Epigenetic regulation of an adverse metabolic phenotype in polycystic ovary syndrome: the impact of the leukocyte methylation of *PPARGC1A* promoter

Hongcui Zhao, M.D., Ph.D., Yue Zhao, Ph.D., Yun Ren, M.D., Ph.D., Min Li, M.D., Ph.D., Tianjie Li, M.M., Rong Li, M.D., Ph.D., Yang Yu, Ph.D., and Jie Qiao, M.D., Ph.D.

Ministry of Education Key Laboratory of Assisted Reproduction, and Beijing Key Laboratory of Reproductive Endocrinology and Assisted Reproductive Technology, Center of Reproductive Medicine, Peking University Third Hospital, Beijing, People's Republic of China

Objective: To investigate *PPARGC1A* promoter methylation and mitochondria DNA (mtDNA) content in the leukocytes of women with polycystic ovary syndrome (PCOS) and analyze the relationship between these indices and metabolic risk for women with PCOS.
Design: Cross-sectional study.
Setting: University hospital.
Patient(s): A total of 175 women with PCOS and 127 healthy controls.
Intervention(s): None.
Main Outcome Measure(s): Women with and without PCOS classified using the typical metabolic risk criteria of the National Cholesterol Education Program's Adult Treatment Panel III report (ATPIII), methylation of *PPARGC1A* promoter tested by methylation-specific polymerase chain reaction, and mtDNA content confirmed by quantitative polymerase chain reaction (PCR).
Result(s): *PPARGC1A* promoter methylation was specifically increased, but mtDNA content was specifically decreased in women with PCOS compared with the control women after adjustment for body mass index. Moreover, in women with PCOS who have increased metabolic risk, the differences in *PPARGC1A* promoter methylation and mitochondrial content were aggravated.
Conclusion(s): In conclusion, *PPARGC1A* promoter methylation and mitochondrial content were found to be potential biomarkers for the prediction of metabolic risk in women with PCOS. (Fertil Steril® 2017;107:467–74. ©2016 The Authors. Published by Elsevier Inc. on behalf of the American Society for Reproductive Medicine. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).)
Key Words: ATPIII, BMI, lipid profile, metabolic risk, mitochondrial biogenesis

Polycystic ovary syndrome (PCOS), a common and heterogeneous endocrine disorder with a prevalence ranging from 5% to 10% (1), is characterized by a clustering of hyperandrogenism, oligomenorrhea, chronic anovulation, and hyperinsulinemia (2). Not only is PCOS a reproductive disorder, but it also enhances the risk of the metabolic syndrome. Metabolic inflexibility is a feature of women with PCOS, and the metabolic sequelae can affect women across their life span (3). The metabolic complications of PCOS are increasingly attracting attention. The National Institutes of Health Office for Disease Prevention-Sponsored Evidence-Based Methodology Workshop on Polycystic Ovary Syndrome even recommended renaming PCOS with important metabolic consequences to manage it effectively across the life span (4). The pathogenesis of PCOS and related metabolic abnormalities are not entirely understood, but they are often associated with some indices, including

Received July 28, 2016; revised and accepted October 28, 2016; published online November 23, 2016.
H.Z. has nothing to disclose. Y.Z. has nothing to disclose. Y.R. has nothing to disclose. M.L. has nothing to disclose. T.L. has nothing to disclose. R.L. has nothing to disclose. Y.Y. has nothing to disclose. J.Q. has nothing to disclose.
H.Z., Y.Z., and Y.R. should be considered similar in author order.
Supported in part by the Ministry of Science and Technology of China Grants (973 program; 2014CB943203), "Reproductive health and major birth defects prevention and control research" specific project (2016YFC1000601), the National Natural Science Funds (31371521, 81571400, 81471427, 81300457, 31501201, and 81300543), Beijing Nova Program (xxjh2015A011), and special projects of the 2015 Science and Technology Innovation Base Cultivation and Development (Z151100001615023).
Reprint requests: Yang Yu, Ph.D., Department of Obstetrics and Gynecology, Peking University Third Hospital, No. 49 HuaYuan North Road, HaiDian District, Beijing 100191, People's Republic of China (E-mail: yuyang5012@hotmail.com).

Fertility and Sterility® Vol. 107, No. 2, February 2017 0015-0282

http://dx.doi.org/10.1016/j.fertnstert.2016.10.039

hyperandrogenism, obesity, insulin resistance (IR), and inflammation.

Recent proteomic and metabolomic studies all suggest abnormal metabolism of carbohydrates, lipids, and proteins in women with PCOS (5–8). All these abnormal metabolisms are involved in energy pathways. Mitochondrial function is fundamental to maintaining metabolic and energy homeostasis. Alterations in mitochondrial function are often associated with peripheral IR and glucose intolerance (9, 10), and are involved in the pathogenesis of PCOS and the metabolic syndrome (11, 12). Mitochondrial DNA (mtDNA) content in body fluids and tissues is a potential biomarker of mitochondrial dysfunction (13). Reduced mtDNA content in peripheral blood leukocytes has been associated with IR in adolescents with features of the metabolic syndrome (14). Moreover decreased mtDNA content in PCOS patients independent of insulin resistance or other metabolic factors has been reported (15). However, the pathophysiologic and clinical significance of the finding are not entirely understood. Therefore, it would be interesting to study the mechanistic reason for the decreased peripheral mtDNA content in PCOS patients and whether mtDNA copy number could be a candidate biomarker for metabolic complications in PCOS.

The transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-α (protein PGC-1α; gene *PPARGC1A*) is an important integrator of the molecular regulatory circuitry involved in mitochondrial function and biogenesis (16, 17). *PPARGC1A* gene expression is regulated by the methylation of its promoter. The abnormal methylation of *PPARGC1A* promoter can result in change of mtDNA content (18–20). In previous studies, *PPARGC1A* promoter methylation in blood at 5 to 7 years old has been shown to predict adiposity from 9 to 14 years old, and the methylation measured in childhood may have utility in predicting cardiometabolic disease risk (21). *PPARGC1A* promoter methylation has also been shown to have a close relationship to metabolic abnormality, but its role in PCOS is still unknown.

In the present study, we investigated *PPARGC1A* promoter methylation and the biogenesis of mitochondria in women with PCOS. Furthermore, we analyzed the relevance between these indices and the metabolic abnormalities of women with PCOS defined by the National Cholesterol Education Program's Adult Treatment Panel III report (ATPIII). The conclusions should provide a new biomarker to predict metabolic risk in women with PCOS.

