Matrix metalloproteinases 2 and 9 and MMP9/NGAL complex activity in women with PCOS

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Abstract

It is believed that matrix metalloproteinases (MMPs) play important roles in follicular development and pathogenesis of polycystic ovary syndrome (PCOS). However, conflicting results are available about the alteration of MMP2 and MMP9 concentrations or activities in PCOS. In fact, there is no study entirely investigating both concentration and activity of these MMPs and serum levels of their tissue inhibitors TIMP2 and TIMP1, as well as lipocalin-bound form of MMP9 (MMP9/NGAL). Therefore, the thoroughness of previous studies is questionable. This study was conducted to determine circulatory concentration of MMP2, MMP9, MMP9/NGAL complex, TIMP1 and TIMP2 as well as gelatinase activities of MMP2, MMP9 and MMP9/NGAL complex in women with PCOS and controls. Mean age and BMI as well as serum levels of total cholesterol, triacylglycerol, HDL-C, LDL-C, fasting blood sugar (FBS), insulin, estradiol and sex hormone-binding globulin did not differ between groups, whereas a marked decrease in FSH and significant increases in LH, LH/FSH ratio, testosterone and free androgen index were observed. Women with PCOS and controls showed closed concentrations of MMP2, MMP9, MMP9/NGAL, TIMP1 and TIMP2. Gelatinase activity of MMP9 was found significantly higher in PCOS than in controls (64.53 ± 15.32 vs 44.61 ± 18.95 respectively) while patients and healthy subjects showed similar activities of MMP2 and MMP9/NGAL complex. Additionally, PCOS patients showed a higher MMP9/TIMP1 ratio compared with control women. Direct correlations were also observed between circulatory MMP9 level and the concentration and activity of MMP9/NGAL complex. In conclusion, based on the results of present study, we believe that MMP9 may be involved in the pathogenesis of PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a prevalent heterogeneous endocrine disorder in women of reproductive age. The worldwide prevalence of 6.5–8% has been estimated for PCOS based on biochemical and/or clinical evidences (Lujan *et al.* 2008). PCOS implies the presence of multiple cystic follicles together with hyperandrogenism, chronic oligo or anovulation, hyperinsulinemia, hirsutism and acne (Mason *et al.* 2008). Remodeling of intra-follicular microenvironment is required for follicle development which is an ordered process coordinately established by various enzymes, cytokines, steroid hormones and growth factors (Dumesic & Abbott 2008). The preovulatory follicle is enclosed in the extracellular matrix (ECM) that consists of collagen and other ECM components and regulates cellular shape and communication (Dumesic *et al.* 2008). Rearrangement of this environment requires a coordinated action of proteases and protease inhibitors. Recently, studies have shown that matrix metalloproteinases (MMPs) which play important roles in ECM

degradation and re-composition may be implicated in the pathogenesis of PCOS through their effects on ovarian tissue remodeling (Liu *et al.* 2007). The role of MMPs in ovarian tissue remodeling has recently been demonstrated, and increased circulating levels of MMP2 and MMP9 have been reported in women with PCOS (Lewandowski *et al.* 2006, Liu *et al.* 2007, 2008) indicating the involvement of the imbalanced activities of MMPs in PCOS (Shalev *et al.* 2001).

MMPs are found in all kingdoms of life and comprise a large family of zinc-dependent endoproteases sharing homologous protein sequences. MMPs can be categorized into six subgroups such as collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others (Amalinei *et al.* 2010). MMP2 (gelatinase A, EC 3.4.24.24) and MMP9 (gelatinase B, EC 3.4.24.35) are constitutively expressed by many cell types and seem to have important roles in degradation of a broad spectrum of ECM molecules (Fanjul-Fernandez *et al.* 2010). It is believed that the observed gelatinolytic activity around the developing follicles and at the apex

of preovulatory follicles corresponds to the MMP2 and MMP9 (Goldman & Shalev 2004). The activity of MMPs is regulated at different levels by interactions with endogenous inhibitors such as α_2 -macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) (Nagase *et al.* 2006), or by proteolytic cleavage of their pro-peptide domain from the zymogen structures yielding the active enzymes. Four different TIMPs have been identified so far such as TIMP1, TIMP2, TIMP3 and TIMP4. Tissue inhibitors of TIMP1 and TIMP2 are natural inhibitors of MMP9 and MMP2 respectively (Murphy 2011). Therefore, orchestration of the growth and remodeling of cells to their extracellular environment is mediated by a balance between MMPs activities and concentrations of their endogenous inhibitors. In addition, Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin 2, is found to be bound to and positively modulates the activity of MMP9 (Bolognani *et al.* 2010, Bouchet & Bauvois 2014). NGAL is an acute phase 25 kDa secretory glycoprotein covalently binds to MMP9 to form MMP9/NGAL complex which triggers an enhancement of the enzymatic activity of MMP9 through protecting the MMP9 from proteolytic degradation (Bolognani *et al.* 2010).

