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ORIGINAL ARTICLE

Expression and regulation of adipocyte fatty acid binding protein in granulosa cells and its relation with clinical characteristics of polycystic ovary syndrome

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Abstract Increased expression of adipocyte fatty acid binding protein (FABP4) is associated with type 2 diabetic, high triglycerides, increased lipid peroxidation, and inflammation markers. To study the expression of FABP4 mRNA in granulosa cells of patients with polycystic ovary syndrome (PCOS) and the impact of testosterone, insulin, and PPARγ agonist rosiglitazone on granulosa cells (GCs), and to investigate the relationship of serum FABP4 levels with clinical characteristics in patients with PCOS. The expression of FABP4 mRNA in GCs of patients with PCOS and normal controls were assayed by RT-PCR. We assessed the level of FABP4 mRNA after treatment with testosterone, insulin, and rosiglitazone in GCs from normal controls. Serum FABP4 were assayed from 96 patients with PCOS (obese and nonobese 48 cases, respectively) and 80 healthy normal controls (obese and the nonobese 40 cases, respectively). The expression of FABP4 mRNA was higher in the GCs of PCOS than that of the controls ($P < 0.05$). FABP4 mRNA expression was up-regulated by testosterone, insulin, and rosiglitazone at different dosages. Serum FABP4 levels were higher in the nonobese PCOS group than that of the nonobese controls (8.9 ± 5.1 ng/ml vs. 4.8 ± 0.7 ng/ml), and in the obese PCOS group than that of the obese controls (28.2 ± 14.0 ng/ml vs. 15.6 ± 6.6 ng/ml), respectively ($P < 0.05$). Multiple linear regression analyses showed that serum FABP4 level was independently associated with HOMA-IR, BMI, and testosterone ($P < 0.05$). Increased FABP4 was related to the clinical characteristics of PCOS.

Keywords Polycystic ovary syndrome · Adipocyte fatty acid binding protein · Granulosa cell

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Introduction

Polycystic ovary syndrome (PCOS) is the most common female endocrinopathy, affecting about 5–10% of reproductive women [1, 2]. It is a heterogeneous disorder and its etiology appears to be complex and multifactorial. Specifically, obesity, insulin resistance (IR), and hyperandrogenism are key features in women with PCOS [3–5]. Epidemiological studies have also confirmed that the incidence of metabolic syndrome (MS), cardiovascular diseases, and type 2 diabetes was significantly higher in PCOS patients than in the controls [6–8]. Recently, there are studies strongly suggesting that PCOS can be defined as a chronic inflammatory disease [9, 10].

FABP4 is a cytoplasmic fatty acid binding protein, mainly presented in adipocytes and macrophages, and also in the circulation. Studies in animal models suggested that FABP4 may be important in glucose homeostasis. Specifically, deletion of the FABP4 gene protected mice from IR and hyperinsulinemia in both diet-induced obesity [11] and genetic obesity [12]. In humans, it has been shown that a promoter polymorphism, T-87C, of the FABP4 gene resulted in decreased adipose tissue FABP4 expression and reduced risk for type 2 diabetes and cardiovascular disease [13]. In addition, previous studies demonstrated that circulating FABP4 predicts the development of the MS [14, 15] and type

2 diabetes [16]. All these results suggested that FABP4 is a regulator of systemic insulin sensitivity and lipid metabolism. Previous study on FABP4 expression in mice ovarian GCs revealed that FABP4 might be involved in the apoptosis of GCs [17]. As to the correlation of FABP4 with the characteristic of PCOS, we reported that FABP4 gene showed significant up-regulation in PCOS patients in a previous study comparing gene expression profiles between PCOS patients and control subjects [18]. The results lead to the hypothesis that FABP4 may have a role in the pathological mechanism of the multiple characteristics of PCOS. To further explore this possibility, the current study was designed (1) to compare the expression levels of FABP4 mRNA on GCs between PCOS patients and normal subjects, (2) to test the effect of testosterone, insulin, and PPARγ agonist rosiglitazone on FABP4 mRNA in GCs in vitro, and (3) to compare the serum FABP4 protein levels by Enzyme-linked Immunosorbent Assay (ELISA) in PCOS patients and controls.

