Study of Association of Global Deoxyribonucleic Acid Methylation in Women with Polycystic Ovary Syndrome

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Background: Polycystic ovary syndrome (PCOS), a common endocrine disorder affecting 5%-10% of reproductive age women worldwide, associated with various metabolic morbidities. One potential molecular mechanism could be epigenetic modifications, such as deoxyribonucleic acid (DNA) methylation.

Aims: The aim is to determine the association of global DNA methylation in peripheral blood leucocyte (PBL) cells and PCOS women. Also to assess abnormal lipid profile, insulin resistance, gonadotropins and reproductive markers in them.

Settings and Design: The study design involves a hospital-based prospective case–control study.

Materials and Methods: Fifty women with PCOS, diagnosed as per Rotterdam criteria and the rest 50 without PCOS or any disease, attending outpatient department were recruited. Serum biochemical markers and Global DNA methylation assay were done by using standardised kit.

Statistical Analysis Used: Data were compared using Independent t-test or Mann–Whitney U test using IBM SPSS version 26.0. P < 0.05 was considered statistically significant.

Results: Majority, 72% of PCOS and 82% non-PCOS women were between 20 and 25 years. Most common presenting symptom was menstrual irregularity. Women with PCOS have high serum cholesterol and triglyceride level, elevated serum luteinising hormone (LH), follicle-stimulating hormone (FSH), LH/FSH ratio and testosterone but low estradiol levels as compared to non-PCOS. Statistically significant high mean Global DNA methylation percentage was found in PBLs of women with PCOS.

Conclusion: Despite study limitations, this study provided insight into Global DNA methylation in PBLs was associated with PCOS. It requires further research to better understand the influence of epigenetic factors including genome-wide DNA methylation profiling in PCOS development.

Keywords: Epigenetic, global deoxyribonucleic acid methylation, polycystic ovary syndrome

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5%-10% of reproductive-age women worldwide.[1] Women with PCOS demonstrate major metabolic morbidities such as obesity, insulin resistance, hyperinsulinaemia, type 2 diabetes, dyslipidaemia, cardiovascular diseases, hypertension and ultimately metabolic syndrome in later lives. It is a leading cause of anovulatory infertility,[2] manifests with a combination of menstrual dysfunction and hyperandrogenism in the adolescent population.[3] There is a complex interaction between genetics and environmental factors. Emerging evidence
supports epigenetic modifications which regulate gene expression without changing the nucleotide sequence of the gene playing a role in driving both the metabolic and reproductive features of PCOS. Deoxyribonucleic acid (DNA) methylation and histone modifications are the most studied epigenetic mechanisms.

DNA methylation is a process in which cytosine is converted to 5-methyl cytosine due to the binding of a methyl unit to the 5’ position in presence of the DNA methyltransferase enzyme. It can occur in the regulatory region of the gene (promoter), intergenic regions and repetitive elements, which regulates gene transcription and expression, by modulating methyl-specific-binding proteins that recruit enzymatic machinery capable of locally altering histone modification. Cytosine bases that are followed by guanidine (CpG) are usually located in the promoter region and in general, it is believed that these CpG regions are protected from methylation. DNA methylation can cause changes in chromatin structure, genomic integrity, stability and the way DNA interacts with proteins, thereby controlling gene expression.\(^4\)

Studies have found that changes in DNA methylation play an important role in transcriptional regulation, X chromosome inactivation, embryonic development, cell differentiation and are closely related to the pathogenesis of many chronic diseases, such as type 2 diabetes, cancer, cardiovascular diseases.\(^5\)\(^-\)\(^7\)

Multiple techniques have been developed to measure DNA methylation: Global methylation, genome-wide loci methylation and single-specific gene methylation. Global methylation status refers to non-sequence dependent measurement of methyl-cytosine content. It does not establish what loci or genes may be affected but can reflect environmental factors. Genome-wide DNA methylation analysis screens all loci or genes across the whole genome, does not give an indication of the precise location of the CpG methylation nor which nucleotide in the loci is methylated or unmethylated. Gene-specific DNA methylation establishes the methylation of individual CpGs at a locus of interest as this is important when evaluating the impact to gene expression and phenotype.\(^8\)