A review of the literature showed conflicting results on circulatory concentrations of MMP2 and MMP9. While increased levels of MMP2 and/or MMP9 have shown in PCOS patients (Lewandowski *et al.* 2006, Liu *et al.* 2007, 2008), no significant differences have been observed between PCOS and control in the study by Gomes *et al.* (2011), or a reduced MMP9 level has been reported by Diamanti-Kandarakis *et al.* (2008). Similar conflicting results are also available about serum TIMP1 and TIMP2 levels (Lewandowski *et al.* 2006, Liu *et al.* 2008, Gomes *et al.* 2011). In addition, a review of the literature showed that not only is there no consistency in the serum levels of MMP2 and MMP9 but also there is no record of serum MMP9 gelatinase activity. Moreover, the only report on MMP2 gelatinase activity showed no difference in MMP2 activity between PCOS patients and healthy subjects (Gomes *et al.* 2011). Likewise, there is no report on the enzymatic activity of MMP9/NGAL complex in the literature. In fact, simultaneous determination of circulatory concentrations of MMP2, MMP9, TIMP1, TIMP2 and MMP9/NGAL complex together with assessing the gelatinase activities of MMP2, MMP9 and MMP9/NGAL has not been previously carried out in women with PCOS.

In view of all the above and based on the hypothesis that as tissue remodeling in PCOS is more intense than in normal ovaries and some of these parameters may differ between two groups, this study was designed to detect any possible alteration in serum levels of MMP2, MMP9, TIMP1, TIMP2 and MMP9/NGAL complex and any imbalance in the enzyme activities of serum MMP2, MMP9 and MMP9/NGAL complex in women with PCOS compared with healthy individuals.

Materials and methods

Subjects

In total, 40 patients with PCOS and 40 healthy, regularly menstruating, women matched for age and BMI, were enrolled in this study. PCOS patients were recruited from the Endometrium and Endometriosis Research Centre of Fatemeh Hospital, Hamadan, Iran. The diagnosis of PCOS was made if there was a history of menstrual irregularity in association with either hyperandrogenism or polycystic ovary morphology, according to the Rotterdam consensus criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Control women had regular menstrual cycles, normal findings on pelvic ultrasound scan and no features of hyperandrogenism. Exclusion criteria for the study were set as age over 35 years, having cardiovascular disease, Cushing's syndrome, thyroid disease, diabetes mellitus or hypertension, currently being a cigarette smoker and taking medications such as oral contraceptives or metformin that could alter the endocrine parameters. Written informed consent for participation was obtained, and the project was approved by the Research Ethics Committee of Hamadan University of Medical Sciences (Iran).

Samples collection and biochemical assays

Fasting blood samples of control and PCOS subjects were collected between 0800 and 1000 h during the early follicular phase (day 2–4 from the day 1 of spontaneous bleeding episode) or any time in an anovulatory phase. Blood samples were centrifuged immediately after collection and aliquots of serum samples were stored at -70°C until analyzed. BMI was calculated as body weight in kilograms divided by height in meters squared (kg/m^2).

Biochemical analysis

The circulating levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol and insulin were determined by commercially available sandwich ELISA Kits (Monobind, Inc., Lake Forest, CA, USA) while serum concentration of sex hormone-binding globulin (SHBG) was measured by solid phase IBL ELISA Kit (IBL International GmbH, Hamburg, Germany). Free androgen index (FAI) was calculated according to FAI equation ($\text{FAI} = \text{Testosterone (nmol/L)} \times 100/\text{SHBG}$), as previously described (Rosner *et al.* 2007), and insulin resistance was determined according to the homeostasis model assessment insulin resistance (HOMA-IR) index, using the equation ($\text{HOMA-IR} = \text{fasting serum glucose (mmol/L)} \times \text{fasting insulin (mU/mL)} / 22.5$) (Matthews *et al.* 1985). Colorimetric methods were performed to measure glucose, total protein, triacylglycerol (TAG), total cholesterol (TC) and HDL-C using Pars Azmoon Kits (Pars Azmoon Co., Tehran, Iran), and Friedewald equation ($\text{LDL} = \text{TC} - (\text{HDL} + \text{TAG}/5)$) was used to calculate LDL-C levels (Friedewald *et al.* 1972).