Subjects and methods

Clinical subject's criteria

We followed the PCOS diagnostic criteria [19] revised on ESHRE/ASRM Rotterdam Conference in October 2003. The recruitment criteria for the controls were (1) regular menstruation, without clinical manifestations of PCOS; (2) detection of normal endocrine; (3) not being detected of polycystic ovarian changes, audio and visual changes of uterine fibroids and endometriosis by ultrasound; and (4) without fever or inflammatory diseases. All patients were not suffering from thyroid and adrenal disease, and they had not used any hormonal drugs for at least 3 months. Informed consents were obtained from subjects for the study and the research protocol had been approved by the Ethics Committee of Peking University Third Hospital.

FABP4 mRNA expression on GCs with PCOS

Eligible patients included five PCOS patients who received in vitro fertilization-embryo transfer (IVF-ET) from May to August in 2007 at the Reproductive Center of Peking University Third Hospital and five normal controls who received IVF-ET at the same Center due to their husbands' infertility during the same period. The five PCOS patients and five normal controls were with an age range between 28 and 31, and body mass index (BMI) ranging from 23 to 24 kg/m^2. On the second day in the last pre-IVF menstrual cycle, serum testosterone concentration was between 2.8 and 3.0 nmol/l in PCOS patients but less than 2.8 nmol/l in controls. Other inter-group differences showed no statistical significance.

Isolation of 22 human ovarian GCs

Human luteinized GCs were picked from all subjects. After ovum was picked up, follicular fluid was rapidly transferred to the lab for isolation of GCs. The layer of GCs was washed in PBS (Sino-American Biotechnology Company) for three times and centrifuged at room temperature (RT) at 1,800 rpm for 8 min. GCs were separated using 50, 90 double layers of Percoll cell separation liquid (Sino-American Biotechnology Company) at RT at 2,000 rpm for 30 min [20]. The GCs layer was collected in the border of two layers Percoll, then drawn into the 0.84% ammonium chloride solution, water bath shaken at 37°C for 20 min, and then centrifuged at 1,800 rpm for 8 min. Then after, the layer was sedimented and suspended with 10 ml PBS containing 0.1% collagenase for 10 min at 37°C, then centrifuged at 1,800 rpm for 8 min again. The sedimented GCs were suspended with 10 ml PBS again, filtrated through 300-mesh cell screen, and centrifuged again at 1,800 rpm for 8 min. The final sedimented GCs were used for either RNA extraction or cell culture.

Detection of FABP4 mRNA expression in granulosa cells

Trizol (Beijing Dingguo Biotechnology Company) was used to extract RNA, which was reverse-transcribed into cDNA (Superscript^{TM} First-Strand Synthesis System for RT-PCR kit: Invitrogen, USA). RT-PCR (Svfa green) system (MJ Research, USA) was then applied to detect FABP4 mRNA in GCs with 18S as internal control. The 18S primer sequence was: 5′-GGA AGG GCA CCA CCA GGA GT-3′ and 5′-TGC AGC CCC GGA CAT CTA AG-3′ (298 bp). The FABP4 primer sequence was: 5′-TCA GTG TGA ATG GGG ATG TGA-3′ and 5′-CCC TTG GCT TAT GCT CTC TCA-3′ (259 bp). PCR parameters were 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min for 28 cycles. PCR products were analyzed by 1% agarose gel electrophoresis and visualized using ethidium bromide. The predicted sizes of RT-PCR products for FABP4 and 18S were 259 and 298 bp, respectively.

The effect of testosterone (Sigma, USA), insulin, and PPARγ agonist rosiglitazone (Sigma, USA) on cultured GCs FABP4 mRNA in vitro, were investigated. Isolation of human ovarian GCs and real-time RT-PCR were same as above mentioned. Ovarian GCs from 22 controls were cultured and treated with testosterone, insulin, and rosiglitazone of different concentrations, respectively.