Global methylation status in peripheral blood leukocytes (PBLs), have been extensively used in the assessment of epigenetic differences, due to their relative accessibility and ease of sampling. However, the study of DNA methylation in PBLs may not reflect the disease-linked epigenetic outcomes addressed by tissue-specific studies. Inappropriate epigenetic programming facilitates the environmental regulation of the gene expression and marked impairment in molecular mechanisms governing the dynamics of follicular development, ovulation and steroidogenesis, as in PCOS, ovaries are the primary target sites of dysgenesis.\(^9\)

However, reports on altered global DNA methylation in ovarian cells and tissues of women with PCOS are limited. So far, only the pioneering study by Xu et al.\(^10\) which has investigated global DNA methylation PBLs in PCOS and found no unique differences between women with and without PCOS. Liu et al.\(^11\) found that numerous important genes are revealed with altered DNA methylation levels that may affect mRNA expression which may provide novel insights into the pathogenesis of PCOS. However, large gaps in knowledge remain as to how epigenetic modifications relate to PCOS and the metabolic, endocrine and reproductive sequelae. Global DNA methylation analysis in PBLs could potentially be useful in finding novel molecular pathways that are associated with PCOS and can be used as a biomarker.

Hence, the primary aim of our study was to investigate and compare global DNA methylation percentage in PBL cells of women with and without PCOS and the association of methylation profiles with biomarkers of key clinical features of PCOS in the Indian context.

The secondary aim was to assess the prevalence and association of abnormal lipid profile, Insulin resistance, gonadotropins and reproductive markers in women with PCOS.

**Materials and Methods**

A prospective case–control study was conducted, comprising 100 women from patients attending our outpatient department of Obstetrics and Gynaecology (O and G). Of these, 50 were cases diagnosed with PCOS according to Rotterdam criteria and the remaining 50 were controls not having any feature of PCOS. The case selection was by using systematic random sampling, every fifth PCOS patient was selected as a study participant and a control participant was selected on the same day. Written, informed consent was obtained from all. The study adhered to the Principles of the Helsinki Declaration (2013). No sample size calculation was performed.

- The study was conducted for 24 months from October 2018 to September 2020 in the Department of O and G, along with molecular genomics and
research laboratory, Department of Biochemistry of our institution.

This study has been approved by our Institutional Ethics Committee, with Ethics Committee Regd. No. ECR/84/Inst/OR/2013/RR-20, vide IEC application No.-195, Dated 05.05.2020.

Inclusion criteria for cases

- Women diagnosed with PCOS according to Rotterdam criteria which required a minimum of two of the following three criteria.
  i. Polycystic ovarian morphology with ultrasound (USG)-verified polycystic ovaries with 12 or more 2–9 mm follicles and/or ovarian volume ≥10 ml in one or both ovaries
  ii. Chronic anovulation: Oligomenorrhea was defined as an intermenstrual interval >45 days and <8 menstrual bleeding in the past year. Amenorrhea was defined as <3 menstruations per year
  iii. Hyperandrogenism: Diagnosed clinically by the presence of excessive acne, androgenic alopecia, or hirsutism; or chemically, by high serum levels of total, bioavailable or free testosterone or dehydroepiandrosterone sulfate.

Exclusion criteria for cases

- Smokers or with alcohol addiction
- The onset of menarche <3 years
- >45 years or postmenopausal
- Any chronic morbid disease.
- Pharmacological treatment within 12 weeks
- Lactating mother.

Inclusion criteria for controls

- Women without any features of PCOS
- No chronic disease.

Exclusion criteria for controls

- Evidence of PCOS according to Rotterdam criteria.

After obtaining the written consent from patients, required data were collected using in-person interviews, including socio-demographic characteristics (age, race/ethnicity and education), height, weight, physical activity and family history of chronic hypertension or diabetes, any past disease. Detailed menstrual history was taken and transvaginal USG was performed. Venous blood was collected in clot activator vials for serum biochemical analysis, in ethylene diamine tetraacetic acid (EDTA) vials for molecular techniques and in oxo-fluoride vials for plasma glucose. They were processed for haemoglobin, packed cell volume, random blood sugar, renal and liver function, thyroid profile, luteinising hormone (LH), follicle-stimulating hormone (FSH) and testosterone in the post-graduate Department of Biochemistry. Tests were done using standard commercial kits and plasma glucose levels were estimated using the Glucose oxidase peroxidase method, adapted to autoanalyser. Serum electrolytes were done by the Ion selective electrode method.

