Assessment of serum fetuin-A and its gene polymorphism as a marker of insulin resistance in polycystic ovary syndrome

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Background/aim
Polycystic ovary syndrome (PCOS) is a prevalent endocrine condition affecting 5–10% of reproductive-aged women, the cause of which is unknown. Chronic anovulation, polycystic ovaries, and hyperandrogenism are symptoms of PCOS. It is linked to hirsutism, obesity, and increased probability of cardiovascular disease, metabolic syndrome, and diabetes mellitus. A risk factor for cardiovascular disease is PCOS that is undiagnosed or untreated. Our aim in this study is to investigate serum fetuin-A level and its gene as potential biomarkers for screening of insulin resistance in PCOS.

Patients and methods
This study involved 100 female participants from outpatient clinic, Department of Obstetrics and Gynecology, Zagazig University Hospital, Egypt. They were split into two groups (each 50). The first group included healthy fertile women without symptoms of hyperandrogenemia as a control. The second group included women with PCOS. Fasting blood sugar levels, cholesterol, high-density lipoprotein cholesterol and triglycerides have been estimated by enzymatic colorimetric technique while low-density lipoprotein cholesterol was calculated. Enzyme-linked immunosorbent assays have been used to measure serum concentrations of luteinizing hormone, follicular-stimulating hormone, testosterone, and fetuin-A, while PCR has been used to extract DNA and genotype common functional polymorphisms in fetuin-A.

Results
The present results revealed a considerable rise in glucose, insulin, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), cholesterol, triglyceride, low-density lipoprotein cholesterol, luteinizing hormone, testosterone, and fetuin-A and significant decrease in follicular-stimulating hormone and high-density lipoprotein cholesterol in PCOS patients. Also, there was significant higher frequency of the fetuin-A gene variant rs1071592 AA genotype and A allele compared to controls.

Conclusions
Fetuin-A has a potential diagnostic value as a biomarker for insulin resistance in PCOS associated with metabolic syndrome. Additionally, 'CG' allele can be considered a risk factor for PCOS.

Keywords:
fetuin-A, gene polymorphism, insulin resistance, polycystic ovary syndrome

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Introduction

Among the most prevalent endocrine and metabolic disorders affecting teenage girls is polycystic ovary syndrome (PCOS). Hyperandrogenism (HA), symptomatology consistent with HA, and oligo or amenorrhea (OA) are the three main markers of this condition. The majority of cases feature obesity and other metabolic abnormalities. Along with HA and problems of reproduction, compensatory hyperinsulinemia and insulin resistance (IR) are increasingly thought to have a major role in how the PCOS may develop [1].

Women with PCOS are more likely to have obesity, dyslipidemia, long-term inflammation, metabolic syndrome, type 2 diabetes mellitus (T2DM), atherosclerosis, and cardiovascular disease (CVD). In general, up to 70% of women with PCOS are impacted by IR. Despite several studies, the processes that cause IR in PCOS individuals are still not clear. PCOS has a complicated pathogenesis, and its exact cause is still unknown. Heterogeneity is a fundamental aspect of PCOS, from its definition to its phenotype, and it also plays a role in its development. The relative contribution of too much androgen and other

factors, like being overweight and IR, to the development of PCOS has also taken different forms in different phenotypes of the condition [2].

The 64 kD glycoprotein known as Alpha 2-Heremans Schmid Glycoprotein or fetuin-A was previously thought to be a hepatokine. Recently, though, it has been discovered by some studies that fat cells can also express, and release fetuin-A, thus it is classified as an adipokine as well as a hepatokine. Obese patients with T2DM, nonalcoholic fatty liver disease, metabolic syndrome, PCOS, and CVD have been found to have either increased, decreased or unchanged circulating fetuin-A levels, with or without a relation to impaired glucose tolerance and IR. Further research is required in light of these contradictory results regarding the connection between metabolic diseases and fetuin-A, as well as IR [3].

