CLINICAL STUDY

Circulating osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in polycystic ovary syndrome: relationships to insulin resistance and endothelial dysfunction

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Abstract

Objective: There is plenty of evidence that osteoprotegerin (OPG) is linked to subclinical vascular damage and predicts cardiovascular disease in high-risk populations. Our aim is to investigate the relationships of OPG/free soluble receptor activator of nuclear factor κB ligand (sRANKL) to insulin resistance, brachial artery flow-mediated vasodilation (FMD), and the carotid artery intima-media thickness (CIMT) in polycystic ovary syndrome (PCOS), a disorder characterized by hyperandrogenism, impaired glucose control, and endothelial injury.

Design: A cross-sectional, observational study.

Methods: Hormonal and metabolic profiles, FMD, CIMT, serum OPG, and ampli-sRANKL were assessed in 64 young PCOS patients and 20 controls of similar age. Body composition was measured by dual energy X-ray absorptiometry.

Results: OPG was significantly lower in PCOS and related negatively to free testosterone and positively to estradiol (E₂) levels. In multivariate analysis, OPG but not ampli-sRANKL correlated positively to fasting insulin, insulin sensitivity indices, and FMD. Neither OPG nor ampli-sRANKL was associated with CIMT. Significantly lower adjusted FMD values were demonstrated in women in the upper OPG quartile group (≥2.65 pmol/l) compared with all other quartile groups together (P = 0.012). In PCOS, multiple regression analysis retained E₂/sex hormone-binding globulin ratio, fat mass, and homeostasis model assessment of insulin resistance as independent predictors of OPG.

Conclusions: In PCOS, circulating OPG is related to both endothelial dysfunction and insulin resistance, independent of obesity and androgen excess, suggesting OPG as a useful biomarker of these effects. Further studies are needed to evaluate OPG in relation to cardiovascular events and cardiovascular mortality in PCOS.

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Introduction

Isolation of osteoprotegerin (OPG), a soluble member of the tumor necrosis factor (TNF) receptor super family, has served as a fundamental stepping stone in the understanding of the close relationship between bone mineralization and vascular pathology. Expression of OPG was demonstrated in human normal vascular smooth muscle and endothelial cells (1, 2) and atherosclerotic lesions (3), however, its role in the vasculature is not entirely clear. In vitro studies are controversial, with OPG enhancing endothelial cells survival in an autocrine manner (4) but more recent evidence suggest that OPG may induce endothelial inflammation (5, 6) and stimulate proliferation of endothelial and vascular smooth muscle cells (7), thus promoting atherogenesis. Taken together, clinical studies in various populations have reported rather elevated circulating OPG levels are associated with subclinical atherosclerosis (8–11) and overall cardiovascular morbidity and mortality (12, 13). Besides reflecting cardiovascular risk, recent data indicated a role of OPG as a metabolic biomarker. Increased serum OPG levels were found in patients with diabetes and poor glycemic control (14–16) and obese nondiabetic subjects (17).

In view of this complex context, we sought to determine whether the OPG/receptor activator of nuclear factor κB ligand (RANKL) cytokine system is related to metabolic and vascular traits of polycystic ovary syndrome (PCOS), a common endocrine disorder of reproductive age. There is substantial evidence that young obese and nonobese patients with PCOS present impaired endothelial function (18–21), insulin resistance (20, 21), and low-grade chronic inflammation (22) and are at risk for cardiovascular disease (23).
Recently, it was shown that serum OPG concentrations are decreased in PCOS and related to hyperandrogenemia (24). However, the relationship of OPG to endothelial injury, one of the initiating events of atherosclerosis, has not been yet described in PCOS. In this prospective case–control evaluation, we demonstrate that serum concentrations of OPG but not of its cognate RANKL are significantly related to fasting insulin levels, insulin sensitivity indices, and endothelium-dependent flow-mediated vasodilation (FMD), suggesting OPG may function as an early-stage marker of cardiometabolic changes associated with PCOS.