The GCs were adjusted to 2–4 $\times$ 10^5/ml with M199 (Sigma, USA) cell culture solution containing 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin, 10 μg/ml streptomycin. They were later seeded in six-well cell culture plates (Corning, USA), each well with

approximately 1–2 × 10^5 cells at 37°C in 5% CO_2 incubator for 48 h. The culture solution was changed after 48 h, and 1 ml M199 cell culture medium was added into each well. The culture medium was supplemented with different concentrations of testosterone (1 and 10 nM), insulin (10 and 100 nM), and rosiglitazone (1 and 10 nM). The culture plates were kept at 37°C in 5% CO_2 incubator for 24 h. At the end of experiments, the culture medium was discarded and the cells were collected and washed for three times with PBS. Then 1 ml Trizol was added, mixed gently, left for 5 min at RT and frozen at −80°C for RT-PCR.

Clinical study

Subjects were recruited from January to August in 2007 at the Reproductive Center of Peking University Third Hospital outpatients. 96 Patients with PCOS were divided into the obese PCOS group and the nonobese PCOS group (48 cases, respectively), and 80 healthy controls were divided into the obese control group and the nonobese control group (40 cases, respectively) based on their BMI. Subjects with BMI $\geq$ 25 kg/m^2 were considered as obese, and BMI $<$ 25 kg/m^2 were considered as nonobese.

Subjects attended each visit after an overnight fast of at least 10 h. Anthropometric measurements (height and weight) and fasting glucose, fasting insulin, total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were completely measured. The homeostasis model assessment index (HOMA-IR) was calculated as fasting glucose (in mmol/l) times fasting insulin (in mIU/l) divided by 22.5. Serum FABP4 was measured with an ELISA (BioVendor Laboratory Medicine, Inc, Modrice, Czech Republic; intra-assay and interassay coefficients of variation of 2.6–5.1% and 3.9–6.6%, respectively). Serum C-reactive protein (CRP) was assayed with Beckman-Coulter ARRAY-360 special protein array system by rate nephelometry.

Statistical analysis

FABP4mRNA analysis was conducted using Prism software package. Results were illustrated as mean ± standard deviation ($\bar{x} \pm$ SEM). We adopted *t*-test for inter-group comparison. $P < 0.05$ was recognized as statistically significant.

SPSS 10.0 package was used for serological analysis. Results were presented as mean ± standard deviation ($\bar{x} \pm$ s). Normal distribution of the two groups was subjected to *t*-test for comparison and Wilcoxon Rank-Sum test for comparison between groups of skewed distribution. Pearson correlations or 1-way ANOVA were used as appropriate for comparisons between 2 groups. We determined partial correlation coefficients between FABP4 and other continuous variable by using a partial correlation test adjusted for BMI. Stepwise multiple linear regression analysis was done to determine the variable with independent significant association with FABP4. Probability (*P*) values less than 0.05 were considered statistically significant.

Results

Expression of FABP4 mRNA in GCs of PCOS patients, and the expression of FABP4 mRNA relation to testosterone, insulin, and rosiglitazone concentration in normal GCs

The FABP4 mRNA levels were increased dramatically, almost six times higher in PCOS patients than in controls ($P = 0.047$) (Fig. 1).

When testosterone concentration was 1 nM, expression of FABP4 mRNA was higher in the GCs compared to the blank control, but the difference was not statistically significant ($P = 0.15$). When testosterone concentration was 10 nM, FABP4 mRNA increased significantly in the GCs compared to the blank control ($P = 0.03$) (Fig. 2).