Fetuin-A has been linked to lipid and glucose metabolism as well as IR, including inhibition of insulin action by blocking IR tyrosine kinase and autophosphorylation of glucose transporter 4; and in conjunction with saturated fatty acids, fetuin-A may induce chronic inflammation via the toll-like receptor 4, resulting in IR; and increased mRNA and protein levels in ob/ob mice. IR is brought on by impaired adipocyte function; elevated endoplasmic reticulum (ER) stress and fetuin-A expression are linked, and ER stress results in IR development [4].

The fetuin-A gene, which is linked to T2DM and cardiometabolic diseases, was found at locus 27 on the (q) arm of chromosome 3 [5].

Despite the fact that numerous studies have examined the mechanism of metabolic abnormalities and IR in PCOS patients over the past few decades, the current diagnostic criteria do not include indicators reflecting these conditions. Therefore, it is crucial to search for circulating biomarkers in PCOS patients that represent IR and metabolic disorders. IR is regarded as a significant pathophysiological component of PCOS, worsening the clinical manifestation of the condition by causing HA. Although IR in lean women manifests mechanistically differently from IR brought on by obesity, being overweight or obese worsens IR and the ensuing hyperinsulinemia. Additionally, weight gain over time and the prevalence of overweight and obesity are both higher in women with PCOS [6].

Fetuin-A directly affects IR while modulating inflammatory responses and causing various metabolic changes. In studies on humans and animal models, the role of fetuin-A in the IR mechanism was demonstrated. Therefore, the current study was against disclosing the connection between fetuin-A levels and ovarian cysts [7].

Our aim in this study was to investigate the association between serum fetuin-A level and its gene as a useful biomarker to identify PCOS in women.

Patients and methods

Patients and experimental design

This study involved 100 female participants. They were divided into two groups (50 each). As follows:

(1) Group 1: healthy fertile women without symptoms of hyperandrogenemia as a control.
(2) Group 2: women with PCOS.

All patients were at the outpatient clinic, Department of Obstetrics and Gynecology, Zagazig University Hospital. All participants have completed detailed questionnaires with information about their medical and surgical history, lifestyle habits and family history.

Inclusion criteria

Patients were adult female volunteers between the ages of 18 and 40, who have regular periods and no signs of hyperandrogenemia, such as hirsutism or acne. Patients who meet at least two of the three criteria for PCOS established by the 2003 Rotterdam Consensus; HA, an ultrasound showing polycystic ovaries, and oligo/anovulation were include [8].

Exclusion criteria

Patients receiving chemotherapy, hormone therapy, and radiotherapy were typically excluded from the study. People with a history of AIDS, liver, kidney, or other infectious or viral infections, as well as those with aberrant karyotypes, were also excluded. Women who had a history of uterine and vaginal surgical correction were disqualified. Additionally, patients who smoke are also not included.

Ethical consideration

In accordance with the values outlined in the Declaration of Helsinki, the current study was carried out in accordance with the World Medical Association's Code of Ethics. The local Ahram Canadian University (ACU), Egypt, Ethics Committee has approved this project with approval number (PBC-2019-04). Each participant gave written informed consent before being enrolled in the study.

Methods

Sample collection

Fasting venous blood samples were taken between days 2 and 5 of their cycles, during the early follicular phase, in totally sterile settings. Each participant contributed 6 ml of blood in one of two tubes. For DNA extraction and genotyping, 2 ml of the sample was obtained in a sterile EDTA-coated vacutainer (BD Vacutainer; Catalog number: 367841). For the purpose of separating the serum, 4 ml was transferred into sterile tubes without the use of an anticoagulant (BD Vacutainer; Catalog No. 368774). While blood vials were appropriately labeled and stored at −80°C, serum was separated as soon as the sample was collected and kept there until hormone profiling. Blood samples from all females were taken during a small window (20th-24th day of the cycle), when the majority of women were in mid-luteal phase, in an effort to limit the hormonal variation during the menstrual cycle.

