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ORIGINAL ARTICLE

Serum concentrations of carboxylated osteocalcin are increased and associated with several components of the polycystic ovarian syndrome

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Abstract Intriguing studies suggest that osteocalcin (OC) and its carboxylated (Gla)/uncarboxylated form are involved in the regulation of insulin secretion and action. Additionally, advanced glycated end products (AGEs) directly regulate the secretion of these osteoblast-derived molecules. In polycystic ovarian syndrome (PCOS), among the pathophysiological aberrations, deregulation of insulin secretion and action as well as elevated AGEs levels have been demonstrated. In this study, we evaluated the serum levels of osteocalcin and Gla osteocalcin and their possible associations with metabolic, hormonal, and ultrasonographic components of PSOS: 97 women were studied, 50 PCOS patients and 47 controls, matched for age and body mass index (BMI). In each subject, the levels of bone metabolism markers have been evaluated, and metabolic and hormonal profiles as well as ovarian ultrasound were carried out. Osteocalcin (4.30 ± 1.74 vs. 6.20 ± 1.78 ng/ml, $P < 0.0005$) values were significantly lower, whereas Gla osteocalcin (37.93 ± 6.87 vs. 9.64 ± 8.21 ng/ml, $P < 0.0005$) and receptor activator for nuclear factor-κB ligand (0.54 ± 0.26 vs. 0.16 ± 0.15 pmol/l, $P < 0.0005$) values were significantly higher in PCOS subjects compared to the control group, independently of obesity. A significant association was disclosed between osteocalcin and Gla osteocalcin with androgens, insulin resistance, AGEs, and ovarian morphology. Receiver operating curve analysis revealed that Gla osteocalcin [AUC, 0.975 (95% CI, 0.93–1.00)] as well as AGEs are significant prognostic factors of PCOS [AUC, 0.986 (95% CI, 0.97–1.00)]. Lower osteocalcin and elevated serum levels of its carboxylated form are displayed in PCOS subjects and are associated with several PCOS components. These findings suggest a potential interaction between bone-derived markers and the metabolic/hormonal abnormalities observed in PCOS. However, the pathophysiological mechanisms and moreover the possible clinical implications require further investigation.

Keywords Osteocalcin · Polycystic ovarian syndrome · Androgens · Osteoblast

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Introduction

Polycystic ovarian syndrome (PCOS) constitutes one of the most common endocrinopathies of unknown etiology [1]. However, the central role of insulin resistance and abnormal carbohydrate metabolism in the pathogenesis of the syndrome has been established [2]. Recently, osteocalcin levels have been positively associated with insulin sensitivity in both animal and human studies, and therefore an interplay between bone tissue and carbohydrate metabolism has been revealed.

Studies by the group of Karsenty and collaborators have shown that knockout mice lacking the protein tyrosine

phosphatase, an enzyme which induces a posttranslational modification of osteocalcin to γ-carboxyglutamic acid (Gla) residues, were protected from obesity, insulin resistance, and glucose intolerance. In this phenotype, an amplification of pancreatic β-cell proliferation, insulin secretion, and increased peripheral insulin sensitivity was observed. In contrast, in mice overexpressing the enzyme, tyrosine phosphatase, and consequently increased Gla osteocalcin/decreased osteocalcin levels, demonstrated reduced β-cell proliferation, insulin secretion, and increased insulin resistance. These intriguing findings suggest that bone tissue appears to have an expanding role, linked with deranged carbohydrate metabolism and insulin resistance [3, 4].

The role of osteocalcin and its carboxylated form and its potential input in insulin resistance in PCOS have not been investigated, to the best of our knowledge. Therefore, in the present study, the levels of osteocalcin and its carboxylated form were evaluated in relationship to metabolic, hormonal, and ultrasound abnormalities in PCOS. Additionally, serum advanced glycated end products (AGEs) were measured, because they have been shown to downregulate mRNA osteocalcin production [5] and, by deregulating intracellular insulin signaling, exacerbate the insulin-resistant state in target tissues; therefore, they could have a role in this circuit of metabolic and hormonal events in PCOS [6].

In the present study, bone metabolic parameters, such as osteocalcin and its carboxylated form, osteoprotegerin (OPG), receptor activator for nuclear factor-κB ligand (RANKL), parathyroid hormone (PTH), calcium, phosphorus, and 1,25-$(OH)_2$-vitamin D levels were evaluated in a cohort of 50 PCOS women and 47 age- and body mass index (BMI)-matched controls. In addition, we searched for possible associations of these parameters with the characteristic features of the syndrome such as androgens, insulin resistance indices, and ovarian morphology.