Determination of serum MMP2, MMP9, MMP9/NGAL, TIMP1 and TIMP2 concentration

Blood samples were centrifuged immediately after collection and aliquots of serum samples were stored at -70°C until

analysis. Serum concentration of MMP2, MMP9, TIMP1, TIMP2 and MMP9/NGAL complex was determined using commercially available solid phase ELISA Kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction. The intra- and inter-assay coefficient of variation values were <5.0 and 7.5% respectively, and all measurements were performed in duplicate.

Substrate gel electrophoresis (zymography) for the determination of MMP2, MMP9 and MMP9/NGAL activities

Gelatinolytic activities of MMP2, MMP9 and MMP9/NGAL complex in serum samples were assessed by zymography, as previously described with some modifications (Lahav-Baratz *et al.* 2003, Goncalves *et al.* 2009, Tsai *et al.* 2011). Briefly, samples were subjected to electrophoresis on a 10% polyacrylamide–SDS gel (PAGE) co-polymerized with 1% gelatin as a substrate. The gels underwent electrophoresis until the marker dye reached the bottom of the gel. After electrophoresis, gels were washed twice by gentle agitation (30 min/wash) in 2.5% Triton X-100 solution at room temperature to enable enzyme renaturation. After decanting the washing solution, gels were immersed in Tris–HCl buffer, pH 7.4 for 30 min at room temperature and were then placed in fresh developing buffer for 16 h at 37 °C. The gels were washed with deionized water and stained with Coomassie Brilliant Blue R-250 (Merck KGaA) in 45% methanol and 10% acetic acid for 35 min at room temperature. The gels were then washed with water and soaked in destaining solution for 3 days until clear bands were seen indicating the MMPs activity of gelatin degradation. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. To confirm gelatinase activities observed in unstained bands (corresponding to MMP2, MMP9, and MMP9/NGAL), experiment was repeated by the immersing of the gels in EDTA buffer which chelates Zn^{2+} on the active site of MMPs and inhibits enzyme activity leaving no unstained area on the gel (data not shown). Gelatinase activities of MMP2, MMP9 and MMP9/NGAL were reconfirmed by comparing the unstained bands to the standard for MMP2 and MMP9 (R&D Systems, Inc.). The gels were scanned by Canon LiDE110 scanner (Canon, Inc., Tokyo, Japan), and images were analyzed using TotalLab TL120 software (Nonlinear Dynamics Ltd, Newcastle, UK) to quantify proteolytic activities of MMP2, MMP9 and MMP9/NGAL complex. MMP2 was detected at 60 kDa and reconfirmed by comparing to the standard for MMP2 (R&D Systems, Inc.). The pro-MMP9 and dimmer forms of MMP9 were identified as bands at 92 and 220 kDa respectively, whereas MMP9/NGAL complex was detected at 135 kDa in comparison with the relative mobility of standard for MMP9 (R&D Systems, Inc.).

Statistical analysis

Data were analyzed by SPSS 16 (SPSS, Inc.), and one-sample Kolmogorov–Smirnov test was applied to determine normal distribution of data. Two-tailed unpaired *t*-test used to compare mean differences. One-way ANOVA was used to analyze

differences between groups, and linear regression analysis was performed to determine correlation between variables. Results were presented as mean $\pm$ s.d. and the *P* value <0.05 was considered as significant.