Biochemical analysis

Evaluation of insulin resistance

Using Centronic Co. (Wartenberg, Germany) kits and an enzyme colorimetric approach, fasting blood glucose levels were calculated using the Trinder method [9]. Enzyme-linked immunosorbent assays (ELISA) based on the technique of Berson and Yalow [10] was used to measure the plasma insulin levels using a BioSource INSEASIA Co. (Nivelles, Belgium) Kit. According to Mathews *et al.* [11], IR was assessed by calculating HOMA-IR using the formula (fasting glucose×fasting insulin)/405.

Estimation of lipid profile

The colorimetric enzymatic analyses depend on the methods of Allain *et al.* [12], Lopes-Virella *et al.* [13], and Glick *et al.* [14] have been used to quantify cholesterol, high-density lipoprotein cholesterol (HDL-C), and triglyceride, respectively. The kits were provided by Biocon Diagnostic (Germany). Low-density lipoprotein cholesterol (LDL-C) has been calculated as total cholesterol−(HDL-C+TG/5) according to the formula proposed by Friedewald *et al.* [15].

Estimation of sex hormone

Serum levels of follicular-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured by ELISA according to the protocols of Nordin *et al.* [16], Harris and Naftolinf [17], and McCann and Kirkish [18].

Quantitative analysis of fetuin-A

ELISA (BioVendor Human Fetuin-A ELISA, Germany) was used to quantify serum fetuin-A levels, as described by Mori *et al.* [19].

Genotyping of the common functional polymorphisms of fetuin-A (Thr256Ser) using PCR-RFLP technique

DNA extraction

Using Fermentas' GeneJET_ Genomic DNA Purification Kit, DNA was isolated from peripheral blood leukocytes from freshly obtained whole blood. Using a Thermo Scientific NanoDrop_ 1000 Spectrophotometer, the purity and concentration of genomic DNA were assessed (Thermo Fisher Scientific, Wilmington, Delaware, USA).

PCR amplification of fetuin-A gene

Five microliters of the extracted DNA were combined with one microliter of each forward and reverse primer and 12.5 µl of DreamTaq_ Green PCR Master Mix (2) to make a total volume of 25 µl for the PCR amplification of the retrieved DNA. The primers were as follows: F5-GTCACCCCTCCTTGTA AC-3 (sense) (Tm=45.60°C) (bought from Fermentas Life Sciences). R5-CCCCAATGAGACC ACA-3 (antisense) (Tm=460°C) is the reverse primer. The following reaction conditions were used for the PCR on the thermal cycler (Biometra): initial denaturation at 950°C for 5 min, followed by 35 cycles of denaturation at 940°C for 1 min, annealing at 590°C for 1 min, extension at 720°C for 1 min, and finally extension at 720°C for 15 min.

Genotyping of fetuin-A gene (Thr256Ser) polymorphism

Fast Digestive SacI restriction enzyme 100 l (from Ferment as Life Sciences) was utilized for the mutational study of fetuin-A Thr256Ser (c.766C>G) at Sac I recognition site. Each 10 µl of PCR product was mixed with 1 µl of Fast Digestive enzyme, 2 µl of Fast Digestive buffer, and diluted to a volume of 30 µl using nuclease-free water. Gently combining them, they were incubated at 37°C for 60 min. On 1.5% agarose gel, the products of digestion were separated. The allele G produces 193-bp and 212-bp fragments while allele C does not have a SacI site and stays undigested as 405 bp fragments. Thr/Thr (allele C), Thr/Ser (heterozygote), and Ser/Ser (allele G) are the three genotypes for fetuin-A that can exist (homozygote for absence of the Sac I site, heterozygote and homozygote for the presence of the Sac I site; respectively) [20].