**Subjects and methods**

**Study protocol**

Informed, written consent was obtained from 84 premenopausal women enrolled for the study. Of the 84 women, 64 fulfilled the criteria of PCOS according to the Androgen Excess Society 2006, defined by clinical and/or biochemical hyperandrogenism and oligoovulation and/or polycystic ovaries. Current or within 6 months use of oral contraceptives, anti-androgens, infertility medications, or drugs known to affect carbohydrate–lipid metabolism was excluded by all subjects. Secondary causes of androgen excess such as androgen-secreting tumors, Cushings’s syndrome, or medication were excluded. To exclude congenital adrenal hyperplasia, ACTH-stimulated and/or basal plasma 17-hydroxyprogesterone levels were assessed. All patients were tested for normal thyroid function and prolactin. The control group consisted of 20 women matched as a group for the age with the PCOS patients with no clinical or biochemical evidence of hyperandrogenism and regular menstrual cycles. The same exclusion criteria as in the test group were used for the control group.

Data on current smoking, first- and second-degree relatives’ history of diabetes mellitus, parental history of cardiovascular disease (documented coronary heart disease, myocardial infarction, or stroke), physical examination, heart rate, blood pressure, body mass index (BMI), waist-to-hip ratio, and the Ferriman–Gallwey score were recorded for each participant. None of the women declared regular strenuous physical exercising for at least 3 months prior to the survey. The study was conducted with the approval of the local ethics committee on clinical investigations.

**Assays**

After an overnight fast, blood samples were collected between 0800 and 1000 h and serum was stored at −80 °C. Blood was drawn during early follicular phase (days 2–5) of a spontaneous or dydrogesterone-induced menstrual cycle.

Glucose (GLU, mg/dl) was determined by the glucose oxidase color method (Diagnosticum Zrt, Budapest, Hungary) while total cholesterol (TC, mg/dl) and triglycerides (TG, mg/dl) were measured by an enzymatic, colorimetric method (Diagnosticum Zrt). Insulin (INS, μU/ml), total testosterone (TT, ng/ml), sex hormone-binding globulin (SHBG, nmol/l), estradiol (E2, pg/ml), and high-sensitive C-reactive protein (hs-CRP, mg/l) were all measured using ELISA kits from DRG Instruments (Marburg, Germany). Serum CrossLaps (ng/ml) and bone-specific alkaline phosphatase (μg/l) were measured using ELISA kits from Immunodiagnostic Systems Ltd (Boldon, UK). Both androstenedione (ng/ml) and DHEA-sulfate (DHEA-S, μg/ml) were measured using ELISA from NovaTec Immunodagnostica GmbH (Dietzenbach, Germany).

Duplicate measurements of serum OPG (pmol/l) were performed using a commercial enzyme immunoassay (Biomedica Gruppe, Vienna, Austria). The sensitivity of the assay was 0.14 pmol/l and the mean intra- and inter-assay coefficients of variation (CV) were 10 and 8% respectively. Single-sample measurements of serum-free soluble RANKL (sRANKL, pmol/l) were performed using a commercially enhanced enzyme immunoassay (ampli-sRANKL, Biomedica Gruppe). The mean intra- and inter-assay CV were below 10%. The sensitivity of the assay was 0.02 pmol/l.

Free testosterone (FT) was calculated based on measured serum TT and SHBG, according to Vemuelen’s formula (25). Hyperandrogenemia was defined as serum calculated FT ≥ 0.028 nmol/l. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) defined as fasting glucose (mg/dl) × insulin (μU/ml)/405 and by the quantitative insulin sensitivity check index (QUICKI) defined as 1/(log (fasting insulin) + log (fasting glucose)). To express the E₂/SHBG ratio, E₂ was converted to pmol/l (multiplied by 3.671).

**Assessment of body composition**

Body composition was assessed on the same morning blood was drawn, in a fasting state, by whole-body dual-energy X-ray-absorptiometry (DXA) with a DPX-NT (GE, Madison, WI, USA) device. Total body bone mineral density was expressed as g/cm². Total body fat mass (TFM) and total body fat-free mass were expressed either as kilograms or as percentages of weight. The precision error for TFM was < 3%.