Results

In total, 40 patients with PCOS and 40 healthy, regularly menstruating, women were enrolled in this study. Serum samples were analyzed, and women with PCOS were compared with normal subjects. Table 1 summarizes the clinical, endocrine and biochemical characteristics of the PCOS and healthy women. Patient and healthy subjects were well-matched for age and BMI with no statistically significant differences in age and BMI. Fasting blood sugar (FBS) and serum TAG, TC, HDL-C, LDL-C and SHBG did not differ between groups. Serum insulin levels were slightly higher in women with PCOS compared with controls (12.19 ± 9.83 vs 9.82 ± 4.52 μ U/ml respectively), but this difference did not reach statistical significance. Similarly, no difference was observed in HOMA-IR index levels between groups. A marked increase was observed in serum testosterone and FAI in women with PCOS compared with control subjects (Table 1) while the higher estradiol level was not statistically significant. A noticeable decline in FSH and an increase in LH levels together with a significant increase in LH/FSH ratio were found in PCOS patients.

The results of the present study showed that there is no significant difference in serum concentration of MMP2 or MMP9 between women with PCOS and control (Table 2). Likewise, we showed that serum levels of TIMP1 and TIMP2 did not differ between two groups, although the TIMP2 level did approach significance. Additionally, although a trend to the reduced circulatory MMP9/NGAL levels was observed in PCOS compared with control (49.90 ± 6.10 vs 63.00 ± 11.02 pg/ml respectively), statistical analysis showed no significant difference between two groups (Table 2).

Table 1 Clinical, endocrine and biochemical characteristics of women with PCOS and controls.

	PCOS (n=40)	Control (n=40)	P value
Age (year)	29.52 $\pm$ 3.10	29.70 $\pm$ 5.40	0.820
BMI (kg/m ²)	27.52 $\pm$ 5.03	27.81 $\pm$ 5.36	0.806
FBS (mg/dl)	92.97 $\pm$ 19.25	90.62 $\pm$ 16.58	0.560
Triacylglycerol (mg/dl)	141.18 $\pm$ 9.07	144.15 $\pm$ 11.53	0.840
Total cholesterol (mg/dl)	173.78 $\pm$ 5.54	173.45 $\pm$ 5.55	0.967
HDL-C (mg/dl)	43.50 $\pm$ 2.40	41.00 $\pm$ 1.81	0.408
LDL-C (mg/dl)	102.04 $\pm$ 4.43	103.62 $\pm$ 5.04	0.814
Insulin (μ U/ml)	12.19 $\pm$ 9.83	9.82 $\pm$ 4.52	0.173
HOMA-IR	2.86 $\pm$ 2.48	2.30 $\pm$ 1.31	0.200
FSH (mU/ml)	5.12 $\pm$ 0.34	11.02 $\pm$ 1.33	0.001
LH (mU/ml)	9.09 $\pm$ 0.57	5.46 $\pm$ 0.74	0.0002
LH/FSH ratio	1.91 $\pm$ 0.59	0.87 $\pm$ 0.51	0.001
Estradiol (pg/ml)	123.52 $\pm$ 6.50	111.70 $\pm$ 7.64	0.242
Testosterone (ng/dl)	3.32 $\pm$ 1.21	0.83 $\pm$ 0.09	0.044
FAI	27.63 $\pm$ 8.51	7.43 $\pm$ 1.24	0.024
SHBG (nmol/l)	55.56 $\pm$ 5.06	60.49 $\pm$ 5.45	0.500

Table 2 Concentration and enzyme activities of MMPs, their tissue inhibitors and MMP9/NGAL in women with PCOS and controls.

	PCOS (n=40)	Control (n=40)	P value
MMP2 (pg/ml)	182.68±36.76	186.90±42.44	0.636
MMP9 (pg/ml)	436.25±167.51	441.67±220.88	0.901
MMP9/NGAL (pg/ml)	49.90±38.58	63.00±69.72	0.303
TIMP1 (pg/ml)	187.52±142.47	169.42±51.16	0.451
TIMP2 (pg/ml)	41.98±8.08	39.08±5.07	0.059
MMP2 activity (AU)	23.99±13.26	20.18±11.14	0.171
MMP9 activity (AU)	64.53±15.32	44.61±18.95	0.002
MMP2act/TIMP2 (AU)	0.59±0.36	0.52±0.28	0.110
MMP9act/TIMP1 (AU)	0.44±0.33	0.29±0.17	0.012
MMP9/NGAL activity (μU/ml)	39.22±35.96	39.67±67.72	0.970

AU: arbitrary unit.

The results of the present study show that women with PCOS and control subjects had relatively similar levels of serum MMP2 gelatinase activities, whereas a great enhancement ($P=0.002$) was detected in serum MMP9 enzyme activity in women with PCOS compared with healthy women (Table 2). Moreover, PCOS patients and control women showed a close serum gelatinase activity of MMP9/NGAL complex with no noticeable difference.