Materials and methods

Subjects

The PCOS group was composed of 50 women who presented to the PCOS endocrine clinic because of menstrual irregularities. All these women were referred from their physicians to our center for evaluation with a running diagnosis of PCOS. The diagnosis of PCOS was based on National Institutes of Health (NIH) consensus criteria. Chronic anovulation was assessed as fewer than eight cycles per year, and serum progesterone levels were below 3 ng/ml during the study period. Hyperandrogenemia was assessed as total testosterone levels above the 95th percentile of the levels detected in the group of normal menstruating women. Other androgen excess disorders (congenital adrenal hyperplasia) were excluded accordingly. A Synacthen test was conducted in each woman with a basal 17-hydroxyprogesterone (17-OHP) plasma level >1.0 ng/ml. The enrolled population was in good health and not suffering from chronic or acute diseases.

Forty-seven healthy women with regular periods and no hyperandrogenemia, hirsutism, or acne served as the control group studied during the follicular phase (progesterone <5 ng/ml). These women have visited our endocrine center with a possible diagnosis of goiter or were healthy wives of infertile couples with male infertility factor. All control subjects were euthyroid, normoandrogenemic, and normoprolactinemic, and none had 17-OHP >1.0 ng/ml. Exclusion criteria for the study included age over 40 years, known cardiovascular disease, neoplasms, current smoking, diabetes mellitus, renal impairment (serum creatinine >120 μmol/l), or hypertension (blood pressure >140/85 mmHg). Oral contraceptives, steroid use, or other drugs or vitamins involved in bone and carbohydrate metabolism, if administered, were discontinued for at least 3 months before the study. None of the study subjects had a history of fracture the past 6 months and none was taking any drug known to affect vitamin K status (warfarin, ketoconazole, etc.).

Because ingestion of lettuce and broccoli affects vitamin K status and subsequently serum intact osteocalcin or Gla osteocalcin levels might be influenced, both PCOS women and controls were advised to avoid these substances for 2 weeks before blood sampling.

The protocol was approved by the Institutional Review Committee of Laiko General Hospital, and written informed consent was obtained from each subject before entry into the study.

Assays

Blood samples were collected from all patients and healthy controls between 0800 and 1000, after an overnight fast. All samples were obtained during the early follicular phase (day 2–4 from the first day of a spontaneous bleeding episode) or at any time in anovulatory subjects with progesterone levels <3 ng/ml. Samples were centrifuged immediately, and serum was stored at −80°C until assayed for glucose, insulin, total testosterone, sex hormone-binding globulin (SHBG), androstenedione (Δ4A), luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17-OHP, dehydroepiandrosterone sulfate (DHEA-S), parathyroid hormone (PTH), 1,25-$(OH)_2$-vitamin D, and AGEs as previously described [7, 8]. 25(OH) vitamin D was measured using a commercial chemiluminescence assay (Diasorin, Saluggia, Italy). The mean intra- and interassay coefficients of variation were

3–5% and 6–10.8%, respectively. Serum intact osteocalcin was measured using an immunoradiometric assay (IRMA) (BioSource, Nivelles, Belgium) with intra- and interassay coefficients of variation of 2–4% and 4–6%, respectively. Gla osteocalcin was measured in duplicate with an enzyme immunoassay (EIA) (Takara Bio, Shiga, Japan) with intra- and interassay coefficients of variation of 2–4% and 1–4%, respectively, and with a sensitivity of 0.5 ng/ml. Because of the difference in molecular weight, the concentrations of Gla osteocalcin are significantly higher than total osteocalcin. Serum osteoprotegerin (OPG) concentrations were measured in duplicate using a commercial enzyme-linked immunoassay (ELISA) (Biomedica Gruppe, Vienna, Austria). The sensitivity of the assay was 1 pg/ml (0.018 pmol/l) and the mean intra- and interassay coefficients of variation were below 10%. Serum RANKL levels were measured in duplicate using a commercial enhanced ELISA (Biomedica Gruppe). The mean intra- and interassay coefficients of variation, as reported by the manufacturer, were below 10%.