Statistical analysis

All information was presented as mean±SE. The standard state test (part of the SPSS software) has verified that the data are distributed normally (version 18, IBM SPSS, IBM Corp., Armonk, N.Y., USA). Statistical significance was determined using one-way analysis of variance and Tukey's post-hoc tests (tests with more than two groups and one variable). A

correlation coefficient calculated by Pearson was found. Statistical significance was determined by the *P* value of 0.05.

Results

The present results reported in Table 1 showed significant increases ($P<0.05$) in the levels of fasting blood sugar (FBS), serum insulin and HOMA-IR in PCOS group when compared with control group. This result denotes the presence of IR in PCOS group.

The results represented in Table 2 showed significant increases ($P<0.05$) of serum cholesterol, TG, LDL-C levels in PCOS group when compared with control group, while significant decrease ($P<0.05$) in serum level of HDL-C was obtained in PCOS group when compared with control group.

The data represented in Table 3 showed significant increases ($P<0.05$) of serum LH, testosterone and fetuin-A levels in PCOS group when compared with control group, meanwhile significant decreases ($P<0.05$) was exhibited in serum FSH level in PCOS group when compared with control group.

The data in Table 4 represented the distribution of fetuin-A gene polymorphism between women with PCOS and healthy control groups and showed PCOS patients had a significant higher frequency of the fetuin-A gene variant CG genotype although, *P* value were not significantly different when compared to controls.

The present study reported that there are significant positive correlation between serum levels of fetuin-A and FBS ($R^2=0.0542$, $P<0.05$), fasting insulin ($R^2=0.0153$, $P<0.05$), HOMA-IR ($R^2=0.0376$, $P<0.05$), total cholesterol ($R^2=0.2371$, $P<0.05$), LDL ($R^2=0.0039$, $P<0.05$), and triacylglycerol ($R^2=0.1876$, $P<0.05$), while significant negative correlation with HDL ($R^2=0.5049$, $P<0.05$) was obtained, as shown in Figs 1 and 2. In addition, the results revealed that fetuin-A level was negatively correlated with FSH ($R^2=0.1507$, $P<0.05$), while it positively correlated with both LH ($R^2=0.183$) and testosterone ($R^2=0.0911$) at *P* value less than 0.05, as shown in Fig. 3.

Discussion

Around 5–8% of reproductive-aged women have PCOS [21]. The fact that both genetics and the environment play a role in its development indicates that it is a multifactorial condition. The condition includes polycystic ovaries, high androgen levels, and persistent anovulation [22]. The systemic and metabolic pathophysiology of this syndrome can lead to CVD, IR, T2DM, hyperinsulinemia, and dyslipidemia [23,24].

In our study, we demonstrate a novel association between human fetuin-A and its gene in PCOS-

Table 1 Fasting blood glucose and serum levels of insulin and HOMA-IR in women with polycystic ovary syndrome and control groups

	Control	PCOS
FBG (mg/dl)	86.27±7.9	91.27±14.6*
Insulin (μIU/ml)	11.45±2.5	20.07±5.6*
HOMA-IR	2.44±0.95	4.53±1.9*

All data are expressed as mean±SE. FBG, fasting blood glucose; PCOS, polycystic ovary syndrome. *Significant difference than control group at *P* value less than 0.05, using Student *t* test.

Table 2 Serum levels of cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in women with polycystic ovary syndrome and control groups

	Control	PCOS
Cholesterol (mg/dl)	176±15.6	194±16.7*
Triglyceride(mg/dl)	70.92±7.7	89.9±8.1*
HDL-C (mg/dl)	75.63±5.4	51.4±4.9*
LDL-C (mg/dl)	85.78±8.7	124.09±11.1*

All data are expressed as mean±SE. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PCOS, polycystic ovary syndrome. *Significant difference than control group at *P* value less than 0.05, using Student *t* test.