MATERIALS AND METHODS

Patients

The study was approved by the institutional review board of Peking University Third Hospital. All participants signed an informed consent form to participate in the study. We calculated the sample size using G*Power calculator (www.gpower.hhu.de/en.html). According to our pilot study results for mtDNA content and *PPARGC1A* promoter methylation, the required size of the study population was calculated to be 102 women per group ($\alpha = 0.05$, and the study power = 0.90).We used a convenience sample with a total of 302 volunteers recruited; 175 women had PCOS diagnosed with the Rotterdam criteria (22), and 127 were healthy women of a similar age who constituted the control group.

The women were selected from the Division of Reproductive Medical Center, Peking University Third Hospital, from March 2012 to May 2013. Exclusion criteria were the same as our previous research (5). In addition, women who had received any hormone treatment or insulin-lowering agent during the preceding 3 months were excluded. The control women were selected from women visiting the clinic as partners for men being treated for azoospermia. All women in the control group had regular menstrual cycles and normal androgen levels.

Overnight fasting blood samples were collected from women with PCOS with amenorrhoea exceeding 3 months without hormone-induced withdrawal bleeding, and at the early follicular phase from women with spontaneous ovulation. The plasma and serum were used to obtain biochemical measurements, and the blood cells were stored for DNA extraction to quantify mtDNA copy number and the promoter methylation of *PPARGC1A*.

Anthropometric and Biochemical Measurements

Anthropometric variables, including waist circumferences, hip circumferences, waist-to-hip ratio, BMI (kg/m^2), and systolic and diastolic blood pressure (SBP and DBP) were evaluated in all of the women. The following biochemical measurements were performed on the plasma samples: insulin, glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides, using standard clinical laboratory techniques. The homeostasis model assessment (HOMA) index was used as an estimator of IR. The cutoff point to determine IR was defined as a HOMA Index ≥ 2.69 (5, 23).

Total testosterone (T) and sex hormone-binding globulin (SHBG) measurements were performed on the serum samples. Total T was measured by liquid chromatography and tandem mass spectrometry. Serum concentrations of SHBG were analyzed by chemiluminometric immunoassay. The free androgen index (FAI) was calculated by total testosterone in nanomoles per liter, multiplied by 100, divided by SHBG in nanomoles per liter.

Bisulfite Treatment of DNA and Methylation-specific Polymerase Chain Reaction

Peripheral blood cell DNA was extracted with the QIAamp Tissue Kit 250 (Qiagen) according to the manufacturer's instructions. The genomic DNA was treated by sodium bisulfite using an EpiTect Bisulfite kit (Qiagen) according to the manufacturer's protocol. The promoter methylation status of the selected CpG dinucleotides in the *PPARGC1A* promoter was measured by the methylation-specific polymerase chain reaction (MSPCR). The primers and method used were as described in Sookoian et al. (24). The experiments were performed in triplicate. The level of methylated DNA was expressed by the ratio of the estimated amount of methylated DNA to the unmethylated DNA levels, calculated for each sample by the

fluorescence threshold cycle (Ct) values for an estimated efficiency of 2 (25). The primer sequences are shown in Supplemental Table 1 (available online).

Quantification of mtDNA Content

The relative amounts of nuclear DNA (nDNA) and mtDNA were determined by real-time polymerase chain reaction (PCR) and performed in triplicate. The mtDNA content was quantified by primers D41 and R56. The nDNA was quantified by gene *GAPDH*. Primer sequences are shown in Supplemental Table 1.

Subgrouping of Patients

To compare the methylation levels of the *PPARGC1A* gene promoter and mtDNA content in blood with metabolic indicators in women with similar metabolic statuses, we subcategorized women with PCOS and healthy women according to the presence or absence of risk factor(s) for metabolic syndromes as defined by the ATPIII (26). We subdivided women into ATPIII negative (ATPIII [−]) and ATPIII positive (ATPIII [+]) with at least one of the ATPIII risk factors. The subcohorts referred to the research of Chang et al. (27), as each of the ATPIII criteria is known to contribute to the future development of metabolic syndromes. The ATPIII risk factors include: [1] waist circumference ≥88 cm, [2] fasting plasma glucose ≥5.6 mmol/L, [3] blood HDL-C level <1.29 mmol/L, [4] triglycerides ≥1.7 mmol/L, and [5] blood pressure ≥130/85 mm Hg (26). With this stratification, we were able to compare the *PPARGC1A* gene promoter methylation levels and the mtDNA content of women with PCOS who were free of signs of metabolic syndromes (ATPIII [−]) with ATPIII [−] healthy women, ATPIII [+] women with PCOS with ATPIII [+] healthy women, ATPIII [−] women with PCOS with ATPIII [+] women with PCOS, and ATPIII [−] healthy women with ATPIII [+] healthy women.

Statistical Analysis

The quantitative data, expressed as the mean ± standard error of the mean (SEM), were analyzed using a two-tailed *t*-test. Analysis of covariance (ANCOVA) was used for adjustment of the BMI. The relationships between the methylation levels of the *PPARGC1A* gene promoter (or mtDNA content) and metabolic indicators were analyzed by Pearson correlation analysis. In addition, the associations of the methylation levels of the *PPARGC1A* gene promoter (or mtDNA content) and adverse metabolic phenotype with PCOS were measured by logistic regression analyses. To perform these analyses, we used SPSS 17.0 software (IBM). $P<.05$ was considered statistically significant.

RESULTS

Opposite Dynamic Changes between *PPARGC1A* Promoter Methylation and mtDNA Content in the PCOS Group

The clinical, metabolic, and hormone characteristics of women with PCOS and healthy women are described in Table 1. As the BMI of the PCOS patients was statistically significantly higher than the controls (25.07 ± 0.35 vs 22.33 ± 0.31, $P<.0001$), all parameters were compared after adjustment for BMI. The PCOS group had statistically significantly higher rates of insulin, IR, total testosterone, FAI, and triglycerides. In addition, the overweight (BMI ≥25) and insulin resistant (HOMA IR ≥2.69) women statistically significantly increased in the PCOS group.

