinsulinemia perpetrate a vicious cycle of ovarian, endocrine and metabolic defects underpinning PCOS.

Emerging reports on the ambiguity of PCOS origins have implicated the involvement of epigenetic alterations in its etiopathogenesis (4). Epigenetic components translate environmental cues into molecular signals by altering DNA methylation at CpG sites and via post-translational histone modifications, thereby introducing transient or permanent changes in gene expression (5). According to Barker's hypothesis on the developmental origins of health and disease, discrepancies in epigenetic regulation of the genome can be broadly explained by intrauterine or environmental fluctuations in gestational and postnatal periods (6). Studies on prenatally androgenized and estrogenized animal models and animals exposed to endocrine disruptors have demonstrated the development of PCOS-like phenotypes in adult stages (7, 8). Insulin excess and hyperandrogenemia have also been strongly proposed to instigate the onset and progression of PCOS via altered DNA methylation (9–11).

Altered global DNA methylation or hypomethylation of transposons and repeat elements, such as long interspersed nucleotide element-1 (LINE1/L1) and *Alu* elements, is indicative of genomic instability characteristic to disease states (12, 13). L1s are a class of autonomously replicating transposons that occupy up to 17% of the human genome and control the mobility of *Alus* through 2 L1-encoded proteins, *i.e.*, open reading frame (ORF)1p and ORF2p (14). Intact L1s (6 kb) contain a CpG-dense 5′-UTR region (Supplemental Fig. 1) harboring promoters that regulate the expression of these proteins. Hypomethylation of L1 5′-UTR is largely responsible for active transcription of these ORF transcripts, which not only increase L1 transposition but also mediate stochastic *trans*-insertions of "processed pseudogenes," (responsible for accumulation of frame-shift mutations and premature stop codons in the genome) and retrogenes (14). Hence, evaluating the differences in methylation across these elements is imperative to the understanding of complex chronic disorders like PCOS.

Several studies have exploited L1 as a surrogate marker for global DNA methylation across several cells or tissues (15–17). Peripheral blood leukocytes (PBLs), due to their relative accessibility and ease of sampling have been extensively used in the assessment of epigenetic differences in cross-sectional and prospective cohort studies. However, PBLs may not necessarily reflect the disease-linked epigenetic outcomes addressed by tissue-specific studies. In PCOS, ovaries are the primary target sites of dysgenesis and show marked impairment in molecular mechanisms governing the dynamics of follicular development, ovulation, and steroidogenesis. However, reports on altered global DNA methylation in ovarian cells and tissues of women with PCOS are limited. So far, only the pioneering studies by Xu *et al.* (18), which used an ELISA-based approach have shown no global methylation differences between PBLs of controls and women with PCOS. Differences in L1 methylation profiles investigated in PCOS-related comorbidities such as obesity, cardiovascular disease, hypertension, and type-2 diabetes mellitus have shown positive association with these risk factors (15, 19, 20). Therefore, we hypothesized that L1 methylation differences in PBLs and ovarian cells may serve as prognostic indicators of PCOS. In this study, we have investigated the global and L1 DNA methylation patterns of PBLs and cumulus granulosa cells (CGCs) in controls and women with PCOS; and studied their association with PCOS risk and its characteristic traits.

Materials and Methods

Study design, participants, and sample collection

This study was carried out at the National Institute for Research in Reproductive Health (NIRRH), Mumbai, India, according to the institututional ethical guidelines. Our study population was comprised of 102 participants recruited as 2 independent groups, each including women with PCOS diagnosed by Rotterdam consensus criteria (21) and age- and body mass index–matched healthy controls. The first group, comprising of 61 women who were not undergoing controlled ovarian hyperstimulation (COH) (non-COH/unstimulated group), was recruited from a larger cohort of couples visiting the Infertility Clinic at NIRRH. This group included 20 unstimulated controls (UC) and 41 women with PCOS (UP). The control group consisted of women whose spouses were diagnosed with male factor infertility and who had regular menses, normal ovaries on ultrasound, and no signs of hyperandrogenism. Women with a history of pelvic inflammatory disorders or surgeries or abnormal menses or who were undergoing treatment for hormonal abnormalities or infertility were excluded. The UP category was further subgrouped into hyperandrogenic (HA) (n = 20) and nonhyperandrogenic (NHA) PCOS (n = 21) based on free androgen index (FAI) levels (>3.21 for HA), to evaluate the effect of hyperandrogenemia on global methylation. The cutoff limits for these indices were based on the 80th percentiles of controls. Fasting blood samples were collected from all 61 women during the early follicular phase (days 3 to 7) of menstrual cycles. The second group consisted of 21 controls and 20 women with PCOS who underwent controlled ovarian hyperstimulation (COH/stimulated group) for IVF/intracytoplasmic sperm injection using a long protocol (see Supplemental Methods) at the Fertility Clinic and IVF Center, Mumbai. Controls were healthy, regularly menstruating women

undergoing IVF due to male factor infertility, recruited as per the previous criteria. Women with PCOS were recruited as per the Rotterdam criteria. Fasting blood samples were collected from all 41 women in this group on the day of oocyte pick-up (d-OPU) to obtain serum and PBLs from stimulated controls (SC) and stimulated women with PCOS (SP) after COH. Follicular fluid (FF) along with CGCs of controls (C-CC) and women with PCOS (P-CC), which were stripped off from the cumulus-oocyte complexes, were collected on the d-OPU. The numbers of preovulatory follicles, total retrieved oocytes, metaphase II (MII) oocytes, and fertilized MII oocytes were obtained from clinical records, and rates of fertilization (ROFs) of MII-oocytes were calculated. Written consent was obtained from participants, and their anthropometric characteristics were recorded.