Table 3 Serum levels of follicular-stimulating hormone, luteinizing hormone, testosterone and fetuin-A in women with polycystic ovary syndrome and control groups

	Control	PCOS
FSH (IU/l)	7.21±1.01	5.57±0.8*
LH (IU/l)	5.19±0.9	7.32±1.05*
Testosterone (nmol/l)	1.01±0.39	1.57±0.45*
Fetuin-A (μg/l)	301.17±18.4	414.6±19.5*

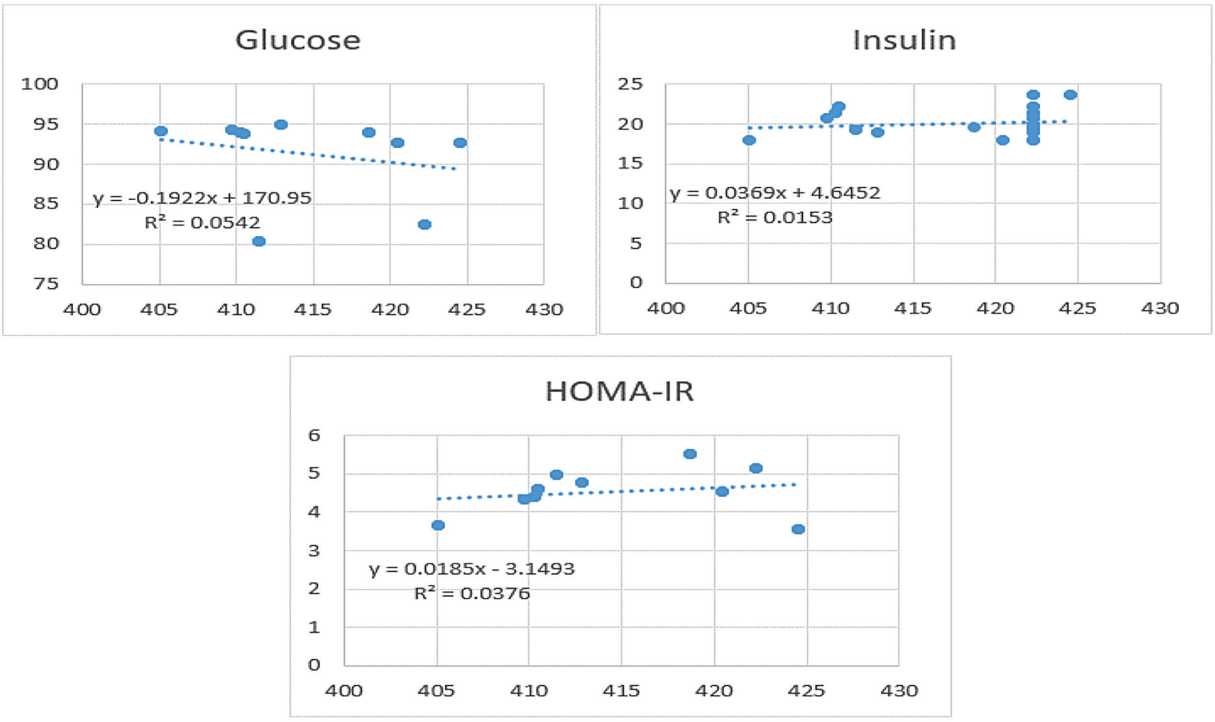
All data are expressed as mean±SE. FSH, follicular-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome. *Significant difference than control group at *P* value less than 0.05, using Student *t* test.