Ultrasound assessment

Transvaginal ultrasound scans of the ovaries were performed during the follicular phase for ovulatory subjects and during the study period for the anovulatory subjects confirmed with progesterone levels <3 ng/ml as previously described [9].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) for continuous variables and as percentages for categorical data. The Kolmogorov–Smirnov test was utilized for normality analysis of the parameters. A comparison of variables between groups was performed using the independent samples *t* test or the Welch test (in case of unequal variances). A two-way analysis of variance (ANOVA) model including as factors the PCOS status (normal vs. abnormal), obesity (BMI <25 vs. BMI >25 kg/m^2), and their interaction was used to analyze the impact of obesity on the relationship between PCOS status and Gla osteocalcin, respectively. The Pearson correlation coefficient was applied to assess the correlation between the variables. A receiver operating curve (ROC) analysis was conducted to obtain cutoff levels of biochemical markers for the classification of patients as PCOS patients or controls by calculating the respective areas under the curve (AUC). The areas under the ROC curves (AUC) with their standard error and 95% confidence interval (CI) were calculated using the maximum likelihood estimation method. Furthermore, the sensitivity and specificity of different cutoff points of biochemical markers were estimated using the PCOS status as the gold standard. All tests are two sided; statistical significance was set at $P < 0.05$. All analyses were carried out using the statistical package SPSS ver. 13.00 (Statistical Package for the Social Sciences; SPSS, Chicago, IL, USA).

Results

Data from 50 PCOS women were compared to data derived from 47 regularly ovulating, normoandrogenemic women (controls). The two groups did not differ in age (26.46 ± 5.86 vs. 27.15 ± 6.72 years; $P = 0.748$) and BMI (26.49 ± 5.00 vs. 26.27 ± 5.30 kg/m^2; $P = 0.833$), respectively. However, waist to hip ratio (WHR) was higher in the PCOS group (0.83 ± 0.06 vs. 0.79 ± 0.07; $P = 0.009$), as well as serum concentrations of gonadotropins (LH, FSH), androgens (testosterone, Δ4, DHEAS, 17-OHP, SHBG), AGEs, insulin, and the insulin resistance index HOMA-IR. Additionally, mean ovarian volume and follicle number were significantly higher in PCOS in comparison to controls. Considering indices of bone metabolism, the concentrations of total osteocalcin was lower, whereas the values of its carboxylated form (Gla) and RANKL were significantly higher, in PCOS compared to controls; no differences between groups were observed considering calcium, phosphorus, PTH, OPG, 25(OH) vitamin D, and 1,25$(OH)_2$ vitamin D levels. The results are analytically depicted in Table 1. Similar results were observed when the two groups were further divided into lean and obese subgroups according to their BMI status (data not shown).

The results of bivariate analysis in the total group are depicted in Table 2. A significant association of Gla with androgens, insulin, HOMA-IR, and follicle number was observed. When two-way ANOVA was carried out to study the effect of PCOS and obesity on bone metabolism parameters, no association was found for either Gla osteocalcin ($F = 0.021$, $P = 0.885$) or osteocalcin ($F = 0.031$, $P = 0.860$). ROC analysis revealed that Gla [AUC 0.975 (95% CI, 0.93–1.00)], as well as AGEs, are significant prognostic factor of PCOS existence [AUC, 0.986 (95% CI, 0.97–1.00)]. These findings are presented in Table 3.

Discussion

In the present study it was found that in the PCOS group osteocalcin values were lower, whereas carboxylated osteocalcin values were higher, in comparison to the age- and BMI-matched control group. Moreover, these parameters were highly correlated with characteristic PCOS components, such as androgens, serum AGEs, insulin