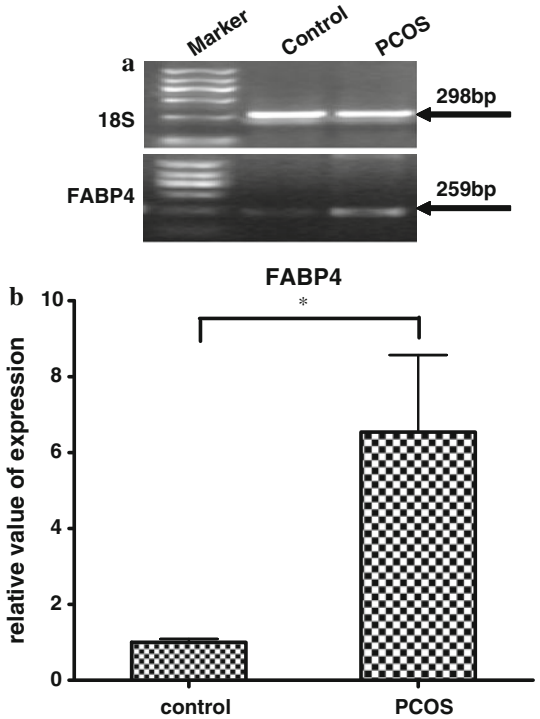

Fig. 1 Expression of FABP4 mRNA in PCOS and controls. **a** PCR products, **b** mRNA levels of FABP4 and 18S (as a control) were analyzed by RT-PCR in GCs from PCOS patients and controls. * $P < 0.05$ compared with medium-only control. Marker sequence (*from bottom to top*): 100, 300, 500, 700, 900, 1200 bp

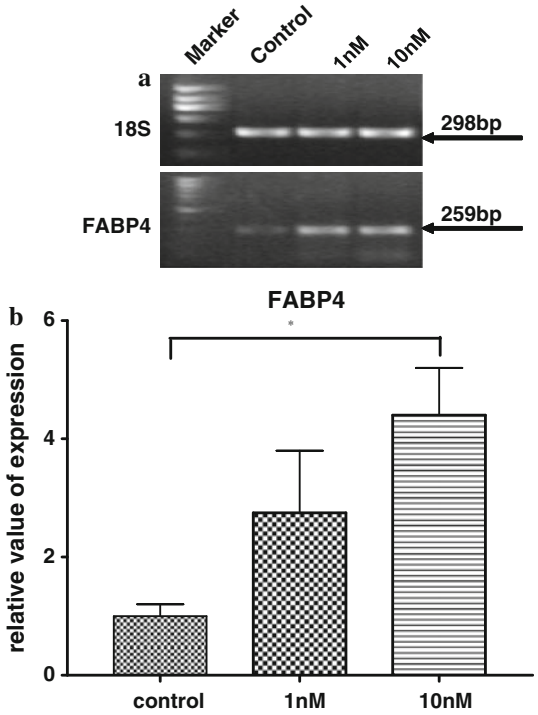

Fig. 2 Upregulation of FABP4 in testosterone-induced GCs. **a** PCR products, **b** mRNA levels of FABP4 and 18S (as a control) were analyzed by RT-PCR in GCs from groups with different testosterone concentration and controls. * $P < 0.05$ compared with medium-only control. Marker sequence (*from bottom to top*): 100, 300, 500, 700, 900, 1200 bp

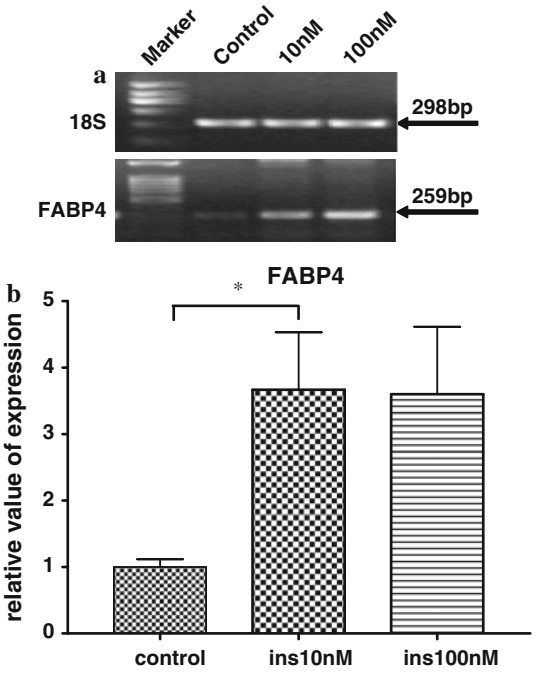

Fig. 3 Upregulation of FABP4 in insulin-induced GCs. **a** PCR products, **b** mRNA levels of FABP4 and 18S (as a control) were analyzed by RT-PCR in GCs from groups with different insulin concentration and controls. * $P < 0.05$ compared with medium-only control. Marker sequence (*from bottom to top*): 100, 300, 500, 700, 900, 1200 bp

When insulin concentration was 10 nM, the expression of FABP4 mRNA was higher in the GCs as compared with the blank control ($P = 0.04$). When insulin concentration was 100 nM, the expression of FABP4 mRNA increased but the difference was not statistically significant ($P = 0.147$) (Fig. 3).