Biochemical and hormonal assays

Serum from women in the non-COH group was assayed for basal/early follicular phase levels of LH, FSH total testosterone (TT), SHBG fasting glucose and fasting insulin. Indices of androgen excess (*i.e.*, free testosterone, bioavailable testosterone, and FAI) and of insulin resistance *i.e.*, homeostatic model assessment of insulin resistance (HOMA-IR) were calculated as reported elsewhere (22, 23). For the COH group, fasting serum and FF samples collected on d-OPU were assessed for SHBG, estradiol (E_2), progesterone (P_4), and TT using commercially available ELISA kits (Diagnostics Biochem Canada Inc., Dorchester, Ontario, Canada) and androgen excess indices were calculated. Basal levels of FSH, LH, TSH, anti-Mullerian hormone (AMH), and prolactin, as well as E_2 and P_4 levels measured before and after administration of human chorionic gonadotropin (hCG) during IVF, were obtained from clinical records. Follicular phase levels of testosterone and insulin were not available in the records as they are not routinely measured before IVF. Moreover, because the stimulated group could be recruited only on d-OPU, we had no access to their follicular phase blood samples before IVF. Therefore, basal androgen–related and insulin-related parameters could not be compared between the COH study groups.

DNA extraction and purification

Genomic DNA from PBLs (n = 102) was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA from CGCs (n = 41) was extracted using the Nucleospin Triprep kit (Macherey-Nagel, Düren, Germany). Quantification and quality assessment of extracted DNA was carried out using nanodrop and by agarose gel electrophoresis.

ELISA for quantification of 5′-methyl-cytosine content in DNA

Global DNA methylation levels were estimated in duplicate in PBLs and CGCs by ELISA using the MDQ1 Imprint Methylated DNA Quantification Kit (Sigma-Aldrich, St. Louis, MO), with few modifications in the manufacturer's protocol (see Supplemental Methods).

Bisulfite conversion and PCR of L1 5′-UTR

One microgram of DNA from PBLs and 500 ng from CGCs was used for bisulfite conversion using the MethylCode bisulfite conversion kit (MECOV-50; Invitrogen, Carlsbad, CA). Bisulfite PCRs (BS-PCRs) were performed using 2 primer sets that amplified the bisulfite-converted L1 5′-UTR as 2 overlapping amplicons, spanning a total of 34 CpG sites in this region (847 bp). Wild-type PCR primers with sequences nearly synonymous to bisulfite primers were used for determining the quality and efficiency of bisulfite reactions. The primer designing and PCR details are outlined in the Supplemental Methods.

Bisulfite sequencing of L1 5′-UTR and CpG site methylation analysis

The BS-PCR products were purified using QIAquick gel extraction kits (Qiagen) and subjected to direct sequencing (Supplemental Methods). Percent methylation levels at individual CpG sites were determined as described elsewhere (24, 25). Individual CpG-site methylation levels were measured using Sequencing Analysis Software v5.4 (Applied Biosystems). Alignment and quality control of bisulfite sequences was evaluated using BiQ Analyzer (26).

Statistical evaluation

Mann-Whitney *U* and Kruskal-Wallis tests were used for univariate analyses of continuous variables between 2 or multiple groups. Association of CpG-site methylation levels with hormonal and oocyte-related parameters were determined using nonparametric measures (*i.e.*, 2-tailed Spearman's correlation constants) followed by linear regression analyses. Association between CpG-site methylation levels and PCOS susceptibility was evaluated by bivariate logistic regression. For all analyses involving CpG sites, multiple testing corrections were applied by controlling the false discovery rate (FDR) at 5% (FDR < 0.05), as described by Benjamini and Hochberg (27). Age and body mass index were used as covariates for all association analyses. Details of the statistical tests are outlined in the Supplemental Methods.

Results

Clinical and biochemical characteristics of study participants

The clinical, hormonal, and biochemical characteristics of women in the non-COH and COH groups are outlined in Tables 1 and 2, respectively. In the non-COH group, basal LH levels, LH/FSH ratio, TT, and androgen-excess indices (free testosterone, bioavailable testosterone, and FAI) were significantly high in all-PCOS women (UP) and in the HA-PCOS subgroup compared to controls and in the HA-PCOS subgroup compared with the NHA subgroup. In the COH group, basal FSH levels were low whereas the LH/FSH ratios, LH and AMH levels were significantly high in women with PCOS (Table 2). Androgen-excess indices were measured in d-OPU serum and FF along with E_2 and P_4 levels (Supplemental Table 1). E_2, TT, and androgen-excess indices were significantly high and SHBG levels were low in serum and FF of women with PCOS compared to controls. P_4 levels were similar in serum but were higher in FF of women with PCOS compared with controls.