| Table 1: General characteristics of study and control groups |
|-----------------------------------------------------------|
| **Baseline characteristics** | **Mean±SD** | **P** |
| **PCOS (n=50)** | **Non-PCOS (n=50)** | |
| Height (cm) | 152.92±3.74 | 153.64±3.24 | 0.307 |
| Weight (kg) | 59.90±5.27 | 54.30±4.52 | 4.5 |
| BMI (kg/m²) | 24.91±1.98 | 23.02±2.07 | 0.001** |

SD=Standard deviation, PCOS=Polycystic ovary syndrome, BMI=Body mass index, *=Statistically significant

| Table 2: Biochemical parameters of study and control groups |
|-----------------------------------------------------------|
| **Biochemical parameters** | **Mean±SD** | **P** |
| **PCOS (n=50)** | **Non-PCOS (n=50)** | |
| Serum lipid profile (mg/dl) | | |
| Cholesterol | 213.16±41.26 | 188.08±31.14 | 0.001** |
| HDL | 52.08±7.33 | 50.54±5.01 | 0.224* |
| LDL | 85.28±20.06 | 91.26±19.48 | 0.427* |
| TG | 108.04±37.06 | 87.91±26.71 | 0.049** |
| Serum GnRH levels (IU/ml) | | |
| LH | 17.94±5.00 | 10.76±2.88 | 0.001** |
| FSH | 8.46±2.63 | 7.08±1.75 | 0.007** |
| LH/FSH | 2.25±0.69 | 1.55±0.40 | 0.001** |
| Serum reproductive markers | | |
| Estradiol (pg/ml) | 64.18±26.85 | 91.22±14.82 | 0.001** |
| Progesterone (ng/ml) | 10.59±3.27 | 8.77±5.18 | 0.159** |
| Testosterone (ng/ml) | 78.88±27.19 | 43.74±15.56 | 0.001** |
| Prolactin (ng/ml) | 14.12±5.99 | 11.96±2.91 | 0.044* |

SD=Standard deviation, PCOS=Polycystic ovary syndrome, HDL=High-density lipoprotein, LDL=Low-density lipoprotein, TG=Triglycerides, GnRH=Gonadotropin-releasing hormone, LH=Luteinising hormone, FSH=Follicle-stimulating hormone, *=Statistically not significant, **=Statistically significant
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Global deoxyribonucleic acid methylation profiling

Using blood samples of 2 ml collected in blood collection tubes with EDTA, Genomic DNA was extracted using a DNA isolation kit according to the manufacturer’s protocol (Genomic DNA isolation Kit, Genxbio) and stored at 40°C. The assay was done by ABNOVA methylated DNA quantification kit (colorimetric) KA1510 version 05. After preparing the DNA sample, it was gently mixed with prepared reagents in strip wells. Strip plates were covered with plate seal or Parafilm M and incubated at 37°C for 90 min. The extracted DNA is bound to strip wells that are specifically treated to have a high-DNA affinity. The binding reaction solution was removed from each well by washing with a sequential amount of the Diluted ME1 1X Wash Buffer followed by incubation each time. Colour change in the sample wells and control wells was monitored after adding reagents and incubation. The ME8 solution will turn blue in the presence of sufficient methylated DNA and yellow in the ME9 solution. The absorbance was red on a microplate reader at 450 nm within 2–15 min. The methylated fraction of DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated DNA is proportional to the OD measured, calculated using the Relative Quantification formula:

\[ 5\text{-mC}\% = \left( \frac{\text{Sample OD} - \text{ME3 OD}}{\text{ME4 OD} - \text{ME3 OD}} \times 2^* + P \right) \times 100 \%
\]

5-mC = 5-methylcytosine level in total DNA.

ME3 = Negative control.

ME4 = Positive control.

S = amount input sample DNA in ng.

*: a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

Statistical analysis

Data were analysed using IBM SPSS statistics software for windows version 26.0 (IBM corporation, Armonk, NY, USA). Clinical characteristics and Global DNA methylation percentage of women with PCOS and controls were compared using Independent t-test or Mann–Whitney U test. Clinical and methylation data were expressed in mean and standard deviation \( P < 0.05 \) was considered statistically significant.