The *PPARGC1A* promoter methylation ratio in the PCOS group was 36.5%, and 26.3% in the control group. Compared with the control group, the methylated DNA/unmethylated DNA ratio of the *PPARGC1A* promoter in the PCOS group was statistically significantly higher (Fig. 1A). As a potential biomarker of mitochondrial dysfunction, mtDNA content, represented by the mtDNA/nDNA ratio, was statistically significantly lower in the women with PCOS compared with that of the healthy women (see Fig. 1B). The mtDNA/nDNA ratio was inversely correlated with the methylation levels of the *PPARGC1A* promoter in both the PCOS (see Fig. 1C) and the non-PCOS group (see Fig. 1D).

Abnormalities of the *PPARGC1A* Promoter Methylation Ratio and mtDNA Content Exacerbated in PCOS ATPIII [+] Women

To determine the relationship between mitochondrial function and metabolic abnormality, we stratified women according to the presence or absence of risk factor(s) for metabolic syndromes as defined by the ATPIII report. Of the 175 women with PCOS, 41.57% (n = 71) were without any ATPIII risk factors (ATPIII [−] PCOS), and risk factors were present (ATPIII [+] PCOS) in 59.43% (n = 104). Of the 127 control women, 83.46% (n = 106) were without any ATPIII risk

TABLE 1

Clinical and biochemical characteristics of women with and without PCOS.

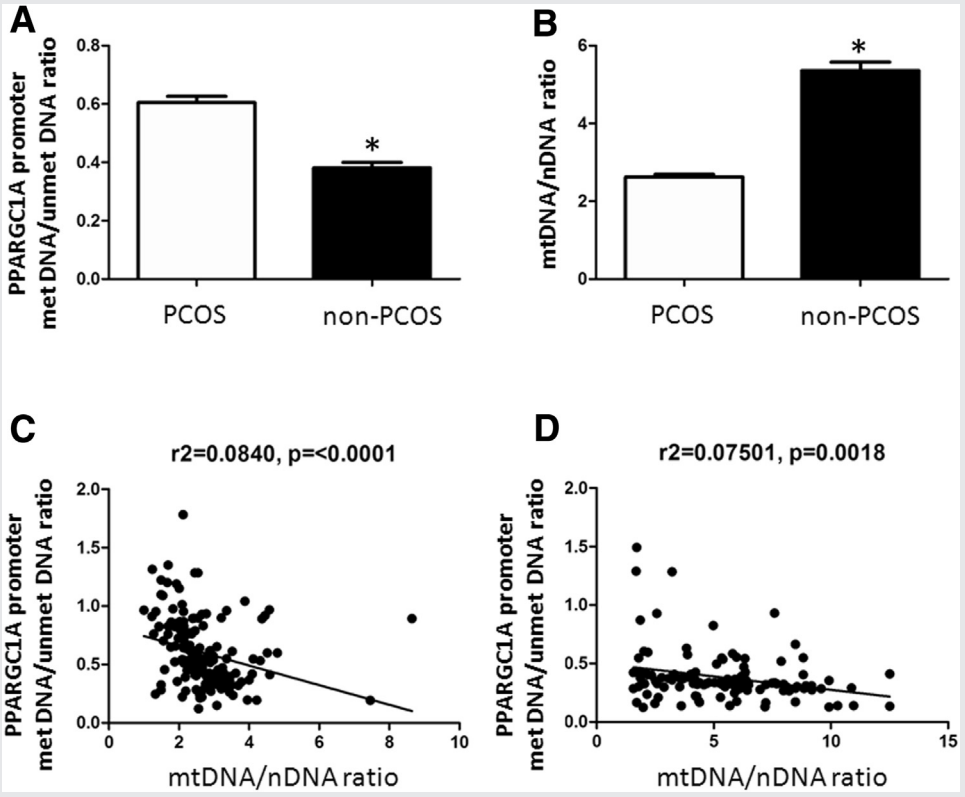
Characteristic	PCOS (n = 175)	Control (n = 127)	*P* value[a]
Age (y)	28.57 ± 0.20	28.28 ± 0.31	.41
BMI (kg/m^2)	25.07 ± 0.35	22.33 ± 0.31	<.0001
BMI >25	85 (48.57%)	27 (21.26%)	<.0001
SBP (mm Hg)	115.53 ± 1.12	114.12 ± 1.03	.13
DBP (mm Hg)	69.53 ± 0.76	68.62 ± 0.68	.24
Waist circumference (cm)	88.43 ± 0.81	78.94 ± 0.84	.19
Waist–hip ratio	0.903 ± 0.004	0.841 ± 0.005	.25
Glucose (mmol/L)	4.82 ± 0.04	4.79 ± 0.03	.63
Insulin (mU/L)	11.64 ± 0.51	9.26 ± 0.26	.0032
HOMA-IR	2.54 ± 0.12	1.98 ± 0.06	.0054
HOMA-IR ≥2.69	54 (30.86%)	11 (8.66%)	<.0001
Total testosterone (nmol/L)	1.22 ± 0.05	0.81 ± 0.05	<.0001
SHBG (nmol/L)	52.33 ± 4.22	59.22 ± 3.89	.078
FAI	4.02 ± 0.46	2.29 ± 0.32	.001
HDL-C (mmol/L)	1.32 ± 0.02	1.30 ± 0.02	.69
LDL-C (mmol/L)	2.79 ± 0.05	2.48 ± 0.05	.28
Triglyceride (mmol/L)	1.68 ± 0.07	1.09 ± 0.05	<.002
Total cholesterol (mmol/L)	4.67 ± 0.05	4.24 ± 0.05	.53

Note: BMI = body mass index; DBP = diastolic blood pressure; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; LDL-C = low-density lipoprotein cholesterol; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

[a] *P* values were calculated after adjustment for BMI.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

FIGURE 1



Expression and correlation analysis of *PPARGC1A* promoter methylation and mitochondrial DNA (mtDNA) content between polycystic ovary syndrome (PCOS) and control group. (**A**) Methylation level in *PPARGC1A* promoter was statistically significantly increased in the PCOS group. **P*<.01. (**B**) The mtDNA content was reversely decreased in PCOS group. **P*<.01. The mtDNA/nDNA ratio was inversely correlated with the methylation levels of the *PPARGC1A* promoter in both the (**C**) PCOS and (**D**) control groups.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

factors (ATPIII [−] healthy), and risk factors were present (ATPIII [+] healthy) in 16.54% (n = 21).