**Carotid artery intima-media thickness measurement technique**

During the early follicular phase of the same menstrual cycle, carotid artery ultrasound imaging was performed by one experienced cardiologist, who was blinded to PCOS status, DXA results, and serum assays, using a color Doppler (AGILENT SONOS 4500) with...
a high-resolution 10 MHz linear probe. Overall, single maximum intima media thickness (IMT) was used as a measurement of carotid artery IMT (CIMT) (26). Longitudinal images were obtained from the distal portion of both common carotid arteries, 2 cm proximal to the carotid bulb, immediately proximal to the origin of the bifurcation, the carotid bulb, and the internal carotid artery on both right and left sides. CIMT was defined as the distance between the junction of the lumen and intima, and that of the media and adventitia. The IMT was measured during end-diastole from the B-mode screen and only the posterior (far) carotid wall measurements were taken. The CV between visits was 5%.

**Hemodynamic studies**

FMD was measured in all subjects by the same cardiologist, on the same visit with carotid IMT. Each patient was taken into a quiet, temperature-controlled room at 20–25 °C. After resting in a supine position for 15 min, the right brachial artery was identified and its position was marked at about 5 cm above the elbow joint. Diameter (measured in mm) of the artery was measured at end-diastole. After the resting measurement, limb flow occlusion (ischemia) was produced by inflating a standard sphygmomanometer cuff on the upper arm to 50 mmHg above systolic pressure for 5 min. Subsequent cuff deflation induced a brief high-flow state through the brachial artery (reactive hyperemia) for the release of the endothelial nitric oxide, to accommodate the dilated resistance vessels. The brachial artery was scanned continuously for 90 s after cuff deflation and the measurements were performed during the 30–90 s interval. The vessel’s diameter was measured at the same point with resting measurement at least twice and the maximal diameter was defined again (diameter during reactive hyperemia). FMD was calculated as the percentage maximum change in the vessel size from baseline. The CV for repeated measurements of resting arterial diameter was 2.3%.

**Statistical analysis**

Results were expressed as mean ± S.E.M. The Kolmogorov–Smirnov test was applied to continuous variables to check for normal distribution. Continuous data were compared using either the t-test for independent samples or the Mann–Whitney U test, as appropriate. Logarithmic transformations were performed as needed to ensure a normal distribution of continuous variables. To analyze the effects of PCOS on serum OPG and RANKL, ANCOVA was applied with family history, smoking, age, BMI, TBFM, HOMA-IR, and E2/SHBG ratio as covariates. Partial correlation analysis was aimed to find correlations between serum OPG or ampli-sRANKL and metabolic and vascular characteristics of subjects after removing the effects of potential confounding variables. A multiple regression analysis was performed to determine which variables predicted OPG levels in PCOS patients. P values ≤ 0.05 were regarded as significant.

**Results**

Table 1 illustrates the anthropometric characteristics and laboratory data of PCOS patients and controls participating in the study. CIMT and FMD measurements and body composition outcomes are presented in Table 2. Women with PCOS reported a borderline higher prevalence of parental history of coronary heart disease (P = 0.07, data not shown) and tended to be more frequent smokers. Prevalence of diabetes in first- and second-degree relatives was similar in both the groups (P = 0.126). As expected, PCOS patients presented with significantly higher TT, FT, androstenedione, and DHEA-S levels and lower SHBG concentrations. Early menstrual cycle E2 levels did not differ between the groups; however, E2/SHBG ratio was