Table 4 Distribution of fetuin-A gene polymorphism between women with polycystic ovary syndrome and control groups

Genotype or allele	Control [*n* (%)]	PCOS [*n* (%)]	χ^2 (*P*)
CC	36 (72)	30 (60)	1.00 (ref.)
CG	10 (20)	18 (26)	2.16 (0.1168)
GG	4 (8)	2 (4)	6.00 (0.6863)

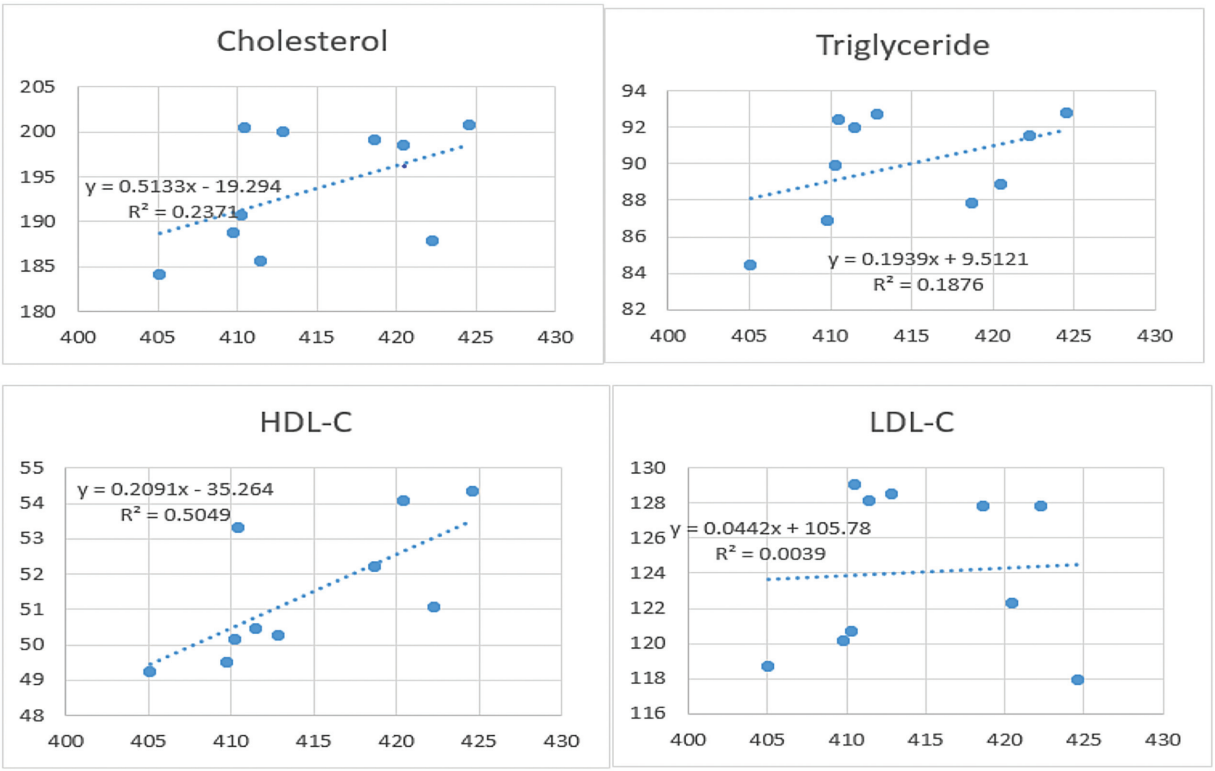
PCOS, polycystic ovary syndrome.