Compared with ATPIII [−] women with PCOS, ATPIII [+] women with PCOS exhibited statistically significantly more severe metabolic problems, manifested as higher BMI, SBP, and DBP; larger waist circumference and waist–hip ratio; higher rates of insulin, IR, LDL-C, triglycerides, and total cholesterol; and lower HDL-C (Supplemental Table 2, available online). ATPIII [+] women with PCOS had a statistically significantly higher *PPARGC1A* promoter methylation ratio and a lower level of mtDNA content compared with ATPIII [−] women with PCOS and ATPIII [+] healthy women (Fig. 2A–D). These results suggest *PPARGC1A* promoter methylation and mtDNA to be potential biomarkers for severe metabolic syndrome in women with PCOS.

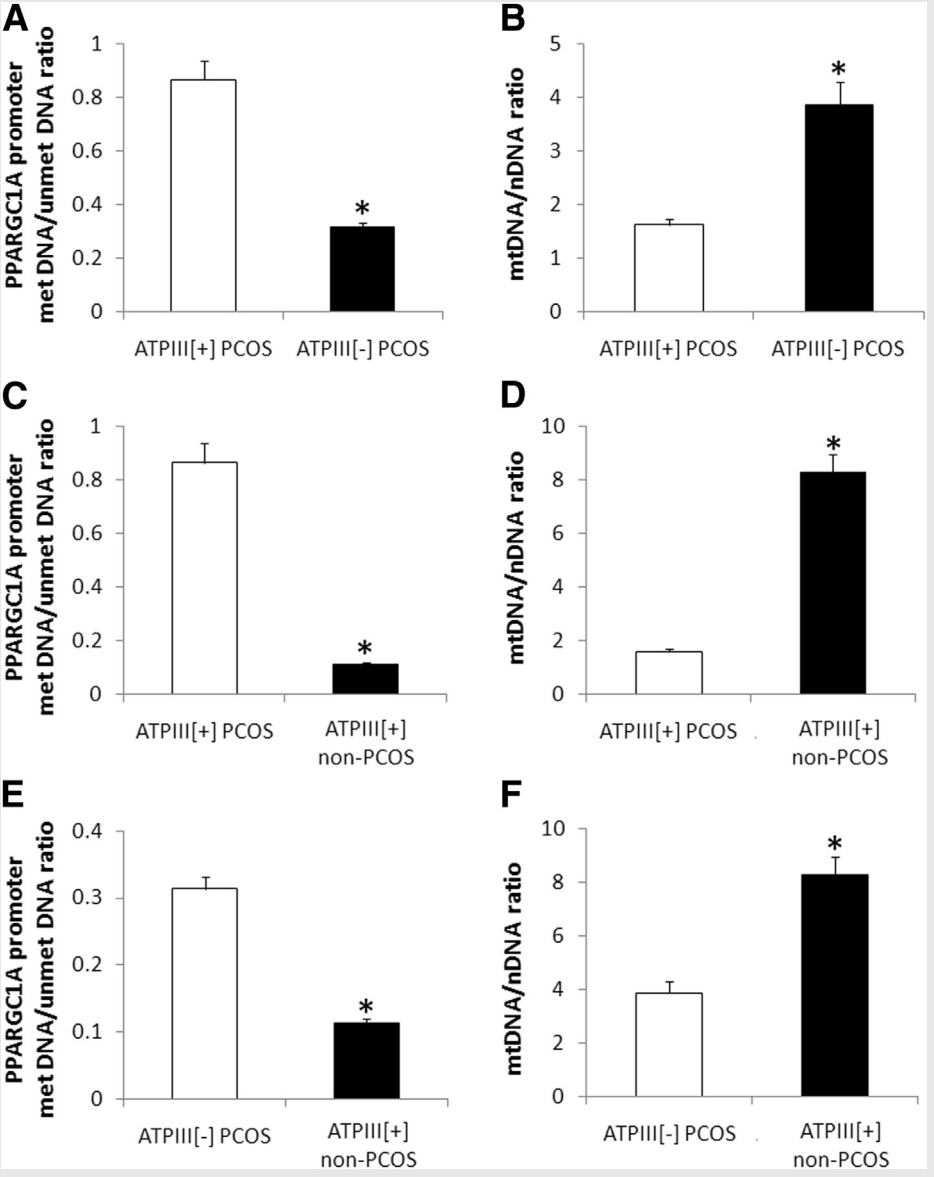
Moreover, the ATPIII [−] women with PCOS and the ATPIII [−] healthy women shared some similar anthropometric and metabolic characteristics, except a larger waist circumference, waist–hip ratio, total testosterone, FAI, total cholesterol and lower HDL-C (Supplemental Table 3, available online). However, the *PPARGC1A* promoter methylation ratio and the level of mtDNA content were statistically significantly different between the ATPIII [−] PCOS and ATPIII [−] non-PCOS subcohorts (see Fig. 2E–H).

In the non-PCOS groups, the *PPARGC1A* promoter methylation ratio and the level of mtDNA content ($P=.6261$, $P=.0539$) were not statistically significantly different between the ATPIII [+] healthy women and ATPIII [−] healthy women (see Fig. 2I and J).

Correlation of *PPARGC1A* Promoter Methylation Ratio and mtDNA Content with Clinical Parameters

To determine whether there was an association between the *PPARGC1A* promoter methylation ratio, mtDNA content, and certain clinical biochemical traits, we performed a Pearson correlation analysis. As shown in Supplemental Table 4 (available online), a statistically significant correlation was found between the *PPARGC1A* promoter methylation ratio and BMI, waist circumference, waist–hip ratio, and rates of insulin and IR in both the PCOS and non-PCOS groups. The *PPARGC1A* promoter methylation ratio statistically significantly correlated with FAI and triglyceride levels in the PCOS group. The mtDNA content statistically significantly correlated with rates of insulin and IR in both the PCOS and non-PCOS groups. In addition, mtDNA content statistically significantly correlated with BMI, waist circumference,