Global DNA methylation assessment by ELISA and L1 bisulfite sequencing

Global DNA methylation was assessed by ELISA for 5′-methyl-cytosine levels. Total L1 methylation was

Table 1. Baseline Characteristics of Study Participants Not Undergoing Controlled Ovarian Hyperstimulation (Non-COH Group)

Characteristics	Control (UC) (n = 20), Median (IQR)	PCOS (UP) (n = 41), Median (IQR)	PCOS (NHA) (n = 21), Median (IQR)	PCOS (HA) (n = 20), Median (IQR)	*P* value			
					UC vs UP	UC vs NHA	UC vs HA	NHA vs HA
Age, y	27.0 (23.50–32.0)	27 (22.0–29.0)	27 (23.5–29.0)	25 (21.0–28.0)	0.104	0.387	0.051	0.189
BMI, kg/m^2	22.72 (19.12–25.20)	23.04 (20.18–26.38)	22.82 (20.8–25.52)	23.82 (20.23–28.98)	0.411	0.886	0.199	0.192
Fasting glucose, mg/dL	91.0 (85.25–95.75)	90.0 (86.0–94.5)	90.0 (85.0–95.5)	89.0 (86.25–94.0)	0.818	0.886	0.807	0.979
Fasting insulin, μIU/mL	9.75 (7.49–11.22)	11.0 (7.9–14.5)	9.85 (7.9–14.0)	11.88 (6.03–14.69)	0.197	0.383	0.185	0.683
HOMA-IR	2.23 (1.73–2.36)	2.63 (1.85–3.13)	2.24 (1.85–2.88)	2.65 (1.36–3.19)	0.141	0.353	0.114	0.622
FSH, μU/mL	6.55 (5.44–8.63)	6.27 (5.35–7.35)	6.64 (5.78–8.38)	5.66 (4.48–7.01)	0.499	0.611	0.083	0.057
LH, μU/mL	5.45 (3.78–7.0)	5.02 (4.81–10.31)	5.71 (4.23–8.91)	9.9 (5.54–20.58)	0.032	0.411	0.0036	0.0164
LH/FSH ratio	0.73 (0.52–1.10)	1.12 (0.77–2.02)	0.97 (0.58–1.17)	1.74 (1.12–3.05)	0.0096	0.328	0.0004	0.0013
TT, ng/dL	29.25 (19.3–38.0)	54.0 (27.5–80.35)	28.0 (20.0–48.7)	79.35 (56.63–97.5)	0.0014	0.638	<0.0001	<0.0001
SHBG, nmol/L	59.5 (50.0–107.5)	41.0 (29.88–66.0)	52.5 (39.75–104.0)	35.5 (25.63–41.38)	0.0036	0.657	<0.0001	0.0004
Free-T, pmol/L	12.3 (8.1–15.58)	24.4 (13.75–46.9)	14.2 (9.71–17.25)	46.9 (39.28–64.95)	0.0006	0.549	<0.0001	<0.0001
Bio-T, nmol/L	0.3 (0.2–0.39)	0.59 (0.32–1.14)	0.33 (0.23–0.41)	1.18 (0.93–1.52)	0.0022	0.896	<0.0001	<0.0001
FAI	1.51 (1.09–2.03)	4.07 (1.76–7.98)	1.76 (1.41–2.59)	7.98 (6.1–12.33)	0.0002	0.167	<0.0001	<0.0001
Menstrual characteristics, n (%)								
Regular cycle	20 (100)	10 (24.4)	8 (38.1)	2 (10)	<0.0001	0.0001	<0.0001	0.042
Oligomenorrhea	0 (0)	22 (53.7)	11 (52.4)	11(55)				
Secondary amenorrhea	0 (0)	9 (22)	2 (9.52)	7 (5)				

Data are represented as median (interquartile range) for anthropometric and hormonal characteristics and comparison was done using Mann-Whitney *U* tests. Menstrual characteristics were assessed by χ^2 analysis. *P* values < 0.05 are considered significant (Mann-Whitney *U* and χ^2 tests).

Abbreviations: BMI, body mass index; Bio-T, bioavailable testosterone; Free-T, free testosterone.

determined by calculating the mean methylation levels for individual L1 CpG sites in each sample and by representing their averages as group methylation levels. In the non-COH group, global methylation levels in PBLs were similar between UC and UP categories (ELISA, $P = 0.26$; L1 sequencing, $P = 0.95$) and between UC, HA, and NHA categories (ELISA, $P^k = 0.4$; L1 sequencing, $P^k = 0.99$, where P^k represents the Kruskal-Wallis statistic) [Fig. 1(A)]. Similarly in the COH group, no difference was observed in methylation levels of PBLs (SC vs SP) or CGCs (C-CC vs P-CC) between controls and women with PCOS [Fig. 1(B)]. Interestingly, we observed tissue-specific hypomethylation in CGCs relative to PBLs in controls (C-CC vs SC: L1 sequencing; $P < 0.0001$) [Fig. 2(A) and 2(B)(i)] and also in women with PCOS (P-CC vs SP: ELISA, $P = 0.042$; L1 sequencing, $P = 0.002$) [Fig. 2(A) and 2(B)(ii)]. This hypomethylation persisted in CGCs even when pooling the data of all CGCs (C-CC + P-CC) in

Table 2. Baseline Characteristics and Parameters Assessed Before and After Initiation of COH in Study Participants Undergoing IVF (COH Group)