Figure 1



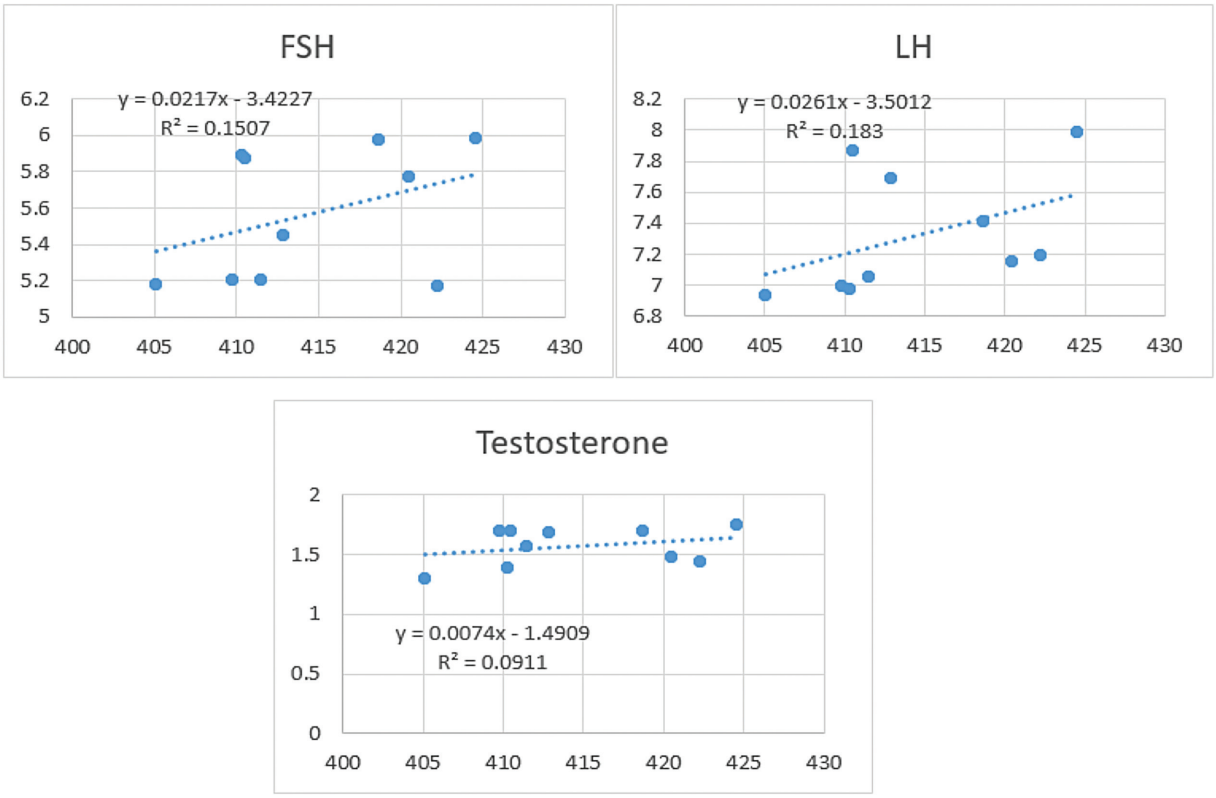
Correlation between serum levels of fetuin-A and glucose, insulin and HOMA-IR in all PCOS patients. PCOS, polycystic ovary syndrome.

Figure 2



Correlation between serum levels of fetuin-A and lipid profile (cholesterol, TG, HDL, and LDL) in all PCOS patients. HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCOS, polycystic ovary syndrome.

Figure 3



Correlation between serum levels of fetuin-A and FSH, LH, and testosterone in all PCOS patients. FSH, follicular-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome.

affected female. Fetuin-A levels were shown to be higher in PCOS-affected women than in healthy ones. This finding is in agreement with Liu *et al.* [3], Enli *et al.* [25], ElSirgany *et al.* [26], and Abali *et al.* [27]. In fact, the main reason of Fetuin-A elevation among PCOS was unknown, but we anticipate that the metabolic problems and HA brought on by IR may encourage the production and release of fetuin-A *in vivo*. Furthermore, the proinflammatory markers like CRP are also elevated in women with PCOS; it is possible that elevated fetuin-A is caused, at least in part, by the presence of low-grade inflammation.

Contrarily, Gulhan *et al.* [28] revealed that the levels of fetuin-A showed insignificant changes between PCOS and control groups. Whereas Díaz *et al.* [29] results were in direct conflict with ours since it demonstrated that PCOS women had considerably lower fetuin-A levels than the control group.