Statistical analysis showed a positive correlation between serum concentration and gelatinase activity of MMP2 in control subjects but not in PCOS patients (Table 3), but no correlation was detected between circulatory concentration of MMP9 and its gelatinase activity. The concentration of TIMP1 did not show any correlation with serum level of MMP9 or its gelatinase activity. The circulatory level of TIMP1 neither correlated with MMP9/NGAL concentration nor with its activity (data not shown). In contrast, a significant correlation was observed between circulatory level of TIMP2 and the concentration of MMP2 ($P=0.028$), but not with MMP2 gelatinase activity (Table 3). To assess net MMP2 and MMP9 activities, the MMP2/TIMP2 and MMP9/TIMP1 ratios were calculated respectively. While PCOS patients and control women did not show different MMP2/TIMP2 ratios, a significant difference was observed in MMP9/TIMP1 ratio between groups (Table 3).

Serum concentration of MMP9/NGAL complex did not have significant correlation with MMP9/NGAL gelatinase activity, according to the linear regression analysis. Serum concentration of MMP9 showed correlation both with MMP9/NGAL concentration and its gelatinase activity, whereas correlation of MMP9 gelatinase activity was observed neither with MMP9/NGAL complex concentration nor with its activity (Table 3).

Discussion

PCOS is one of the most common endocrine disorders occurring in women of reproductive age. The underlying etiology of PCOS remains elusive despite being the focus of intense basic and clinical studies (Lujan *et al.* 2008, Mason *et al.* 2008). Because of the diverse clinical and

metabolic manifestations, considerable debates remain regarding definitive biochemical markers found in PCOS, i.e. although the characteristic abnormalities including hypersecretion of androgens and LH, dyslipidemia, hyperinsulinemia and insulin resistance are known as common laboratory findings in PCOS, these findings have not been consistent in all studies. For instance, while no alteration has been reported in TC, TAG, HDL-C and LDL-C in some studies (Diamanti-Kandarakis *et al.* 2008, Cakal *et al.* 2011, Gomes *et al.* 2011), a recent report by Sahin *et al.* (2014) shows significant dyslipidemia in patient with PCOS. Inconsistent findings are also available in the literature showing no alteration (Panidis *et al.* 2010, Sahin *et al.* 2014) or increased concentration of insulin in PCOS (Liu *et al.* 2007, Cakal *et al.* 2011, Gomes *et al.* 2011). In contrast to inconsistent reports on blood lipids and insulin, a common surge has been reported in the serum concentration of LH, testosterone and FAI in all conducted studies (Liu *et al.* 2007, 2008, Panidis *et al.* 2010, Gomes *et al.* 2011, Sahin *et al.* 2014), but the alteration has not been shown in the serum concentration of FSH or HOMA-IR (Liu *et al.* 2007, 2008, Panidis *et al.* 2010, Sahin *et al.* 2014).

The results of the present study showed increased serum levels of LH, testosterone FAI and LH/FSH ratio in line with previous reports (Liu *et al.* 2007, 2008, Panidis *et al.* 2010, Gomes *et al.* 2011, Sahin *et al.* 2014). We have also showed that circulatory concentrations of TC, TAG, HDL-C, LDL-C, E_2 , SHBG, insulin and HOMA-IR index did not differ between healthy subjects and women with PCOS. It is plausible that age and BMI are the main players in determining the aforementioned biochemical and hormonal features; therefore, differences in the mean age or BMI of patients (in different studies) may lead to inconsistent results, as seen for insulin resistance which is higher in overweight and obese women than in lean individuals (Livadas *et al.* 2014, Moran *et al.* 2015).

Remodeling of intra-follicular microenvironment and follicle development requires a coordinated action of proteases and protease inhibitors. It is believed that

Table 3 Correlations between concentration and enzyme activities of MMPs, their tissue inhibitors and MMP9/NGAL.