Anthropometric and Baseline Characteristics Recorded in Early Follicular Phase Before Initiation of COH				Hormones and Oocyte Parameters Recorded After Initiation of COH			
Parameters Assessed	Control (n = 21), Median (IQR)	PCOS (n = 20), Median (IQR)	*P* Value	Parameters Assessed	Control (n = 21), Median (IQR)	PCOS (n = 20), Median (IQR)	*P* Value
Age, y	27.0 (24.0–30.5)	29.5 (26.25–31.0)	0.14	Total recombinant FSH (rFSH) administered (IU)	1960 (1685–2500)	1880 (1371–2263)	0.40
BMI, kg/m^2	23.5 (22.65–25.13)	24.77 (22.15–25.77)	0.53	E_2 (pg/mL) before hCG administration	1696 (1281–2692)	3086 (1451–3273)	0.08
Basal FSH levels, μU/mL	6.98 (5.09–8.45)	5.06 (3.78–5.49)	0.004	E_2 (pg/mL) on day of hCG administration	2654 (1617–3780)	4025 (2850–4462)	0.045
Basal LH levels, μU/mL	3.63 (2.96–6.13)	9.12 (6.18–14.5)	0.0006	P_4 (ng/mL) before hCG administration	0.57 (0.3–1.0)	0.36 (0.2–0.62)	0.12
Basal LH/FSH ratio	0.66 (0.42–0.83)	1.89 (1.38–3.19)	<0.0001	P_4 (ng/mL) on day of hCG administration	3.6 (2.33–6.63)	4.88 (2.75–6.85)	0.48
Prolactin, ng/mL	17.52 (13.65–20.42)	19.0 (13.73–23.38)	0.47	Preovulatory follicles (n)	21.0 (17.0–28.0)	16.5 (12.25–27.5)	0.18
TSH, mIU/mL	1.70 (1.07–2.39)	1.75 (1.35–2.86)	0.49	Mature or MII oocytes (n)	16.0 (11.5–19.5)	15.5 (8.5–19.0)	0.73
AMH, ng/mL	3.94 (2.31–5.86)	6.78 (3.63–11.25)	0.019	MII oocytes (%)	85.19 (70.59–94.43)	88.56 (73.77–90.23)	0.99
Menstrual characteristics, n (%)							
Regular cycle	21 (100)	3 (15)		Fertilized MII oocytes (n)	14.0 (7.5–17.0)	11.5 (6.25–15.75)	0.54
Oligomenorrhea	0 (0)	11 (55)	<0.0001	% Fertilized MII oocytes (% ROF)	83.33 (67.5–100.0)	72.38 (62.26–82.6)	0.045
Secondary amenorrhea	0 (0)	6 (30)					

Data are represented as median (interquartile range) for anthropometric, hormonal and oocyte related characteristics. Comparison was done using Mann Whitney *U* tests. Menstrual characteristics were assessed by χ^2 analysis. *P* values <0.05 are considered significant (Mann-Whitney *U* and χ^2 tests).

Abbreviations: BMI, body mass index; ROF, rate of fertilization.

comparison with all PBLs (SC + SP) (ELISA, $P = 0.013$; L1 sequencing, $P < 0.0001$) [Fig. 2(A) and 2(B)(iii)].

Individual CpG-site methylation assessment in L1 5′-UTR

Of the 34 CpG sites spanned by the 2 BS-PCR primer sets, we could analyze methylation at 22 sites after sequencing. In the non-COH group, PBLs of UP, NHA and HA-PCOS showed consistent hypomethylation at a single CpG site (CpG-4) in relation to UC subjects after Mann-Whitney *U* tests, whereas CpG-635 was hypomethylated only in the NHA-PCOS subgroup. However, at FDR < 0.05, none of these sites remained significant. Similarly in the COH group, CpG-4 showed hypomethylation in PBLs (SP) and CGCs (P-CC) of PCOS compared with controls (SC and C-CC, respectively) on performing Mann-Whitney *U* tests but not after adjusting the FDR. Additionally, CpG-138, CpG-464, CpG-580, CpG-611, and CpG-620, which also initially showed differential methylation in P-CCs relative to C-CCs, did not show significance at FDR < 0.05.

We evaluated the tissue-specific methylation status of ovarian cells (CGCs) in relation to the PBLs of controls, PBLs of women with PCOS and PBLs of controls and women with PCOS pooled together. At FDR < 0.05, CGCs of controls (C-CCs) and women with PCOS (P-CCs) revealed differential methylation in a total of 10 and 9 CpG sites, respectively, compared to their PBL counterparts SC and SP [Fig. 2(C)(i) and (ii)]. Of these, 5 CpG sites, CpG-414, CpG-460, CpG-464, CpG-588, and CpG-620) [Fig. 2(C)] showed hypomethylation in CGCs compared to PBLs, irrespective of their control or PCOS status and were therefore identified as ovary-specific, hypomethylated marks. On comparing the pooled CGC population (C-CC + P-CC) with PBLs pooled from controls and women with PCOS (SC + SP), we detected 4 additional hypomethylated CpG sites (CpG-191, CpG-234, CpG-536, and CpG-585) at the ovarian level [Fig. 2(C)(iii)].