In our study, we discovered that, as compared to healthy group, PCOS had higher levels of glucose, insulin, and HOMA-IR. Plasma fetuin-A levels, particularly in those with elevated plasma glucose, are predictive of the development of T2DM regardless of other recognized risk factors. In addition, there is proof that fetuin-A can impact adipose tissue, stimulate the production of inflammatory cytokines by macrophages and adipocytes, and eventually induce IR according to Norbert *et al.* [30]. Additionally, fetuin-a produces IR and changes metabolism in insulin-sensitive tissues by inhibiting the autophosphorylation of tyrosine kinase; thus, the liver, fatty tissues, muscles, and hypothalamus are the tissues most severely impacted, where it affects cell signaling pathways and homeostasis. It has been demonstrated that circulating fetuin-A levels are associated with IGT, IR, and T2DM [31].

In this current study, we found that PCOS had high levels of cholesterol, triglycerides, and LDL concomitant with a lower level of HDL-C. These findings were in agreement with Dursun *et al.* [32] who indicated that PCOS patients are more likely to have impaired lipid profiles and Liu *et al.* [3] who found that PCOS have dyslipidemia.

Uncertainty exists regarding the processes behind the link between dyslipidemia and fetuin-A. The inhibitory impact of fetuin-A on the tyrosine of the

insulin receptor, which increases lipolysis and causes the free fatty acids to be released from adipose tissue, has been used in previous research to assess these alterations. This may then result in more very LDL containing apolipoprotein B being produced [33]. Additionally, HDL-C content may drop as a result of hypertriglyceridemia, enhancing HDL clearance from the bloodstream [34], and perhaps contributing to the atherogenic lipid profile seen in our study. Additionally, demonstrated that the IR increases hepatic de novo lipogenesis and hyperlipidemia by increasing free fatty acids and triglycerides and decreasing the inhibition of adipocyte lipolysis [35].

Stefan *et al.* [36], and Ou *et al.* [37] indicated that Fetuin-A levels were positively correlated with FBS, fasting insulin, HOMA-IR total cholesterol, LDL, and triacylglycerol, while negatively correlated with HDL as was found in our current study.

Our results demonstrated that, as compared to healthy females, women with PCOS had elevated LH and testosterone levels, and lower levels of FSH. Thus, elevation of fetuin-A as well as hyperinsulinemia affect the hypothalamic–pituitary–gonadal axis in women with PCOS, and increase the secretion of LH over FSH, decrease follicular maturation and sex hormone-binding globulin, resulting in excessive androgen production in the ovaries [23]. Here, we correlated fetuin-A to some additional hormones that are typically evaluated in PCOS situations, particularly in those trying to conceive. Our results revealed that fetuin-A level was negatively correlated with FSH, while it positively correlated with both LH and testosterone. Additionally, PCOS patients are more likely to have other metabolic disorders like obesity [38] that plays a crucial role in the pathophysiology and clinical characteristics of PCOS through the elevation of the blood's free androgen circulation, affecting the way that ovarian granulose cells and growing follicle's function [39].

In this study, we attempted to examine the association of fetuin-A polymorphism among PCOS. Our study results revealed that, 'CC' and GG allele are concomitant in control group more than PCOS; contrarily, 'CG' allele was related with PCOS.

To our knowledge, only one study investigated the distribution of fetuin-A gene polymorphism in PCOS [40]; they found that PCOS patients had a significant higher frequency of the fetuin-A gene variant rs1071592 AA genotype and A allele compared to controls although, allele and genotype frequencies of rs2593813 were not significantly different between PCOS and controls. So that individuals with CG variations in the fetuin-A gene may be more predisposed to develop PCOS, however, the fetuin-A gene and PCOS are not significantly related.

Conclusion

The present study concluded that serum level of fetuin-A in women's with PCOS significantly increased comparing with the control healthy women's and correlated significantly with IR, lipid profile and related hormones. Fetuin-A could be considered as an important marker of insulin resistance associated with PCOS. Additionally, fetuin-A 'CG' allele can be considered as a risk factor for PCOS.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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