FIGURE 2

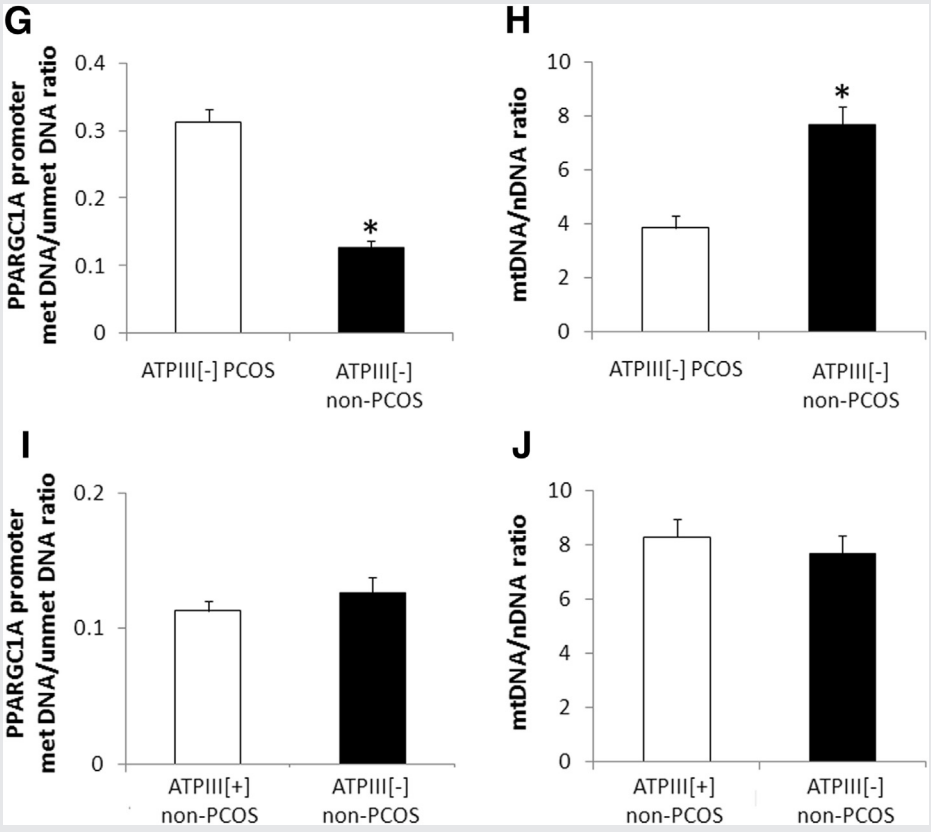


PPARGC1A promoter methylation and mitochondrial DNA (mtDNA) content in polycystic ovary syndrome (PCOS) and non-PCOS groups with or without ATPIII characteristics. (**A**, **B**) Statistically significantly higher *PPARGC1A* promoter methylation ratio and lower mtDNA content can be observed in ATPIII [+] women with PCOS compared with ATPIII [−] women with PCOS. (**C**, **D**) A similar tendency was suggested in ATPIII [+] women with PCOS compared with ATPIII [+] healthy women. Statistically significantly higher *PPARGC1A* promoter methylation ratio and lower mtDNA content can also be observed in ATPIII [−] women with PCOS compared with (**E**, **F**) ATPIII [−] healthy women,

(*Continued on next page*)

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

FIGURE 2 Continued



and even (**G**, **H**) ATPIII [+] healthy women. (**I**, **J**) No differences were found for *PPARGC1A* promoter methylation ratio and mtDNA content in the non-PCOS group with or without ATPIII characteristics. *$P<.01$.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

waist–hip ratio, FAI, and triglyceride in the PCOS group (Supplemental Table 5, available online).

To predict the metabolic risk for PCOS, we performed a multiple logistic regression analysis. As Table 2 shows, the corresponding odds ratios (95% confidence interval [CI]) for ATPIII [+] PCOS by *PPARGC1A* promoter methylation ratio, mtDNA content, and HOMA-IR were 3.33 (1.39–6.22), 0.16 (0.05–0.53), and 4.16 (1.49–11.56), respectively, after adjustment for BMI. This implies that *PPARGC1A* promoter methylation and mtDNA content are metabolic risk factors of PCOS.

DISCUSSION

This study revealed that women with PCOS have an overtly higher *PPARGC1A* promoter methylation ratio and lower mtDNA content compared with healthy women, even after

TABLE 2

Odds ratios and 95% confidence intervals for ATPIII [+] PCOS with *PPARGC1A* promoter methylation ratio, mtDNA content and metabolic factors.

	Model 1[a]		Model 2[b]	
Variable	**Odds ratio (95% CI)**	***P* value**	**Odds ratio (95% CI)**	***P* value**
PPARGC1A promoter methylation ratio	2.02 (1.25–3.89)	.0456	3.33 (1.39–6.22)	.0102
mtDNA content	0.34 (0.23–0.79)	.0123	0.16 (0.05–0.53)	.0096
HOMA-IR	2.32 (1.13–5.22)	.0298	4.16 (1.49–11.56)	.0083
FAI	1.21 (0.73–1.36)	.36	1.21 (0.73–1.36)	.36

Note: ATPIII = National Cholesterol Education Program's Adult Treatment Panel III report; FAI = free androgen index; HOMA-IR = homeostatic model assessment of insulin resistance; mtDNA = mitochondrial DNA; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

[a] Model 1: unadjusted.

[b] Model 2: adjusted for body mass index.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

controlling for confounding factors such as ATPIII metabolic syndrome risk factors or BMI. We found that PCOS was associated with an abnormal *PPARGC1A* promoter methylation ratio and lower mtDNA content. A positive correlation was found between the *PPARGC1A* promoter methylation ratio and the FAI in the PCOS group, but the correlation of mtDNA content and the FAI was negative. In addition, *PPARGC1A* promoter methylation and mtDNA content had associations with ATPIII [+] PCOS. All these findings suggest that *PPARGC1A* promoter methylation ratios and mtDNA content may be part of one of the putative links between PCOS and its metabolic abnormalities.

The mechanistic reason for *PPARGC1A* promoter hypermethylation in the PCOS group is still unknown. Polycystic ovary syndrome is a heterogeneous disease with many different phenotypes and metabolic aspects. There is no clear consensus on the diagnosis and pathogenesis of PCOS, but hyperandrogenism is considered to be one key factor (28). The relationship of hyperandrogenism and *PPARGC1A* promoter hypermethylation in the PCOS group is manifested by our Pearson correlation analysis, but the causal relationship was not clear.

Androgens could induce epigenetic alterations in the genome and may induce *PPARGC1A* promoter hypermethylation (29, 30). On the other hand, PGC-1α, a protein encoded by *PPARGC1A*, may affect the synthesis of androgens. PGC-1α can up-regulate the expression of uncoupling protein 2, which can also increase the production of testosterone (31, 32). Moreover in the analysis of clinical parameters of *PPARGC1A* promoter methylation and mitochondrial content, we found a specific correlation between the two indices and lipid profiles (FAI and triglyceride). Sex hormone-binding globulin and triglycerides can be regulated by androgens. Women with PCOS commonly have elevated triglycerides, and it is the most common metabolic abnormality in young women with PCOS (33). Therefore, *PPARGC1A* promoter methylation and mitochondrial content may be used to predict the lipid metabolism status of women with PCOS. Androgen disorder usually results in an abnormal metabolic status in women with PCOS (34).