Evaluation of the relationship between differential methylation of L1 CpG sites and:

PCOS susceptibility and its related traits

CpG-4 showed consistent hypomethylation in the PBLs and CGCs of women with PCOS. Therefore, we evaluated its relationship with risk of PCOS development even though it did not reach significance at FDR < 0.05 (Table 3). Hypomethylation at CpG-4 in PBLs was associated with increased risk of PCOS in UP, HA, and NHA categories of the non-COH group and in PBLs and CGCs of the COH group (Table 4). However, no association was observed with any of the hormonal or biochemical traits related to PCOS in the non-COH group (Supplemental Table 2). In the COH group, hypomethylation at CpG-4 in PBLs showed a strong association with androgen-excess indices in d-OPU serum (Supplemental Table 3). Association of status of CpG-4 methylation in CGCs was evaluated with hormones measured in serum as well as FF since FF is composed of both plasma derived factors and secretions of follicular cells. In CGCs, hypomethylation of CpG-4 again showed strong association with androgen-excess indices in d-OPU serum and with low SHBG in FF. High levels of E_2 and P_4 in serum were also strongly associated with decreased methylation at this site.

Percentage of total retrieved MII oocytes and their ROFs

Of all the oocyte parameters assessed in COH group (Table 2), only the ROFs of oocytes were found to be low in PCOS. Hypomethylation at CpG-4 showed no association with ROFs of MII oocytes [$P = 0.166$; $R^2 = 0.007$; $\beta = (+)\ 0.233$; 95% confidence interval, -0.243 to 1.36].

Discussion

Global DNA methylation is altered in several disease states and is highly affected by environmental and lifestyle factors (28). Because circumstantial evidence supporting the developmental origins of PCOS implicate the involvement of epigenetics in its pathophysiology, we investigated the global methylation changes in ovarian tissues and PBLs of women with PCOS. As the procurement of ovarian biopsies poses ethical and statistical limitations, we have used CGCs of women undergoing IVF as a subset of ovarian cells. Tissue-specific epigenetic studies provide a direct link to extrapolate the effects of localized molecular aberrations that sire developmental or *de novo* reprogramming of germline and somatic cells in disease states (29). However, PBLs have also been widely used as noninvasive biological samples to detect global methylation differences. In PCOS, a pilot study of PBLs by Xu *et al.* (18) demonstrated no change in global DNA methylation levels compared with controls, using only ELISA to quantify these changes. Although this ELISA-based method can effectively detect a total change in 5′-methyl-cytosine methylation levels, the results rely heavily on the specificity of methylation capture antibodies. On the contrary, bisulfite sequencing generates locus-specific information at a single-base resolution and is more specific to ELISA. We have used both these strategies for extensive analysis of methylation levels in PBLs and CGCs. CGCs are largely homogeneous compared with PBLs and play a vital role in ovarian functions, such as relay of signals from mural granulosa cells to