<table>
<thead>
<tr>
<th>Table 1 Clinical characteristics, metabolic, and hormonal laboratory parameters in study groups. Results are expressed as mean ± S.E.M.</th>
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<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Smokers (n%)</td>
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<tr>
<td>Heart rate (beats/min)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>BMI (kg/m²)</td>
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<tr>
<td>WHR</td>
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<tr>
<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<tr>
<td>TT (nmol/l)</td>
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<td>DHEA-S (µg/ml)</td>
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<td>Androstenedione (ng/ml)</td>
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<td>SHBG (nmol/l)</td>
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<td>FT (nmol/l)</td>
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<td>E2 (pg/ml)</td>
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<tr>
<td>E2/SHBG ratio</td>
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<tr>
<td>BAP (µg/l)</td>
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<td>CrossLaps (ng/ml)</td>
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<td>Fasting glucose (mg/dl)</td>
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<td>Fasting insulin (µU/ml)</td>
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<tr>
<td>HOMA-IR</td>
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<tr>
<td>QUICKI</td>
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<tr>
<td>hs-CRP (µg/ml)</td>
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<tr>
<td>Total COL (mg/dl)</td>
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<td>TG (mg/dl)</td>
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BMI, body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; TT, total testosterone; DHEA-S, DHEA sulfate; FT, free testosterone; SHBG, sex hormone-binding globulin; E2, estradiol; BAP, bone-specific alkaline phosphatase; HOMA-IR, homeostasis model assessment for insulin resistance; QUICKI, quantiative insulin sensitivity check index; hs-CRP, high sensitivity C-reactive protein; COL, cholesterol; TG, triglycerides. FT was calculated according to Vermeulen’s formula (25). *P < 0.051 in BMI and TBFM-adjusted analysis, †P = 0.051 in BMI and TBFM-adjusted analysis.
markedly increased in PCOS patients, as highlighted in Table 1. In addition, fasting insulin and HOMA-IR values were higher in PCOS patients than in controls (Table 1) and the significance persisted after adjusting for BMI and TBFM, emphasizing altered insulin sensitivity featuring PCOS.

Relationships of serum OPG and ampli-sRANKL to lipid and glucose metabolism parameters

Both OPG and ampli-sRANKL failed to be significantly related to either TC or TG levels (data not shown). After controlling for familial history, smoking, age, BMI, TBFM, and E2/SHBG, serum OPG correlated with insulin sensitivity indices HOMA-IR and QUICKI and fasting glucose concentration in PCOS patients (with fasting glucose: \( r = 0.294, P = 0.029 \); with HOMA-IR: \( r = 0.311, P = 0.021 \); and with QUICKI: \( r = 0.295, P = 0.028 \)). Notably, these correlations persisted after adjusting for serum bone turnover markers, FT, and hs-CRP (Table 3). Ampli-sRANKL relationship to insulin sensitivity markers was less consistent, as evidenced by the significant correlation with fasting insulin, HOMA-IR, and QUICKI revealed in all women (Table 3) and in PCOS by simple analysis (with fasting insulin: \( r = 0.327, P = 0.008 \); with HOMA-IR: \( r = 0.267, P = 0.033 \); and with QUICKI: \( r = 0.255, P = 0.042 \)). In PCOS, the correlation was lost when multivariate analysis was performed (Table 3).


table 3

Partial correlation coefficients \((r)\) of serum OPG and ampli-sRANKL respectively, with glucose metabolism parameters, hemodynamic studies (flow-mediated vasodilation, FMD %), and structural changes of the carotid artery wall CIMT in PCOS patients and all the study population. Variables with nonparametric distribution were log transformed for the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Serum OPG (pmol/l)</th>
<th>Serum ampli-sRANKL (pmol/l)</th>
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<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( P )</td>
</tr>
<tr>
<td>Glucose* (mg/dl)</td>
<td>+0.190</td>
<td>0.117</td>
</tr>
<tr>
<td>Insulin* (( \mu U/ml ))</td>
<td>+0.245</td>
<td>0.042</td>
</tr>
<tr>
<td>HOMA-IR*</td>
<td>+0.247</td>
<td>0.040</td>
</tr>
<tr>
<td>QUICKI*</td>
<td>−0.231</td>
<td>0.055</td>
</tr>
<tr>
<td>FMD* (%)</td>
<td>−0.394</td>
<td>0.002</td>
</tr>
<tr>
<td>CIMT* (mm)</td>
<td>+0.018</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>−0.123</td>
<td>0.314</td>
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<tr>
<td></td>
<td>−0.312</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>−0.262</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>+0.244</td>
<td>0.043</td>
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<tr>
<td></td>
<td>+0.085</td>
<td>0.520</td>
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<tr>
<td></td>
<td>+0.110</td>
<td>0.400</td>
</tr>
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</table>

Multivariate adjustment was performed to correct for the confounding effects for age, family history, BMI, TBFM, E2/SHBG, FT, bone markers, and hs-CRP (*) and age, family history, BMI, TBFM, E2/SHBG, FT, bone markers, hs-CRP, and glucose metabolism (*) respectively. \( P \) value \( \leq 0.05 \) was considered statistically significant.