In this study, metabolic risk was assessed following ATPIII criteria. Both these standards have indicated metabolic risk factors in studies related to type II diabetes and cardiovascular disease (35, 36). ATPIII [+] women with PCOS have a higher *PPARGC1A* promoter methylation ratio and lower mtDNA content. The results imply that the *PPARGC1A* promoter methylation ratio and mtDNA content may be new biomarkers for PCOS metabolic risk assessment. *PPARGC1A* promoter methylation measured in childhood may have utility in predicting cardiometabolic disease risk (21). The long-term metabolic risk prediction value of *PPARGC1A* promoter methylation in PCOS needs further exploration by longitudinal studies.

In the results of the correlation analysis of *PPARGC1A* promoter methylation, mtDNA content, and clinical parameters, we found that *PPARGC1A* promoter methylation and mtDNA content all statistically significantly correlated with insulin and IR levels not only in PCOS but also in non-PCOS groups. This finding was consistent with recent observations of epigenetic regulation of insulin resistance in the metabolic syndrome (20, 24, 37). Evidence has showed that insulin resistance plays a central role in the pathogenesis of PCOS and obesity, and their associated metabolic complications (38). Our multivariate model shows the association between metabolic abnormality with PCOS and *PPARGC1A* promoter methylation and mtDNA content. All those results provide further evidence that *PPARGC1A* promoter methylation and mtDNA content may have utility in predicting metabolic risk of PCOS.

PPARGC1A has displayed a key role in regulating physiological processes, including blood pressure and cellular cholesterol homoeostasis, and can also reflect the clinical phenotypes, including obesity, type II diabetes, and nonalcoholic fatty liver (24, 39). Moreover, some genes that regulate energy and substance metabolism, thermogenesis, and even fatty cell differentiation are orchestrated by *PPARGC1A* (40). The relationship of *PPARGC1A* promoter methylation, mtDNA content, and PCOS may also imply the role of it in the pathogenesis of PCOS and its associated metabolic abnormalities. More importantly, *PPARGC1A* promoter methylation and mtDNA content was worse in women with PCOS with increased metabolic risk, so they may act as a potential biomarker for an adverse metabolic phenotype in PCOS.

The present results were derived from the whole blood of women with PCOS, but not from organs related to reproductive or metabolic function. Whole blood is easier to obtain in the clinic, and some biomarkers for some diseases have been screened from it; evidence has suggested there were statistically significant associations between individual CpG loci in human cells from different embryonic tissue lineages (41, 42). In consideration of dynamic epigenetic modification in different organs, the study of local *PPARGC1A* methylation levels should be discussed in future.

The *PPARGC1A* promoter methylation ratio was associated with hyperandrogenism and IR, two main factors associated with the pathogenesis of PCOS. *PPARGC1A* promoter hypermethylation in blood may be involved in the pathogenesis of PCOS and could be a new biomarker for an adverse metabolic phenotype in women with PCOS. More importantly, *PPARGC1A* promoter methylation is a dynamic and flexible process. It not only may be involved in the regulation of conditions associated with IR but can also be reverted by pharmacologic or lifestyle interventions. Further research needs to be undertaken to explore the possible biologic mechanisms of *PPARGC1A* promoter methylation in the pathogenesis of PCOS. This study may also bring a more integrated understanding of this pathogenesis and possibly contribute to the development of new therapeutic opportunities for women with PCOS and its associated metabolic abnormalities.

REFERENCES

1. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. Nat Rev Endocrinol 2011;7:219–31.
2. Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic ovary syndrome. Lancet 2007;370:685–97.