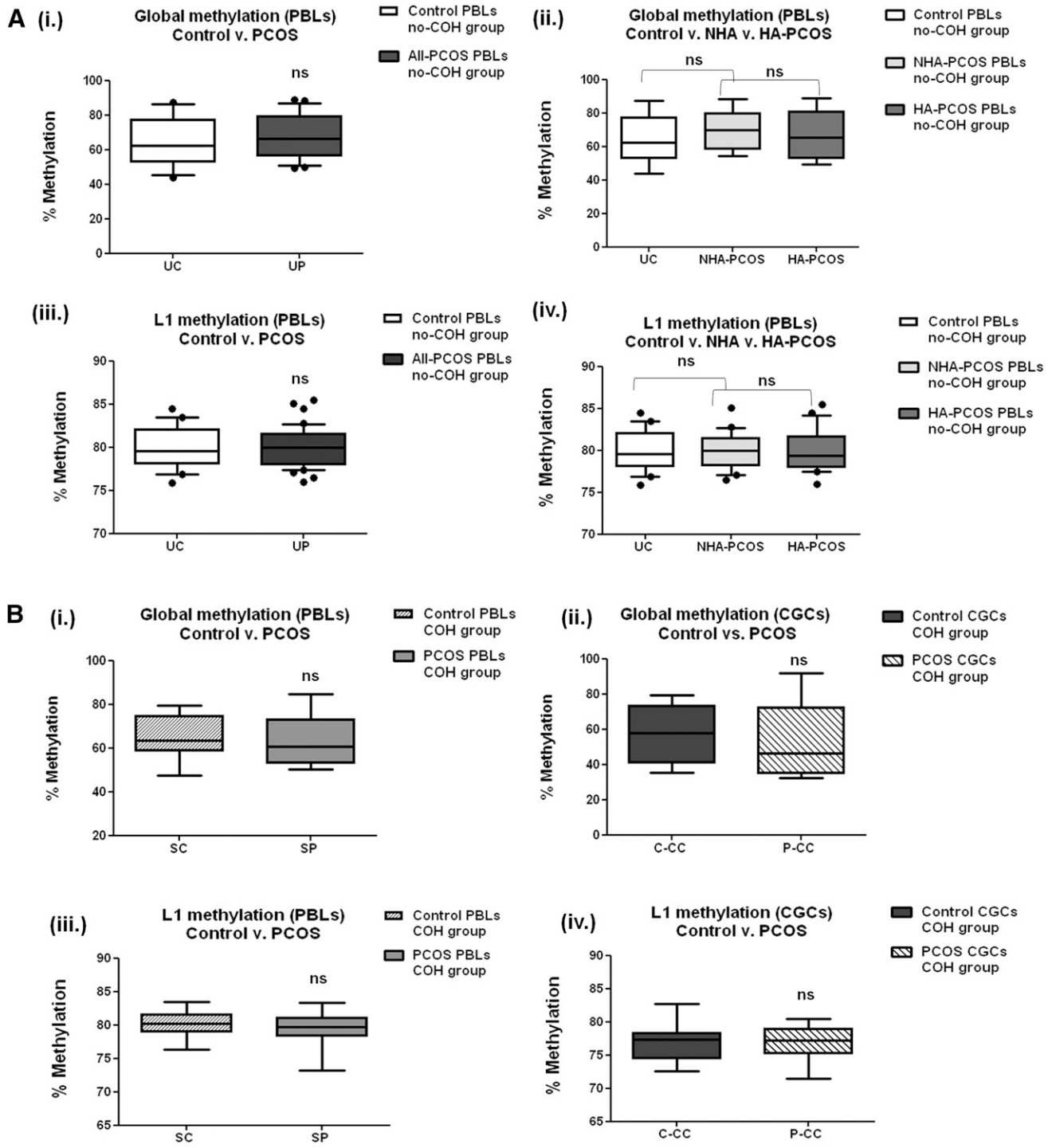

Figure 1. Comparative analyses of global DNA methylation levels between controls and women with PCOS in non-COH and COH groups. (A) Percent global methylation levels measured in PBLs of non-COH group by ELISA and L1 bisulfite sequencing between UC and UP categories [(i) and (iii)] and between UC, NHA, and HA PCOS categories [(ii) and (iv)]. (B) Percent global methylation levels in the COH group measured in PBLs [(i) and (iii)] and in CGCs [(ii) and (iv)] by ELISA and bisulfite sequencing of L1 5′-UTR, respectively. Data for each group are represented as medians ± SD for all box-and-whisker plots. Mann-Whitney *U* tests were performed for evaluation of statistical significance between 2 groups, and Kruskal Wallis tests were used for comparison between multiple groups. ns, nonsignificant ($P > 0.05$).

maturing oocytes on LH surge and facilitation of oocyte developmental competence (30). Thus, CGCs, which can be easily procured during IVF cycles are highly suited as candidate cells for tissue-specific methylation studies in PCOS.

Our results demonstrated no change in global DNA methylation levels between controls and women with PCOS in both non-COH and COH groups, which is in agreement with Xu *et al* (18). We therefore extended our search to identify the differentially methylated CpG sites