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concentration in either group (Table 3). Neither FMD nor CIMT was associated with ampli-sRANKL levels.

It is demonstrated that adjusted FMD values decreased in a step-wise manner by quartiles of OPG (P=0.033, by ANCOVA, Fig. 1). Women in the upper OPG quartile group (QIV > 2.65 pmol/l) presented with significantly lower adjusted FMD levels compared with all other quartile groups together (QI–III, P=0.012, by ANCOVA, Fig. 1).

Eventually, multiple regression analysis was carried out in patients with PCOS to identify the parameters independently associated with OPG levels. When OPG was entered as a dependent variable and HOMA-IR, FT, and E₂/SHBG ratio were considered as independent variables, the model (R²=0.171, F=4.133, P=0.01) retained E₂/SHBG ratio (β=0.501, P=0.004) as the only significant predictor of OPG, explaining 12.99% of the variation in circulating OPG levels. Adding TBFM as an independent variable expressed the best prediction of the variation in circulating OPG levels. Adding TBFM as an independent variable expressed the best prediction of serum OPG (R²=0.254, F=5.032, P=0.001), with E₂/SHBG ratio (β=0.621, P=0.0004), HOMA-IR (β=0.482, P=0.043), FT (β=0.472, P=0.038), and fat mass (β=0.00002, P=0.012) as significant determinants, explaining 20.38% of OPG variability. When bone markers were added as independent variables, E₂/SHBG ratio (β=0.511, P=0.006), HOMA-IR (β=0.461, P=0.057), and fat mass (β=-0.00002, P=0.010) were further retained as statistically significant predictors of OPG, whereas FT (β=-0.341, P=0.158) was excluded from the model (R²=0.242, F=2.99, P=0.013).

Discussion

PCOS patients exhibited significantly lower FMD values compared with controls, which is in good agreement with previously published data, emphasizing impaired endothelial dysfunction, an early-stage marker of atherosclerosis, as a trait of PCOS. As shown for the first time in this study, circulating OPG was significantly related to FMD and to be kept in mind women with serum OPG > 2.65 pmol/l presented significantly altered endothelial function compared to all the rest. Although OPG was negatively related to FT, to confirm the previously described deleterious effect of androgen excess on OPG (24), the pathophysiological role of this relationship is not yet clarified. In fact, high serum OPG levels were reported in several states associated with vascular damage such as diabetes, ischemic stroke (27), and coronary heart disease (28, 29) and, recently, serum OPG was reinforced as an independent predictor of cardiovascular disease and mortality in high-risk populations (13). Moreover, in previous studies, elevated plasma OPG concentrations were negatively related to endothelium-dependent arterial dilation in newly diagnosed diabetes (8, 9, 14) and hypothyroidism (30). Nevertheless, it is unclear whether, as thought, the relationship between OPG and FMD reflects a compensatory response of the endothelium to injury or if it should rather be regarded as a direct involvement of OPG in the pathogenesis of endothelial dysfunction. Human endothelial cell experiments were used to demonstrate the ability of OPG to upregulate inflammatory markers at the endothelial level (6). In turn, treatment of endothelial cells with pro-inflammatory cytokines was followed by a rapid response of OPG secretion (31), suggesting a role of OPG to modulate the vascular damage process. When administered topically, in experiments performed on rat postcapillary venules, human recombinant OPG induced leukocyte and endothelial cell adhesion (5), thus promoting endothelial inflammation and dysfunction. Since patients with important insulin resistance and severely impaired FMD presented with highest OPG levels, we might speculate that, in PCOS, OPG may link disturbances in insulin sensitivity to impaired endothelial dysfunction and progression of atherosclerosis, independently from hyperandrogenia. There is evidence of OPG stimulating endothelial cell proliferation (7) and dose dependently increasing, in the presence of TNF-α, the expression of cell adhesion molecules (CAMs) such as intercellular adhesion molecule-1 (ICAM-1), vascular CAM-1 (VCAM-1), and E-selectin (6). To be mentioned, previous reports have demonstrated in patients with PCOS increased concentrations of the above mentioned pro-inflammatory CAMs (32–34), with ICAM-1 and VCAM-1 adequately decreasing in response to metformin (33, 34) or statins (34).
In this study, we found a positive correlation between circulating OPG and insulin resistance in PCOS, although weak and more difficult to interpret. Studies in patients with type 1 and type 2 diabetes provide striking evidence for increased OPG concentrations in both serum and tunica media samples (12, 14–16). Moreover, in diabetes, OPG is related not only to glycemic control but also to cardiovascular disease and mortality (14, 35), compatible with the hypothesis of OPG as a marker or mediator of diabetic vascular complications. In PCOS, the association between OPG and insulin resistance, also reported in young obese non-PCOS individuals (17) and apparently healthy subjects (36), might reflect the low-grade chronic pro-inflammatory and pro-atherogenic state associated with all these clinical conditions. This hypothesis is emphasized by HOMA-IR functioning as an independent predictor of serum OPG in obese nondiabetic patients (17) and, as we have shown, in PCOS. It should be kept in mind, however, that variables studied in regression analysis, including HOMA-IR, explained no more than 20% of circulating OPG, suggesting that several potentially contributing factors have not been elucidated yet.