Table 1 Comparison of biochemical markers between groups

	PCOS ($n = 50$)	Controls ($n = 47$)	P value
Age (years)	26.46 ± 5.86	27.15 ± 6.72	0.748
BMI (kg/m)	26.49 ± 5.00	26.27 ± 5.30	0.833
WHR	0.83 ± 0.06	0.79 ± 0.07	0.009
Ca (mg/dl)	9.88 ± 0.41	10.03 ± 0.50	0.117
P (mg/dl)	3.81 ± 0.46	3.99 ± 0.66	0.133
PTH (ng/l)	38.32 ± 15.67	33.71 ± 16.23	0.158
1,25-(OH)2-vitamin D (ng/ml)	29.12 ± 6.39	31.04 ± 8.11	0.196
Osteocalcin (ng/ml)	4.30 ± 1.74	6.20 ± 1.78	<0.0005
OPG (ng/ml)	3.41 ± 1.19	3.98 ± 1.49	0.052
RANKL (pmol/l)	0.54 ± 0.26	0.16 ± 0.15	<0.0005
Gla (ng/ml)	37.93 ± 6.87	9.64 ± 8.21	<0.0005
Glucose (mg/dl)	91.85 ± 8.44	92.76 ± 8.16	0.602
Insulin (μU/ml)	14.92 ± 8.81	10.16 ± 6.74	0.004
HOMA-IR	3.29 ± 1.87	2.20 ± 1.15	0.002
FSH (IU/l)	5.83 ± 1.50	6.75 ± 2.32	0.021
LH (IU/l)	9.39 ± 5.85	5.81 ± 2.43	<0.0005
Testosterone (ng/dl)	87.65 ± 23.91	42.36 ± 9.96	<0.0005
Δ4 (ng/ml)	3.18 ± 1.03	1.71 ± 0.46	<0.0005
DHEAS (ng/ml)	3098 ± 1388	2127 ± 883	<0.0005
17-OHP (ng/ml)	1.14 ± 0.49	0.81 ± 0.40	<0.0005
SHBG (nmol/l)	40.10 ± 19.71	54.92 ± 17.90	0.003
AGEs (U/ml)	8.85 ± 1.15	5.95 ± 0.76	<0.0005
Ovarian volume (cm)	10.02 ± 6.10	6.13 ± 3.27	0.001
Follicle number	28.21 ± 10.64	14.19 ± 4.06	<0.0005

All values are presented as mean ± SD

OPG osteoprotegerin, *RANKL* receptor activator for nuclear factor κB ligand, *Gla* carboxylated form of osteocalcin, *AGEs* advanced glycated end products, *PCOS* polycystic ovary syndrome, *BMI* body mass index, *WHR* waist to hip ratio, *PTH* parathyroid hormone, *HOMA-IR* homeostatic model assessment of insulin resistance, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *Δ4* androstenedione, *DHEAS* dehydroepiandrosterone sulfate, *17-OHP* 17 hydroxyprogesterone, *SHBG* sex hormone-binding globulin

Table 2 Correlation coefficients between bone metabolism parameters and features of PCOS in the total group

	BMI	HOMA-IR	Insulin	Testosterone	Δ4	AGEs	Ovarian volume	Follicle number
GLA								
r	−0.002	0.289	0.281	0.706	0.620	0.748	0.295	0.598
P value	0.987	0.005	0.005	<0.0005	<0.0005	<0.0005	0.016	<0.0005
OC								
r	−0.124	−0.266	−0.215	−0.396	−0.278	−0.435	−0.017	−0.349
P value	0.225	0.010	0.034	<0.0005	0.006	<0.0005	0.889	0.001
RANKL								
r	0.205	0.401	0.355	0.361	0.296	0.305	0.114	0.243
P value	0.044	<0.0005	<0.0005	<0.0005	0.003	0.004	0.357	0.019
OPG								
r	−0.251	−0.225	−0.259	−0.196	−0.115	−0.201	−0.087	−0.177
P value	0.014	0.031	0.011	0.056	0.265	0.064	0.485	0.091

OPG osteoprotegerin, *RANKL* receptor activator for nuclear factor κB ligand, *Gla* carboxylated form of osteocalcin, *OC* osteocalcin, *AGEs* advanced glycated end products, *PCOS* polycystic ovary syndrome, *BMI* body mass index, *HOMA-IR* homeostatic model assessment of insulin resistance, *Δ4* androstenedione, *DHEAS* dehydroepiandrosterone sulfate

resistance index HOMA-IR, and ovarian follicle number (see Table 1).

Osteocalcin is one of the most abundant noncollagenous bone matrix proteins synthesized exclusively by osteoblasts [10]. Experimental and human studies have explored a new role of osteocalcin as a "rheostat regulating energy metabolism." Specifically, uncarboxylation/carboxylation of osteocalcin appears to play a central role linking bone with carbohydrate metabolism. In wild-type and transgenic mice, osteocalcin may undergo, via the action of tyrosine

Table 3 ROC analysis (most important predictors)