When rosiglitazone concentration was 1 nM, the expression of FABP4 mRNA in ovarian GCs significantly increased compared with the blank control ($P = 0.007$). When rosiglitazone concentration was at 10 nM, the FABP4 mRNA expression significantly increased compared to the concentration at 1 nM ($P = 0.049$) (Fig. 4).

Serum FABP4 correlation with the clinical characteristics of PCOS

Nonobese subjects with PCOS had significantly higher FABP4, TC, and HOMA-IR than controls ($P < 0.05$); obese subjects with PCOS had significantly higher FABP4, CRP, and HOMA-IR than controls ($P < 0.05$), and adverse HDL-C ($P < 0.05$) (Table 1). The level of serum FABP4 was found to be significantly increased in the obese group of control and PCOS compared with the nonobese groups of control and PCOS, respectively ($P < 0.05$) (Table 1).

Univariate analysis revealed that serum FABP4 levels were positively correlated with BMI ($r = 0.531$, $P = 0.000$), testosterone ($r = 0.247$, $P = 0.020$), HOMA-IR ($r = 0.549$, $P = 0.000$), and CRP ($r = 0.474$, $P = 0.000$), but inversely with serum HDL-C ($r = -0.317$, $P = 0.003$).

Partial correlation showed that serum FABP4 levels were positively correlated with testosterone ($r = 0.242$, $P = 0.026$), HOMA-IR ($r = 0.409$, $P = 0.000$) and CRP ($r = 0.318$, $P = 0.003$), however, they had no correlation with other clinical index ($P > 0.05$).

Multiple linear regression analyses showed that serum FABP4 level was independently associated with BMI ($P = 0.000$), HOMA-IR ($P = 0.000$), and testosterone ($P = 0.037$) (Table 2).

Discussion

In our previous study, it was found that FABP4 mRNA expression significantly increased in PCOS patients compared to controls by using gene chip analysis technique [18]. In the current study, we verified the result by demonstrating that FABP4 mRNA expression was higher in PCOS patient's ovarian GCs than in the controls with real-time RT-PCR. The role of FABP4 in GCs and in the

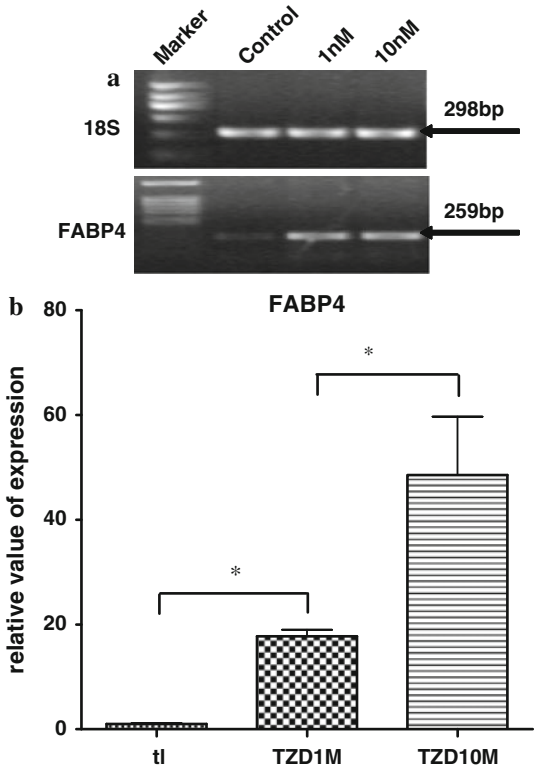

Fig. 4 Upregulation of FABP4 in rosiglitazone-induced GCs. **a** PCR products, **b** mRNA levels of FABP4 and 18S (as a control) were assayed by RT-PCR in GCs from groups with different rosiglitazone concentration and controls. * $P < 0.05$ compared with rosiglitazone concentration at 1 nM and medium-only control. Marker sequence (*from bottom to top*): 100, 300, 500, 700, 900, 1200 bp

pathologies of PCOS was further explored in the current study.