RESULTS

In our study, the most common presenting complaint was menstrual irregularity, i.e., Oligomenorrhea (50%) and amenorrhea (16%). Other complaints were acne (24%), hirsutism (18%) and infertility (14%).

Mean fasting blood sugar levels were 87.56 mg/dl and 71.83 mg/dl, mean 2 h postprandial blood sugar levels were 114.74 mg/dl and 121.84 mg/dl and mean serum fasting insulin levels were 6.06 m IU/L and 6.48 mIU/L in study and control groups, respectively, both groups were comparable.

The HOMA IR were 1.31 and 1.40 in the study and control groups, respectively, the difference is statistically insignificant.

We found that women with PCOS have high total cholesterol, elevated serum LH, FSH level and LH/FSH ratio, low serum estradiol value, high serum testosterone and marginally high prolactin level in our study group, when compared with control by Mann–Whitney U test, \( P \) values were statistically significant.

The mean DNA Methylation percentage of the study group was 11.39% and that of the control group was 5.87%. On comparing both the groups by Mann–Whitney U test the \( P = 0.001 \) which was statistically significant.

DISCUSSION

Our study identified that the mean age of study and control group were 22.08 years and 21.70 years, respectively [Figure 1], which was comparable with the study by Xu et al.\(^{10}\) Gill et al.,\(^{12}\) and Joshi et al.,\(^{13}\) in their study, selected the age range which were similar to our study.

Fifty percent of cases in our study presented with oligomenorrhea, similar to a study by Kim and Choi,\(^{14}\) in which the most common presenting symptom of South Asian patients was irregular menstruation (92.3%), and half (49.5%) of them had oligo/amenorrhea since menarche.

Previous studies included women with similar body mass index to ours [Table 1].\(^{10,12}\)

The mean fasting blood sugar and serum fasting insulin levels of our study and control groups were similar to that reported by Xu et al.\(^{10}\)

| Table 3: Mean global DNA methylation percentage of study and controls |
|------------------|------------------|-----------------|----------|
|                  | Mean±SD          | \( P \)         |
| **PCOS** (\( n=50 \)) | 11.39±3.61       |               |
| **Non-PCOS** (\( n=50 \)) | 5.87±1.42       | 0.001**        |

SD=Standard deviation, PCOS=Polycystic ovary syndrome, **=Statistically significant
Similar to our study, Xu et al.,\textsuperscript{10} and Hiam et al.,\textsuperscript{15} have reported no statistically significant difference in HOMA IR in women with PCOS and their matched controls.

Our study [Table 2] and Kiranmayee et al.,\textsuperscript{16} both showed, that women with PCOS have statistically significantly high total cholesterol and serum triglyceride levels than control. However, the study by Hiam et al.,\textsuperscript{15} showed no difference in lipid profile among the two groups.

We found elevated serum LH and FSH levels in women with PCOS [Table 2], unlike Lewandowski et al.,\textsuperscript{17} who found no difference in baseline LH and FSH among both groups. However, there is a statistically significant increase in LH/FSH ratio in their study group 1.59 than matched controls 0.76 similar to our study.

Hiam et al.,\textsuperscript{15} in their study, found elevated mean testosterone level 1.72 nmol/L and 1.03 nmol/L among cases and controls, respectively, with \((P \leq 0.001)\) similar to our study. Delcour et al.,\textsuperscript{18} in their study, found hyperprolactinaemia in cases of PCOS patients.

The mean DNA methylation percentage in our study group was 11.39% and that of the control group was 5.87% [Table 3], which was a statistically significant \(P = 0.001\). Hence, we find there was a significant difference of global DNA methylation percentage in PBLs of women with PCOS and matched controls in our study. Xu et al.,\textsuperscript{10} and Hiam et al.,\textsuperscript{15} reported that there was no statistically significant difference in the Global DNA methylation percentage in whole blood peripheral leucocytes of women with PCOS and controls. However, Hiam et al.,\textsuperscript{15} found Women with PCOS had lower global DNA methylation in monocytes, T helper, T cytotoxic and B cells.

Sagvekar et al.,\textsuperscript{19} suggested that epigenetic dysregulation of ovarian genes may contribute to abnormal folliculogenesis in women with PCOS.