3. Di Sarra D, Tosi F, Bonin C, Fiers T, Kaufman JM, Signori C, et al. Metabolic inflexibility is a feature of women with polycystic ovary syndrome and is associated with both insulin resistance and hyperandrogenism. J Clin Endocrinol Metab 2013;98:2581–8.
4. Dunaif A, Fauser BC. Renaming PCOS–a two-state solution. J Clin Endocrinol Metab 2013;98:4325–8.
5. Zhao Y, Fu L, Li R, Wang LN, Yang Y, Liu NN, et al. Metabolic profiles characterizing different phenotypes of polycystic ovary syndrome: plasma metabolomics analysis. BMC Med 2012;10:153.
6. Sun L, Hu W, Liu Q, Hao Q, Sun B, Zhang Q, et al. Metabonomics reveals plasma metabolic changes and inflammatory marker in polycystic ovary syndrome patients. J Proteome Res 2012;11:2937–46.
7. Zhao X, Xu F, Qi B, Hao S, Li Y, Li Y, et al. Serum metabolomics study of polycystic ovary syndrome based on liquid chromatography-mass spectrometry. J Proteome Res 2014;13:1101–11.
8. Insenser M, Montes-Nieto R, Murri M, Escobar-Morreale HF. Proteomic and metabolomic approaches to the study of polycystic ovary syndrome. Mol Cell Endocrinol 2013;370:65–77.
9. Cheng Z, Tseng Y, White MF. Insulin signaling meets mitochondria in metabolism. Trends Endocrin Met 2010;21:589–98.
10. Serra D, Mera P, Malandrino MI, Mir JF, Herrero L. Mitochondrial fatty acid oxidation in obesity. Antioxid Redox Sign 2013;19:269–84.
11. Victor VM, Rocha M, Banuls C, Alvarez A, de Pablo C, Sanchez-Serrano M, et al. Induction of oxidative stress and human leukocyte/endothelial cell interactions in polycystic ovary syndrome patients with insulin resistance. J Clin Endocrinol Metab 2011;96:3115–22.
12. Pirola CJ, Gianotti TF, Burgueno AL, Rey-Funes M, Loidl CF, Mallardi P, et al. Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. Gut 2013;62:1356–63.
13. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? Mitochondrion 2013;13:481–92.
14. Gianotti TF, Sookoian S, Dieuzeide G, Garcia SI, Gemma C, Gonzalez CD, et al. A decreased mitochondrial DNA content is related to insulin resistance in adolescents. Obesity 2008;16:1591–5.
15. Lee SH, Chung DJ, Lee HS, Kim TJ, Kim MH, Jeong HJ, et al. Mitochondrial DNA copy number in peripheral blood in polycystic ovary syndrome. Metabolism 2011;60:1677–82.
16. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 2004;18:357–68.
17. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 1998;92:829–39.
18. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci USA 2003;100:8466–71.
19. Croce MA, Eagon JC, LaRiviere LL, Korenblat KM, Klein S, Finck BN. Hepatic lipin 1β expression is diminished in insulin-resistant obese subjects and is reactivated by marked weight loss. Diabetes 2007;56:2395–9.
20. Ling C, Del Guerra S, Lupi R, Ronn T, Granhall C, Luthman H, et al. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia 2008;51:615–22.
21. Clarke-Harris R, Wilkin TJ, Hosking J, Pinkney J, Jeffery AN, Metcalf BS, et al. PGC1α promoter methylation in blood at 5–7 years predicts adiposity from 9 to 14 years (EarlyBird 50). Diabetes 2014;63:2528–37.
22. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19–25.
23. Tai ES, Lim SC, Chew SK, Tan BY, Tan CE. Homeostasis model assessment in a population with mixed ethnicity: the 1992 Singapore National Health Survey. Diabetes Res Clin Pr 2000;49:159–68.
24. Sookoian S, Rosselli MS, Gemma C, Burgueno AL, Fernandez Gianotti T, Castano GO, et al. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1α promoter. Hepatology 2010;52:1992–2000.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\ C_T}$ method. Methods 2001;25:402–8.
26. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C, American Heart A, et al. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation 2004;109:433–8.
27. Chang CL, Huang SY, Soong YK, Cheng PJ, Wang CJ, Liang IT. Circulating irisin and glucose-dependent insulinotropic peptide are associated with the development of polycystic ovary syndrome. J Clin Endocrinol Metab 2014;99:E2539–48.
28. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab 2006;91:4237–45.
29. Gamez-Del-Estal MM, Contreras I, Prieto-Perez R, Ruiz-Rubio M. Epigenetic effect of testosterone in the behavior of *C. elegans*. A clue to explain androgen-dependent autistic traits? Front Cell Neurosci 2014;8:69.
30. Ammerpohl O, Bens S, Appari M, Werner R, Korn B, Drop SL, et al. Androgen receptor function links human sexual dimorphism to DNA methylation. PLoS One 2013;8:e73288.
31. Oberkofler H, Klein K, Felder TK, Krempler F, Patsch W. Role of peroxisome proliferator-activated receptor-gamma coactivator-1α in the transcriptional regulation of the human uncoupling protein 2 gene in INS-1E cells. Endocrinology 2006;147:966–76.
32. Liu Y, Jiang H, Xing FQ, Huang WJ, Mao LH, He LY. Uncoupling protein 2 expression affects androgen synthesis in polycystic ovary syndrome. Endocrine 2013;43:714–23.
33. Roe A, Hillman J, Butts S, Smith M, Rader D, Playford M, et al. Decreased cholesterol efflux capacity and atherogenic lipid profile in young women with PCOS. J Clin Endocrinol Metab 2014;99:E841–7.
34. Nisenblat V, Norman RJ. Androgens and polycystic ovary syndrome. Curr Opin Endocrinol Diabetes Obes 2009;16:224–31.
35. Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. Circulation 2005;112:3066–72.
36. Meigs JB, Wilson PW, Fox CS, Vasan RS, Nathan DM, Sullivan LM, et al. Body mass index, metabolic syndrome, and risk of type 2 diabetes or cardiovascular disease. J Clin Endocrinol Metab 2006;91:2906–12.
37. Sookoian S, Pirola CJ. Epigenetics of insulin resistance: an emerging field in translational medicine. Curr Diab Rep 2013;13:229–37.
38. Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev 1997;18:774–800.
39. Santos JM, Tewari S, Benite-Ribeiro SA. The effect of exercise on epigenetic modifications of PGC1: the impact on type 2 diabetes. Med Hypotheses 2014;82:748–53.
40. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Endocrinol Metab 2006;116:615–22.
41. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G, et al. Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. FASEB J 2010;24:3135–44.
42. Wong J, McLennan SV, Molyneaux L, Min D, Twigg SM, Yue DK. Mitochondrial DNA content in peripheral blood monocytes: relationship with age of diabetes onset and diabetic complications. Diabetologia 2009;52:1953–61.

SUPPLEMENTAL TABLE 1

Primer sets used for methylation-specific PCR and quantification of mtDNA copy number.

Gene	Primer sets (5′ to 3′)	Product size (bp)
Methylation-specific PCR		
PPARGC1A-M	Forward: ATTTTTTATTGTTATGGGGGTAGTC Reverse: AAAAATATTTAAAAACGCAAACGAA	143
PPARGC1A-U	Forward: TTTTATTGTTATGGGGGTAGTTGA Reverse: AAAAAATATTTAAAAACACAAACAAA	141
mtDNAand nDNA amplification		
mtDNA	D41: CGAAAGGACAAGAGAAATAAGG D56: CTGTAAAGTTTTAAGTTTTATGCG	158
GAPDH	Forward: CCACCATGGAGAAGGCTGGGGC Reverse: AGTGATGGCATGGACTGTGGTC	286

Note: PCR = polymerase chain reaction; M = methylated-specific; mtDNA = mitochondrial DNA; nDNA = nuclear DNA; U = unmethylated-specific.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

SUPPLEMENTAL TABLE 2

Clinical and biochemical characteristics of ATPIII [+] and ATPIII [−] PCOS patients.