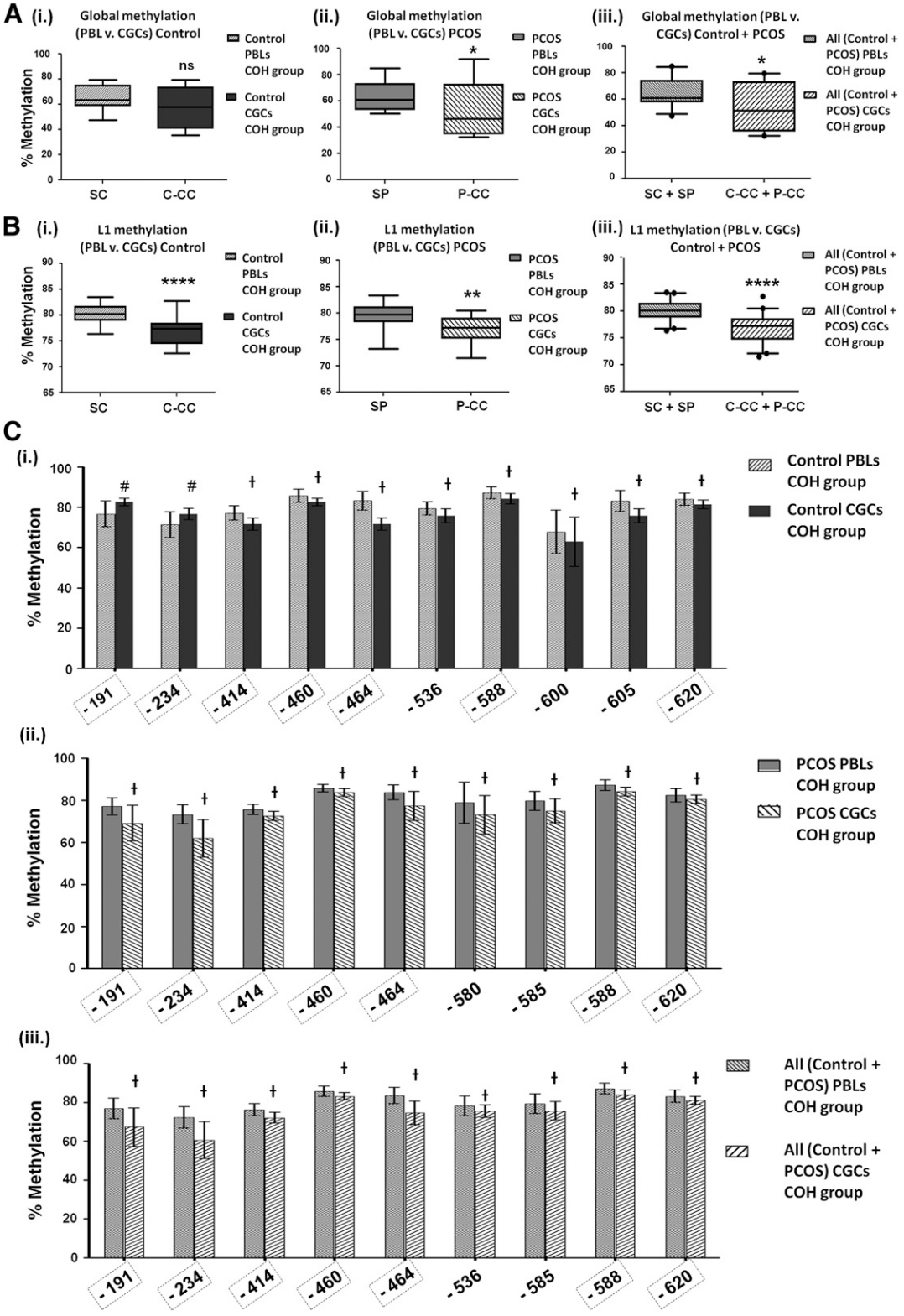

Figure 2. Comparative analyses of global DNA methylation levels between PBLs and CGCs of women in the COH group (ovary-specific methylation). (A) Percent global DNA methylation levels measured by ELISA for 5′-methyl-cytosine content showing (i) no significant difference in C-CCs compared with their PBL counterparts (SC), and (ii) hypomethylation in P-CCs compared with their PBL counterparts (SP) and (iii) hypomethylation in pooled CGCs (C-CC + P-CC) compared with pooled PBLs (SC + SP) of both categories. (B) Average L1 methylation levels measured by bisulfite sequencing of the L1 5′-UTR region showing hypomethylation in (i) C-CCs compared with SC, (ii) P-CCs compared with SP, and (iii) C-CC + P-CC compared with SC + SP categories. (C) Representative bar diagram showing CGC-specific, differentially methylated CpG sites in L1 5′-UTR with respect to PBLs (i) SC vs C-CC, (ii) SP vs P-CC, and (iii) SC + SP vs C-CC + P-CC. CpG sites highlighted by broken boxes (CpG-191, CpG-234, CpG-414, CpG-460, CpG-464, CpG-588, and CpG-620) showed differential methylation in all 3 CGC categories and were interpreted as ovary-specific marks. Data for each group are represented as medians ± SD for all box-and-whisker plots and as means ± SD for

Table 3. L1 CpG-Site Methylation Levels in PBLs and CGCs of Women With PCOS Compared with Controls (Non-COH and COH Groups)

Percent Methylation in Non-COH/Unstimulated Group (PBLs)				Percent Methylation in COH/Stimulated Group (PBLs)			
CpG Site	**Median (IQR)**	**Median (IQR)**	***P* Value**	**CpG Site**	**Median (IQR)**	**Median (IQR)**	***P* Value**
CpG-4	(UC, n = 21)	(UP, n = 41)		CpG-4	(SC, n = 21)	(SP, n = 20)	
	88.71 (80.62–92.55)	80.43 (75.34–87.71)	0.007		86.96 (81.44–91.71)	79.51 (77.50–85.23)	0.019
CpG-4	(UC, n = 21)	(HA, n = 20)					
	88.71 (80.62–92.55)	81.71 (74.70–83.69)	0.038		**Percent methylation in COH/stimulated group (CGCs)**		
					(C-CC, n = 21)	(P-CC, n = 20)	
CpG-4	(UC, n = 21)	(NHA, n = 21)		CpG-4	85.22 (79.69–91.81)	79.22 (77.05–83.84)	0.006
	88.71 (80.62–92.55)	79.73 (77.07–81.82)	0.029	CpG-138	86.31 (79.83–88.81)	88.92 (90.77–86.62)	0.029
				CpG-464	76.26 (74.86–78.19)	77.7 (77.16–78.93)	0.029
CpG-635	(UC, n = 21)	(NHA, n = 21)		CpG-580	81.23 (79.37–82.15)	77.89 (64.26–81.17)	0.005
	78.85 (76.53–82.20)	75.95 (74.15–79.70)	0.048	CpG-611	70.46 (68.70–73.61)	67.3 (66.35–70.58)	0.019
				CpG-620	81.44 (80.67–82.63)	80.52 (79.44–81.83)	0.018

Data for each group are represented as median (interquartile range). After adjusting for multiple testing corrections at FDR < 0.05 using Benjamini-Hochberg method, none of the *P* values (Mann-Whitney *U* test) was significant.

in L1 elements in PCOS. Hypomethylation at individual CpG sites in transposons and repeat elements has been frequently associated with disease states (31–33). Interestingly, of the 22 CpGs analyzed, a single CpG site at position -4 relative to the L1 TSS showed a consistent pattern of hypomethylation in PBLs and CGCs of women with PCOS. Single CpG sites are being increasingly used as surrogate predictors of methylation and gene expression patterns in adjacent genes (34, 35). Thus, with a proximity to TSS and a consistent pattern of hypomethylation in both PCOS groups, CpG-4 can be further explored as a putative, epigenetic target in these women.