Pan et al.,\textsuperscript{20} found that hypomethylated genes related to the synthesis of lipid and steroid could partially explain mechanisms of hyperandrogenism in PCOS as they may promote the synthesis of androgen by dysregulation of the expression of these genes.

Alterations in the DNA methylene of immune cells could have consequences in their functioning and contribute to the low-grade inflammation in PCOS. Immune cells could infiltrate many tissues (skeletal muscle, ovaries, adipose tissue) and integrate any physiological and pathophysiological changes that are occurring in these tissues\textsuperscript{21} or the DNA methylene of immune cells can adapt to their environmental milieu and then influence the epigenome of surrounding tissues.\textsuperscript{22} Lifestyle factors such as a healthy diet and exercise can remodel DNA methylation. Alterations to DNA methylation patterns after an intervention could be a key factor contributing to improvements in metabolic status. Studies have shown skeletal muscle DNA methylation is remodeled by exercise and has a unique intensity-dependent exercise effects on DNA methylation.\textsuperscript{23}

Thymoquinone, the main component of black bean grass interferes with the production of reactive oxygen species during \textit{in vitro} maturation can help to improve epigenetic modifications, including DNA methylation. Benrick et al.,\textsuperscript{24} and Kokosar et al.,\textsuperscript{25} showed that a single low-frequency electroacupuncture therapy changed skeletal muscle DNA methylation and gene expression in PCOS adipose tissue, thereby improving metabolic disorders in women with PCOS.\textsuperscript{24,25}

Homocysteine through activation of single-carbon metabolism can cause hypermethylation of mitochondrial DNA (mtDNA), resulting in dysfunction of oocytes and oocyte quality. However, treatment with 5’Azacytidine, a DNA methyltransferase inhibitor can improve oocyte quality by inhibiting active carbon metabolism and hypermethylation of mtDNA.\textsuperscript{26} Recently, Mimouni et al.,\textsuperscript{27} used S-adenosylmethionine, the universal methyl donor in their study to treat 6-month-old female offspring of PCOS, which partially restored metabolic and reproductive functions. Therefore, supplementing the offspring of PCOS with folic acid or a methyl donor may improve their metabolism and reproductive functions. Drugs used in PCOS, such as metformin and their effects on the DNA methylation of PCOS are still relatively scarce. Recently, Amani Abkenari et al.,\textsuperscript{28} confirmed that metformin reduces oxidative stress and epigenetic changes, including DNA methylation and can enhance the developmental ability of PCOS oocytes. Whether exercise, lifestyle modification and drug intervention in women with PCOS can alter DNA methylation patterns in skeletal muscle, peripheral blood or adipose tissue needs to be investigated. Gaining a better understanding may improve future management of PCOS through the identification of new therapeutic targets and/or effective lifestyle interventions.

We in India reported that there was a significant difference in the percentage of global DNA methylation among the women with PCOS and their matched control, unlike the previous study by Xu et al.,\textsuperscript{10} which may be due to different ethnicity. As mentioned by Zhang et al.,\textsuperscript{29} there occurs significant differences in global genomic DNA methylation by gender and race in peripheral blood. This study in the case of epigenetics of PCOS may be helpful in the Indian population.
The strengths of this study were, that we used a well characterised otherwise healthy cases and controls, and validated methods to assess all parameters from glucose metabolism, liver and kidney function, electrolyte and hormonal assay. We did not observe any significant difference in blood cell counts between women with and without PCOS that rules out the potential masking effect by altered blood counts. However, the limitations of our study include a small sample size. Inability to assess the effect of DNA methylation and which pathway it affects in PCOS. This limits our capacity to clearly conclude whether methylation changes have any causal impact on molecular pathways and also affected our capacity to fully explore potential confounding variables in order not to violate the assumptions of the statistical model. Further studies with a large number of participants and genome-wide methylation are needed for a detailed analysis of epigenetics of PCOS.

CONCLUSION

Despite study limitations, this study provides some insight into global DNA methylation in PBLs and its association with PCOS. The influence of epigenetic factors in the development of PCOS is of increasing interest to explain the complexity and challenges of the inheritance and pathophysiology of the syndrome. Further research is needed to better understand epigenetics in PCOS including genome-wide DNA methylation profiling.

Ethical approval

Done by institutional ethical committee.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

Data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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