Low serum OPG levels were previously reported by Escobar-Morreale et al. (24) but in contrast to our findings, the authors failed to detect any association between OPG and glucose metabolism parameters in PCOS women of similar age. It should be pointed out, however, that in our study the HOMA was used, primarily expressing hepatic insulin resistance, whereas in Escobar-Morreale’s research, the insulin sensitivity index during oral glucose tolerance test according to Matsuda & DeFronzo was chosen, which rather provides information on whole-body (hepatic and peripheral, in particular muscle) insulin sensitivity. Although these indices were generally found to correlate well, different methods of expressing insulin sensitivity may display variations for associations with metabolic risk factors (37). Additionally, multivariate adjustments for E2/SHBG ratio, bone turnover markers, and body fat mass were performed in this study.

One of the striking differences between the above mentioned study and our data is the highly significant positive association of OPG and E2 levels we have observed. Moreover, among parameters considered in regression analysis in this study, E2/SHBG ratio was the most important contributor to serum OPG levels. In keeping with experimental data, showing E2 stimulates OPG production in human osteoblastic cells (38) our findings may reflect the anti-resorptive activity of E2/SHBG in bone metabolism in order to preserve bone mass. Controversy exists, nevertheless, as to whether or not increased bone mineral density is constantly found in PCOS (39, 40). Furthermore, several factors such as androgen excess, insulin resistance, and increased fat mass may all intervene as potential determinants of bone mass in PCOS.

There are limitations that need to be addressed regarding this study. First, the small number of controls could raise questions about the comparability of the groups. However, with the proposed number of patients, the likelihood was good (power between 80 and 92%) to yield statistically significant results for differences in serum OPG concentration, insulin sensitivity indices, and FMD, in addition to serum testosterone and SHBG levels (power of 100%). It should be pointed out that 30% and higher differences in CIMT could be well detected in this study and have been previously reported in similar populations (19). Nevertheless, smaller differences in CIMT between controls and women with PCOS may have been overlooked. Secondly, dydrogesterone given to induce withdrawn bleedings could more or less affect insulin secretion in PCOS patients (41). Nevertheless, in our patients, dydrogesterone was given for a maximum of 5–10 days, whereas in all demonstrations performed in postmenopausal women, hormone replacement therapy was administered for at least 3 months. Thirdly, we measured body composition by DXA, whose main disadvantage is the impossibility of differentiating subcutaneous from visceral abdominal fat. However, there are data showing parallel changes in the trunk fat as measured by DXA and visceral adipose fat measured by magnetic resonance imaging (42).

To conclude, in PCOS both endothelial dysfunction and insulin resistance are related to circulating OPG, independent of obesity and androgen excess, thus suggesting its role as a useful biomarker of these traits in this specific population. Further studies are needed to evaluate OPG in relation to cardiovascular events and cardiovascular mortality in women with the 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.

Funding

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