In vitro studies in adipocytes demonstrated that FABP4 mRNA is regulated by fatty acids, PPARγ, and insulin [17, 21–24]. FABP4 is known to solubilize lipophilic fatty acids and facilitate their intracellular transport, positioning lipid ligands of PPARγ in close proximity to the nuclear receptor allowing selective enhancement of PPARγ transcriptional activity [25]. Evidence suggests that FABP4 expression is regulated by PPARγ through the PPRE element presented in the FABP4 gene promoter [26]. PPARγ is located in the nucleus of luteal cells where it may affect luteal cell survival via regulating the expression of bcl-2 [27]. Moreover, PPARγ agonist rosiglitazone, either through systemic improvements to specific metabolic parameters or by direct modulation of PPARγ-regulated gene expression (CD36, FABP4) in ovarian cells, is able to reverse the deficits in oocyte quality resulted from DIO [28]. Our current results showed that rosiglitazone increased FABP4 mRNA in a dose-related manner in human GCs. Namely, we identified significant up-regulation of FABP4 gene, which is known to possess PPARγ response elements (PPREs) in proximal promoter regions and to be regulated in response to PPARγ activation in human GCs. In addition, we also found that low concentration of insulin can up-regulate the expression of FABP4 mRNA in human GCs. Similarly, FABP4 mRNA expression significantly increased compared with the control in the presence of high concentration of testosterone (T).

Table 1 Serum FABP4 and clinical characteristics of the group of PCOS and control group ($\bar{x}\pm s$)

Clinical characteristics	BMI < 25 (kg/m^2)		BMI ≥ 25 (kg/m^2)	
	Control ($n = 40$)	PCOS group ($n = 48$)	Control ($n = 40$)	PCOS group ($n = 48$)
Age (year)	30.3 ± 3.4	28.5 ± 2.8	30.0 ± 2.1	30.8 ± 3.3
BMI (kg/m^2)	21.5 ± 1.8	22.3 ± 1.5	26.9 ± 1.9	27.6 ± 1.8
FABP4 (ng/ml)	8.9 ± 5.1	$12.7 \pm 4.2^{\Delta\Delta\Delta}$	15.6 ± 6.6	$28.2 \pm 13.8^{\Delta\Delta}$
PRL (μg/l)	14.7 ± 6.2	$17.6 \pm 8.2^{\Delta}$	15.3 ± 4.5	13.9 ± 4.8
E2 (pmol/l)	107.2 ± 43.2	$117.6 \pm 47.9^{\Delta}$	102.7 ± 25.9	135.0 ± 47.1
LH/FSH	0.5 ± 0.3	$2.2 \pm 1.9^{\Delta\Delta\Delta}$	0.5 ± 0.2	$1.6 \pm 1.0^{\Delta\Delta}$
T (nmol/l)	1.1 ± 0.3	$2.6 \pm 1.8^{\Delta\Delta\Delta}$	1.3 ± 0.5	$2.6 \pm 1.2^{\Delta\Delta\Delta}$
A (nmol/l)	5.4 ± 1.7	$12.5 \pm 5.5^{\Delta\Delta\Delta}$	7.1 ± 1.8	$10.3 \pm 4.5^{\Delta\Delta\Delta}$
TC (mmol/l)	3.8 ± 1.5	$4.8 \pm 0.7^{\Delta\Delta}$	4.5 ± 0.9	4.5 ± 0.8
TG (mmol/l)	0.9 ± 0.3	$1.4 \pm 1.6^{\Delta}$	1.2 ± 0.7	2.6 ± 3.4
HDL-C (mmol/l)	1.5 ± 0.4	1.4 ± 0.5	1.5 ± 0.3	$1.2 \pm 0.2^{\Delta\Delta\Delta}$
LDL-C (mmol/l)	2.6 ± 0.9	2.7 ± 0.7	3.0 ± 0.7	2.7 ± 0.7
HOMA-IR	1.0 ± 0.3	$2.3 \pm 1.0^{\Delta\Delta\Delta}$	1.2 ± 0.5	$5.0 \pm 4.6^{\Delta\Delta\Delta}$
CRP	0.92	1.41^{Δ}	1.23	$3.89^{\Delta\Delta\Delta}$