Correlation of variables		R ²	P value
MMP2 conc	TIMP2 conc	0.245	0.028
MMP2 conc	MMP2 act	0.147	0.196
MMP2 act	TIMP2 conc	0.168	0.139
MMP9 conc	TIMP1 conc	0.092	0.418
MMP9 conc	MMP9 act	0.082	0.472
MMP9 conc	MMP9/NGAL conc	0.274	0.014
MMP9 conc	MMP9/NGAL act	0.224	0.048
MMP9 act	TIMP1 conc	0.008	0.944
MMP9 act	MMP9/NGAL conc	0.172	0.128
MMP9 act	MMP9/NGAL act	0.022	0.189
MMP9/NGAL conc	MMP9/NGAL act	0.092	0.421

act: activity; conc: concentration.

MMPs may be implicated in the pathogenesis of PCOS through their effect on ovarian tissue remodeling (Liu *et al.* 2007). It is postulated that the orchestration of the growth and remodeling of follicles to their extracellular environment is mediated by a balance between MMPs activities and concentrations of their endogenous inhibitors (TIMPs). However, conflicting results have been reported during the last few years about the involvement of MMPs and/or their tissue inhibitors in the pathogenesis of PCOS. There is no extensive body of literature investigating the role of MMP2 in women with PCOS, and the circulatory concentration of MMP2 has only been investigated in two studies indicating an increased level of MMP2 in PCOS (Lewandowski *et al.* 2006, Liu *et al.* 2007). Intriguingly, here we showed a close concentration of MMP2 in healthy women and women with PCOS. The observed disagreement between our results and the results of previous studies might be due to the low number of subjects recruited in previous works by Lewandowski *et al.* (2006) and Liu *et al.* (2007) (35 and 45 subjects respectively) compared with 80 subjects enrolled in the present study.

Although an increased serum level of MMP9 has been reported in some studies (Lewandowski *et al.* 2006, Liu *et al.* 2008), there are also evidences in literature indicating a marked decline (Diamanti-Kandarakis *et al.* 2008) or even no alteration in the plasma MMP9 concentration in PCOS patients (Gomes *et al.* 2011). These conflicting results may indicate that in comparison to the enzymatic activities, the concentration of MMPs may not play a pivotal role in the pathogenesis of PCOS. Concordant with this explanation, we showed that there is no difference in MMP9 concentration between healthy subjects and women with PCOS. We also showed that the concentrations of TIMP1 and TIMP2 did not differ between groups.

The balance between MMPs and their tissue inhibitors is many-sided. For instance, although TIMP1 and TIMP2 inhibit MMP9 and MMP2 respectively, the pro-enzyme (zymogen) forms of these MMPs may also bind to and inhibited by TIMP3 in the body (Murphy 2011). Therefore, consistent correlations have not been observed between the concentration of MMP2 or MMP9 and the serum levels of their corresponding tissue inhibitors in previous works. Accordingly, it has shown that there is no synchronized changes between serum MMP9 and TIMP1 (Lewandowski *et al.* 2006, Liu *et al.* 2008, Gomes *et al.* 2011), or the concentration of TIMP2 is not following the alteration in serum level of MMP2 (Lewandowski *et al.* 2006). Likewise, we showed that the concentration of TIMP1 is not correlated to serum MMP9 level. This observation is in line with the results reported by Gomes *et al.* (2011) confirming neither changes in the concentrations of MMP9 and TIMP1 nor existence of any correlation between these concentrations in PCOS. Unlike MMP9, in the present study, MMP2 has evinced a significant correlation with its

corresponding tissue inhibitor (TIMP2). A similar correlation between the concentrations of MMP2 and TIMP2 has recently been reported in follicular fluid (Baka *et al.* 2010) but not in the serum of women with PCOS (Lewandowski *et al.* 2006). However, there is no compelling evidences in the literature to show concomitant change of MMPs concentrations/activities in serum and follicular fluid. It is plausible that in PCOS, the concentration of MMPs and TIMPs is more prone to be affected in follicular fluid than in circulation. To assess net MMP2 and MMP9 activities, the MMP2/TIMP2 and MMP9/TIMP1 ratios were calculated respectively. The MMP2/TIMP2 ratio was slightly higher in women with PCOS compared with controls, but this difference did not reach statistical significance, whereas a marked difference was observed in MMP9/TIMP1 ratio between groups (Table 3). A similar increased MMP9/TIMP1 ratio has previously been reported in serum (Liu *et al.* 2008) and follicular fluid (Gomes *et al.* 2011) samples in PCOS patients. Together these observations confirm alteration of MMP9 activity in PCOS patients and, therefore, provide its potential role in the pathogenesis of the disease.