Rank	Area	SE	Significance	Cutoff points	Sensitivity (%)	Specificity (%)	95% CI	
Testosterone[a]	1.000	0.00	<0.0005	58.00	100	100	1.00	1.00
Gla[a]	0.975	0.02	<0.0005	23.47	100	98	0.93	1.00
HOMA-IR[a]	0.673	0.06	0.008	2.39	67.50	65	0.55	0.79
AGEs[a]	0.986	0.01	<0.0005	7.06	97.5	99.5	0.97	1.00
Osteocalcin[b]	0.806	0.05	<0.0005	4.8	72	83	0.72	0.90
Follicle number[a]	0.940	0.027	<0.0005	19.50	85	98	0.89	0.99

[a] Larger test result indicates more positive test

[b] Smaller test result indicates more positive test

Gla carboxylated form of osteocalcin, *AGEs* advanced glycated end products

phosphatase, OST-PTP, a posttranslational modification where glutamic acid residues are carboxylated to form Gla residues. Lee et al. have demonstrated for the first time that mice lacking OST-PTP are hypoglycemic and protected from obesity and glucose intolerance, whereas mice lacking osteocalcin display decreased β-cell proliferation, glucose intolerance, and insulin resistance [3, 4, 11].

In the present study, decreased osteocalcin and increased Gla levels were demonstrated in insulin-resistant PCOS women to be strongly associated with the insulin resistance index, HOMA-IR. These findings suggest an interaction between these factors modified by carboxylation osteoblastic molecules and insulin action in PCOS, as has been implicated in other insulin resistance states, such as aging, obesity, and diabetes mellitus type 2 [12–17]. Furthermore, the association of decreased osteocalcin/increased Gla osteocalcin with characteristic PCOS features, such as androgens, ovarian morphology, and oxidative stress markers, suggests that the modification of osteocalcin by carboxylation may be involved in PCOS pathophysiological abnormalities.

Although the role of osteocalcin in PCOS remains to be clarified, the possible role of the carboxylated form of osteocalcin has not been investigated in this syndrome, to the best of our knowledge. In the present study, it was found that PCOS women displayed higher levels of Gla osteocalcin compared to controls and that these findings remained unchanged when data from lean and overweight subjects were analyzed separately. From bivariate analysis, a significant association of Gla with all characteristic features of PCOS was found. The strong positive association of Gla osteocalcin with insulin levels and HOMA-IR in the present study, is compatible with the findings of Lee et al. [11], which have displayed an association of insulin resistance with Gla overexpression in mice. Moreover, ROC analysis put emphasis on the very strong association of Gla with the existence of PCOS (see Table 2). Because osteocalcin synthesis is directly regulated from 1,25-$(OH)_2$-vitamin D and parathyroid hormone [see 22], the values of these hormones have also been evaluated, but no difference was observed between them, and accordingly the decreased levels of osteocalcin values found in the PCOS group could not be attributed to alterations of these factors.

Another interesting finding of the present study is that the elevated AGEs levels in PCOS women are significantly associated with osteocalcin and its carboxylated form. This finding is in accordance with in vitro studies that demonstrated that AGEs decrease osteocalcin mRNA production through the activation of their receptor pathway (RAGE), and that the administration of metformin reverses the effects of AGEs on osteoblasts [6, 18]. The possible interaction of AGEs in this context is also suggested by the elevated RANKL values in PCOS women in the present study. This finding may be explained from the fact that enhanced RANKL mRNA expression has been demonstrated in human osteoblast cultures exposed to AGEs [6]. However, further studies are required to elucidate the role of bone tissue in PCOS because, at present, data are rather limited and to some extent contradictory in women with this heterogeneous syndrome.

Escobar-Morreale et al. [19] demonstrated statistically significant lower OPG values and no difference in RANKL values in PCOS compared to controls, whereas in two other studies comparable osteocalcin values were observed between normal ovulating and PCOS women [20, 21]. A possible explanation for these discrepancies could be attributed to ethnic and/or methodological variations. Nevertheless, it should be mentioned that in our PCOS patients, although OPG levels were also lower compared to controls, the difference did not reach statistical significance.

In conclusion, in the present study, low levels of osteocalcin and elevated levels of its carboxylated form were found in a group of PCOS women in comparison to controls. In addition, one may assume that these bone-derived molecules may participate in the pathophysiological mechanisms of PCOS. Although one should be cautious with mechanistic interpretations of cross-sectional association studies, our

data support recently published experimental studies of osteocalcin-mediated crosstalk between the skeleton and energy metabolism. Clearly, further studies are required to unravel the intercommunication of bone and carbohydrate metabolism in PCOS women and the impact of bone tissue in abnormalities characterizing this multifaceted syndrome.

Conflict of interest All authors have nothing to disclose.

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