Note: The group of PCOS compared with the control: $^{\Delta}$ $P < 0.05$; $^{\Delta\Delta}$ $P < 0.01$; $^{\Delta\Delta\Delta}$ $P < 0.001$

Table 2 Stepwise multiple regression analysis showing the parameters with significant independent associations with FABP4

	Coefficient B	SE (B)	*P* value
HOMA-IR	1.346	0.263	0.000
BMI	1.056	0.194	0.000
T	1.066	0.128	0.037

Multiple linear regression analyses showed that serum FABP4 level was independently associated with BMI ($P = 0.000$), HOMA-IR ($P = 0.000$) and testosterone ($P = 0.037$)

FABP4 is one of the most abundant proteins secreted from adipocytes and our study confirmed the presence of circulating FABP4 in humans. The physiological function of plasma FABP4 is not well known. But there is increasing evidence about the relationships between FABP4 concentrations and MS, obesity and type 2 diabetes [14–16, 29–31]. Obesity and IR are clinical characteristics of women with PCOS. Möhlig et al. [32] reported that circulating FABP4 was correlated with markers of obesity, but had no major impact on IR, inflammation, or hyperandrogenemia in PCOS women. Our present case–control study indicated that serum FABP4 levels were significantly higher in the obese and nonobese PCOS group than that of the obese and nonobese controls, respectively. Stepwise multiple linear regression analysis showed that serum FABP4 level was independently associated with BMI. Given the fact that approximately 50% of PCOS women are overweight or obese [33] and adipose tissue is the major contributor of circulating FABP4 [13], circulating FABP4 may well contribute to the disorders associated with obesity in PCOS patients.

IR is definitely a key component of PCOS [34]. Both lean and obese women with PCOS have peripheral IR and hyperinsulinemia [35]. Studies in both animals [11, 12] and humans [13–15] suggest FABP4 to be an important mediator of IR. In the present study, serum FABP4 level in PCOS patients was associated with HOMA-IR and stepwise multiple linear regression analysis results showed that serum FABP4 level was independently associated with HOMA-IR. Combined together, the above evidences suggest that FABP4 may have a role in IR in patients with PCOS.

Our previous study found that increased serum FABP4 level in the PCOS patients was correlated with high androgen level [36]. In our present study with increased sample size, serum FABP4 levels were shown to be significantly and positively correlated with testosterone. Stepwise multiple linear regression analysis showed that serum FABP4 level was independently associated with testosterone. In PCOS, markers of obesity are associated with hyperandrogenemia [3–5]. But the exact relationship of circulating FABP4 with testosterone remains to be determined.

FABP4 is proinflammatory, in that the ablation of FABP4 in macrophages leads to reduced IκB kinase and NF-κB activity, diminished cyclooxygenase-2 and inducible nitric-oxide synthase expression, and impaired production of proinflammatory cytokines [37, 38]. Studies reported that serum FABP4 levels correlated positively with serum CRP levels in humans [15]. In present study, we also showed that serum FABP4 levels were positively correlated with CRP. Serum FABP4 may participate in the development of chronic inflammation in PCOS patients.

FABP4 is proposed to contribute to dyslipidemia [39]. In humans, decreased adipose tissue expression of FABP4 in subjects homozygous for a functional polymorphism, which is associated with a reduced risk of hypertriglyceridemia [12]. This hypothesis is supported by our present study showing that serum FABP4 levels were significantly and negatively correlated with HDL-C. Based on these results, it can be suggested that plasma FABP4 concentrations should be taken as a marker of dysmetabolic condition in patients of PCOS.

Although the role of FABP4 on GCs is unknown, we confirmed the increased expression of FABP4 mRNA in ovary GCs and its regulation by testosterone, insulin, and rosiglitazone in PCOS patients. We also showed that circulating FABP4 levels were increased in PCOS and high FABP4 levels were related with dysmetabolic. Obesity, hyperandrogenism, and IR could be major factors in increasing FABP4 levels in PCOS.

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Conflict of interest All authors declare that there is no conflict of interest.

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