Because PCOS is predominantly a state of hormonal imbalance, we investigated the association of altered L1-CpG methylation in both cell types with hormonal features of PCOS. Androgen excess has been implicated as 1 of the principal factors that could trigger the onset of PCOS by propagating epimutations (7–10). In the stimulated group, hypomethylation at CpG-4 showed strong association with hyperandrogenemia, whereas in the unstimulated group there was no such association in UP or even the HA-PCOS subcategory. However, there was no clear explanation for the disparity observed in these association results in the 2 study groups. Additionally, insulin and the ovarian steroids E_2 and P_4, which generally show increased tendencies in PCOS, were explored as modulators of L1 methylation. Although insulin indices showed no association with CpG-4, their possible role in altering L1 methylation can be explored in larger sample sets. Further, the association of elevated E_2 and P_4 levels with hypomethylation of CpG-4, as seen in women with PCOS in the stimulated group, suggests that multiple hormonal factors rather than any single factor influence the L1 CpG-site methylation patterns.

In addition to prospective studies on differential DNA methylation in diseased cohorts and comparative methylation studies in case-control set-ups, the detection of tissue-specific methylation has been recognized as an effective strategy to identify disease-linked genes (36). Several research groups have resorted to studies that combine the analysis of disease-associated and tissue-specific differentially methylated regions, often comparing the methylation profiles of PBLs with tissues that are severely affected in a disease condition. Although our study did not investigate such multiple loci in PCOS, we have attempted the assessment of ovary-specific methylation in whole DNA and L1 elements, which constitute a major bulk of the human genome. A salient finding of our study was the state of global hypomethylation of CGCs relative to their PBL counterparts, which was also affirmed by sequence-based validation of L1 5′-UTR. These observations prompted us to speculate that hypomethylation of CGCs may be crucial in maintaining a relatively more "open chromatin" structure to enable expression of tissue-specific gene sets governing complex ovarian functions. However, our limited sample sizes may have hindered the identification of several disease-linked methyl-CpG marks in PCOS, which otherwise showed consistent hypomethylation at the ovarian level (e.g., CpG-464, CpG-580, and CpG-620).

Figure 2. (Continued). bar graphs. ns, denotes no significance between compared groups. *$P < 0.05$, **$P < 0.01$, ***$0.001 > P > 0.0001$, and ****$P < 0.0001$ (Mann-Whitney *U* tests). CpG-sites that were significant (Mann-Whitney *U* tests) after adjustment for multiple testing corrections at FDR < 0.05 (Benjamini-Hochberg method) are shown. [#]Hypermethylation; [†]hypomethylation. Median (interquartile range) values and *P* values at FDR < 0.05 for (i), (ii), and (iii) are provided in Supplemental Table 4.

Table 4. Association of CpG-4 Methylation Levels with PCOS Susceptibility in PBLs and CGCs of Women in Non-COH and COH Groups

	Association with PCOS, R^2 (direction) β	Odds Ratio	P^a
Non-COH Group			
PBLs (UC + UP)	0.376 (–) 1.560	0.210	0.006
PBLs (UC + HA)	0.538 (–) 2.00	0.135	0.006
PBLs (UC + NHA)	0.358 (–) 1.911	0.148	0.037
COH group			
PBLs (SC + SP)	0.353 (–) 1.531	0.216	0.009
CGCs (C-CC + P-CC)	0.367 (–) 1.685	0.185	0.008

Association of CpG-site methylation status with PCOS susceptibility was analyzed by logistic regression. The coefficients of determination (R^2), beta coefficients (β) after adjustment for age and body mass index, and odds ratios are indicated. The directions of associations (beta coefficients) are given in parentheses.

[a] Age- and BMI-adjusted *P* values for logistic regression. All values were significant after adjusting for multiple testing corrections at FDR < 0.05 using Benjamini-Hochberg method.

We believe that identification of such CpG signatures in larger sample sets would provide valuable information on patho-mechanisms at the ovarian level in PCOS.

ROFs of oocytes have been reported to be low in women with PCOS compared with healthy control subjects (37, 38). Likewise, our study showed reduced ROFs in women with PCOS, although this parameter showed no correlation with CpG-4 methylation status. Large-scale studies are needed to provide clearer insights on the plausibility of altered L1 methylation affecting oocyte and embryo-quality outcomes prior to IVF.

Although we successfully identified a single disease associated CpG mark and ovary-specific methyl-CpG differences in L1 elements of women with PCOS, our study has several limitations. Detection of global methylation differences and differentially methylated sites between CGCs of control sunjects and women with PCOS may have been limited due to the relatively small study groups. This drawback was mainly encountered due to unwillingness of several subjects to give consent, the quantity and quality of collected samples, and loss of samples due to logistical issues. A *post hoc* analysis calculating the statistical power and sample size of our study groups has been provided, giving an estimate of the required population size (Supplemental Table 5). However, recruitment of enough subjects for each group would pose practical limitations. Further, 12 out of 34 CpG sites spanned by the bisulfite primers could not be analyzed after sequencing.

Several groups have focused on epigenetics to explain the pathophysiology of PCOS at tissue- or cell-specific levels using array-based and epigenome-wide analysis tools. Such studies have been mainly carried out in Chinese populations (39–41). Our findings on global DNA methylation studies in the ovary emphasize the need to conduct large-scale, epigenome-wide association studies in combination with gene expression studies at the ovarian level. With ethnicity playing a major role in the phenotypic variability that prevails in PCOS, it is important that research groups across the globe conduct similar studies to explain these differences.

In this study, we explored epigenetic changes in Indian women with PCOS. We believe that our preliminary findings lay an essential groundwork to support whole methylome and candidate-gene–based methylation studies in this population and contribute to understanding the role of plausible epigenetic factors associated with reproductive outcomes in PCOS.

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