Considering the literature data, the present study is the first study that simultaneously determines circulatory concentrations of MMP2, MMP9, TIMP1, TIMP2 and MMP9/NGAL complex together with the determination of gelatinase activities of MMP2, MMP9 and MMP9/NGAL in women with PCOS. We showed that the serum concentration and activity of MMP9/NGAL did not differ between groups. Although the decreased concentration of MMP9/NGAL complex has recently been reported in PCOS (Diamanti-Kandarakis *et al.* 2008), there is no information available in the literature about the gelatinase activity of MMP9/NGAL complex. Supposedly, the disagreement between our findings and previous report by Diamanti-Kandarakis *et al.* (2008) can be explained by the fact that the concentration of NGAL, which binds to MMP9 and protects the MMP9 from proteolytic degradation (Bolignano *et al.* 2010, Bouchet & Bauvois 2014), is closely correlated with insulin, insulin resistance and testosterone (Cakal *et al.* 2011). Therefore, differences in hormonal features of subjects in different studies may affect the concentration and/or activity of MMP9/NGAL complex.

Here for the first time, we also showed that women with PCOS exhibit much higher gelatinase activity of MMP9 than control subjects, whereas there was no difference in the activity of MMP2 between groups. Our later observation is supported by the previous reports indicating that the activity of MMP2 in serum (Gomes *et al.* 2011) or follicular fluid (Lahav-Baratz *et al.* 2003) does not alter in PCOS. However, there is no such a record in the literature about MMP9, since the activity of MMP9 has not been previously investigated in PCOS. Therefore, regarding the higher MMP9 activity in PCOS, we have strengthened the hypothesis of involvement of

MMP9 gelatinase activity in the pathogenesis of PCOS. This finding is of immense importance, since it is the first report on the association of PCOS with MMP9 activity. The importance of contribution of MMP9 in PCOS, as observed here, is boosted by augmenting results showing significant correlation of serum MMP9 level with either MMP9/NGAL concentration or activity.

There are, however, arguments in literature on using serum samples to assess MMP9 concentration or activity. Some authors have argued that blood coagulation process results the release of MMPs by platelets or leukocytes during platelet activation and, thus, leads to artificially higher MMP9 levels in serum compared to citrate and heparin plasma samples (Gerlach *et al.* 2005, 2007, Souza-Tarla *et al.* 2005). This notion was supported by further observations indicating that serum MMP9 levels did not correlate with the levels found in plasma samples (Gerlach *et al.* 2007). However, we still believed that serum MMP9 levels, as determined in the present study, may reasonably be valid, since first, MMP9 levels have been determined in serum samples of both patients and control women; therefore, any methodological pitfalls or artifact increment in MMP9 levels may occur evenly in all samples. Secondly, very recent study has revealed the existence of good correlation between serum and plasma concentrations of MMP9 when evaluating alterations in the concentration of this marker in disease conditions (Gerlach *et al.* 2009). Therefore, serum samples could simply be used instead of plasma samples when comparing concentrations in a particular study (Gerlach *et al.* 2009).

Taking in to account the limitations present in our study, we believe that the usage of citrate or heparin plasma samples instead of serum samples, determination of circulatory lipocalin-2 (NGAL) in its free unbound form, assessment of active forms of MMPs rather than total MMPs (pro-MMPs plus active MMPs) and measurement of MMP2, MMP9, TIMP1 and TIMP2 gene expression levels may strengthen the results. Considering the literature data, as i) age and BMI which are two important players in determining hormonal and biochemical features, differed in previous studies, ii) the low number of subjects recruited in previous studies and iii) the outcomes of previous studies are conflicting, conducting further investigations which entirely overcome all confounder factors by the enrolment of larger number of subjects, pair matching of control and patients in age and BMI and simultaneous determination of gelatinase activity as well as concentration and gene expression levels of MMPs is required. In addition, since most of the clinical studies have determined only the total MMP2 and/or MMP9 and it is not clear whether the total MMP forms or the activated forms are of diagnostic value for diseases, separate analysis of these markers is needed.

In conclusion, our results have shown that PCOS patients exhibit higher MMP9 gelatinase activity and

MMP9/TIMP1 ratio than healthy control women. In addition, direct associations have been observed between the serum concentration of MMP9 with either MMP9/NGAL concentration or its activity. Therefore, MMP9 may involve in the pathogenesis of PCOS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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