Characteristic	ATPIII [+] PCOS (n = 104)	ATPIII [−] PCOS (n = 71)	*P* value
Age (y)	28.88 ± 0.24	28.11 ± 0.33	.06
BMI (kg/m^2)	27.27 ± 0.41	21.84 ± 0.36	< .0001
SBP (mm Hg)	116.63 ± 1.23	113.92 ± 1.05	.0011
DBP (mm Hg)	70.02 ± 0.63	68.81 ± 0.72	.0022
Waist circumference (cm)	94.07 ± 0.89	80.15 ± 0.80	< .0001
Waist–hip ratio	0.93 ± 0.004	0.87 ± 0.006	< .0001
Glucose (mmol/L)	4.83 ± 0.06	4.81 ± 0.05	.85
Insulin (mU/L)	13.64 ± 0.73	8.72 ± 0.51	< .0001
HOMA-IR	2.98 ± 0.18	1.88 ± 0.12	< .0001
Total testosterone (nmol/L)	1.24 ± 0.08	1.19 ± 0.12	.56
SHBG (nmol/L)	48.19 ± 3.56	58.39 ± 3.89	.064
FAI	4.23 ± 0.48	2.49 ± 0.37	.046
HDL-C (mmol/L)	1.22 ± 0.03	1.46 ± 0.03	< .0001
LDL-C (mmol/L)	2.96 ± 0.06	2.55 ± 0.07	< .0001
Triglyceride (mmol/L)	2.13 ± 0.08	1.01 ± 0.05	< .0001
Total cholesterol (mmol/L)	4.79 ± 0.07	4.49 ± 0.08	.0069

Note: ATPIII = National Cholesterol Education Program's Adult Treatment Panel III report; BMI = body mass index; DBP = diastolic blood pressure; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; LDL-C = low-density lipoprotein cholesterol; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

SUPPLEMENTAL TABLE 3

Clinical and biochemical characteristics of ATPIII [−] women with PCOS and ATPIII [−] healthy women.

Characteristic	ATPIII [−] PCOS (n = 71)	ATPIII [−] control (n = 106)	*P* value
Age (y)	28.11 ± 0.33	28.07 ± 0.34	.93
BMI (kg/m^2)	21.84 ± 0.36	21.49 ± 0.28	.43
SBP (mm Hg)	113.92 ± 1.05	114.12 ± 1.03	.49
DBP (mm Hg)	68.81 ± 0.72	68.62 ± 0.68	.67
Waist circumference (cm)	80.15 ± 0.80	76.57 ± 0.75	.0018
Waist–hip ratio	0.87 ± 0.006	0.83 ± 0.005	< .0001
Glucose (mmol/L)	4.81 ± 0.05	4.77 ± 0.03	.47
Insulin (mU/L)	8.72 ± 0.51	8.67 ± 0.21	.91
HOMA-IR	1.88 ± 0.12	1.84 ± 0.05	.73
Total testosterone (nmol/L)	1.19 ± 0.12	0.79 ± 0,08	.0032
SHBG (nmol/L)	58.39 ± 3.89	59.47 ± 3.53	.087
FAI	2.49 ± 0.37	1.95 ± 0.58	.037
HDL-C (mmol/L)	1.32 ± 0.02	1.46 ± 0.03	.0003
LDL-C (mmol/L)	2.55 ± 0.07	2.42 ± 0.05	.15
Triglyceride (mmol/L)	1.01 ± 0.05	0.94 ± 0.03	.18
Total cholesterol (mmol/L)	4.49 ± 0.08	4.19 ± 0.05	.0014

Note: ATPIII = National Cholesterol Education Program's Adult Treatment Panel III report; BMI = body mass index; DBP = diastolic blood pressure; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; LDL-C = low-density lipoprotein cholesterol; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

SUPPLEMENTAL TABLE 4

Partial Pearson's correlation coefficients of the methylation ration of *PPARGC1A* promoter (the methylated DNA/unmethylated DNA ratio of the *PPARGC1A* promoter) and participant characteristics.

	Non-PCOS		PCOS	
Variable	***r***	***P* value**	***r***	***P* value**
Age (y)	−0.0629	.4822	0.0870	.2525
BMI(kg/m^2)	0.2387	.0069	0.2918	<.0001
SBP (mm Hg)	0.1045	.1456	0.1156	.1498
DBP (mm Hg)	0.1327	.0781	0.1372	.0724
Waist circumference (cm)	0.2291	.0096	0.3238	<.0001
Waist–hip ratio	0.2283	.0098	0.2123	.0048
Glucose (mmol/L)	−0.0015	.9866	0.1137	.1342
Insulin (mU/L)	0.3331	.0001	0.4145	<.0001
HOMA-IR	0.3134	.0003	0.4	<.0001
Total testosterone (nmol/L)	0.0320	.7234	0.0967	.1517
SHBG (nmol/L)	0.0346	.5123	0.0546	.4236
FAI	0.0722	.3821	0.3024	.0039
HDL-C (mmol/L)	−0.0028	.9748	−0.1448	.0560
LDL-C (mmol/L)	0.0548	.5408	0.0632	.4061
Triglyceride (mmol/L)	0.0010	.9907	0.1637	.0304
Total cholesterol (mmol/L)	0.1305	.1436	0.0349	.6470

Note: BMI = body mass index; DBP = diastolic blood pressure; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; LDL-C = low-density lipoprotein cholesterol; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.

SUPPLEMENTAL TABLE 5

Partial Pearson's correlation coefficients of mtDNA content (mtDNA/nDNA ration) and participant characteristics.

	Non-PCOS		PCOS	
Variable	***r***	***P* value**	***r***	***P* value**
Age (y)	−0.1088	.2234	0.0245	.7480
BMI (kg/m^2)	0.1387	.1199	−0.2531	.0007
SBP (mm Hg)	0.1267	.1357	−0.1174	.0995
DBP (mm Hg)	0.1044	.2181	0.1202	.2524
Waist circumference (cm)	−0.1226	.1698	−0.3049	<.0001
Waist–hip ratio	−0.0865	.3334	−0.2926	<.0001
Glucose (mmol/L)	0.0759	.3966	−0.1291	.0886
Insulin (mU/L)	−0.3311	<.0001	−0.3019	<.0001
HOMA-IR	−0.3193	.0003	−0.3034	<.0001
Total testosterone (nmol/L)	0.0043	.9261	0.0346	.6966
SHBG (nmol/−)	0.0046	.8923	0.0496	.6254
FAI	−0.0623	.4926	−0.3822	<.0001
HDL-C (mmol/L)	−0.0058	.9486	0.0880	.2471
LDL-C (mmol/L)	−0.0620	.4888	−0.0512	.5014
Triglyceride (mmol/L)	−0.0550	.5392	−0.2611	.0005
Total cholesterol (mmol/L)	−0.1118	.2109	−0.0616	.4719

Note: BMI = body mass index; DBP = diastolic blood pressure; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; LDL-C = low-density lipoprotein cholesterol; mtDNA = mitochondrial DNA; nDNA = nuclear DNA; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SHBG = sex hormone-binding globulin.

Zhao. Epigenetic biomarker in PCOS. Fertil Steril 2016.