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# Metformin decreases bone turnover markers in polycystic ovary syndrome: a post hoc study

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**Objective:** To study the effects of metformin treatment on bone turnover in women with polycystic ovary syndrome (PCOS), as measured by serum concentrations of bone turnover markers.

**Design:** Post hoc study of a previously conducted prospective multicenter, placebo-controlled, randomized study.

**Setting:** University clinic.

**Patient(s):** The study cohort consisted of 74 non-obese women (body mass index $< 27$ kg/m$^2$) and 44 obese women (body mass index $\geq 27$ kg/m$^2$) diagnosed with PCOS, with a mean age of $27.6 \pm 4.0$ (SD) years.

**Intervention(s):** Randomization to receive metformin or placebo for 3 months.

**Main Outcome Measure(s):** Serum levels of bone formation marker procollagen type I amino-terminal propeptide (PINP) and bone resorption marker carboxy-terminal cross-linking telopeptide of type I collagen (CTX) at baseline and after metformin/placebo treatment.

**Result(s):** Serum levels of PINP and CTX were similar between the metformin and placebo groups at baseline in the whole study population. Obese women, when compared with non-obese, had lower baseline levels of PINP and CTX. Levels of PINP and CTX were significantly reduced in the whole study population, as well as in both non-obese and obese women after 3 months of metformin treatment, whereas no significant changes were observed in the placebo group.

**Conclusion(s):** Metformin treatment, when compared with placebo, was associated with reduced bone turnover, as suggested by reductions in markers of bone formation and resorption, leading to slower bone remodeling in premenopausal women with PCOS.

**Clinical Trial Registration Number:** NCT00994812. (Fertil Steril® 2019;■:■–■. ©2019 by American Society for Reproductive Medicine.)

**Key Words:** Bone turnover markers, carboxy-terminal cross-linking telopeptide of type I collagen (CTX), metformin, polycystic ovary syndrome, procollagen type I amino-terminal propeptide (PINP)

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Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age, with a prevalence of 6%–15%, depending on the diagnostic criteria used (1). Women with PCOS show heterogeneity of characteristics, including oligo/amenorrhea, hyperandrogenism, obesity, insulin resistance, and hyperinsulinemia. Peak skeletal mass is attained from late adolescence to the early thirties, and menstrual dysfunction during this period might influence the bone mass accrued.

Furthermore, both androgens and estrogens have an independent and possibly additive association with peak bone mass attainment and maintenance (2). It has been postulated that hormonal imbalance in women with PCOS might have a negative effect on bone formation and bone mineral density (BMD) (3), but whether it predisposes to osteoporosis in later life remains elusive. Furthermore, few studies have even reported lower BMD in women with PCOS compared with their healthy counterparts (4, 5).

Metformin is one of the widely used drugs for the treatment of PCOS and acts by inhibiting hepatic glucose production and increasing peripheral

ORIGINAL ARTICLE: REPRODUCTIVE ENDOCRINOLOGY

tissue sensitivity to insulin. Long-term treatment with metformin has been shown to normalize ovulation, menstrual cyclicity, and hyperandrogenism [6]. Although the exact mechanism is not fully understood, it is thought that metformin lowers glucose production via activation of the $5'$ adenosine monophosphate-activated protein kinase (AMPK) pathway [7]. Further, AMPK subunits are highly expressed in bone tissue, osteoblasts, and osteoclasts. Cellular and animal studies have reported that metformin has a direct osteogenic effect and bone loss inhibiting effect [8]. Few clinical studies have evaluated the effects of metformin on bone metabolism and bone turnover in diabetics, suggesting a beneficial effect on bone [9]. However, there are only limited data as regards its effect on bone metabolism and measures of bone turnover in women with PCOS.

Bone, being a metabolically active tissue, undergoes continuous remodeling, wherein bone formation by osteoblasts is coupled to bone resorption by osteoclasts. Bone formation and resorption can be determined indirectly by the measurement of serum concentrations of various biomarkers (i.e., bone matrix components released into the circulation during bone formation or resorption). The serum concentrations of bone turnover markers (BTMs) reflect bone remodeling and can be used as markers of the rate of bone formation and resorption. These markers allow noninvasive assessment of bone turnover and are sensitive enough to reflect acute changes in it, providing a more representative view of overall bone loss than that obtained by measuring the rates of change in BMD at specific skeletal sites [10].

Ninety percent of the bone matrix is composed of type I collagen, which is synthesized as a precursor procollagen, cleavage of which releases procollagen type I aminoterminal propeptide (PINP) into the circulation. The carboxy-terminal cross-linking telopeptide of type I collagen (CTX) is released from the bone matrix during resorption and reflects the degradation of type I collagen. Thus, PINP and CTX reflect the rates of bone formation and resorption, respectively [11]. The International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine have recommended the use of serum PINP and CTX as reference biochemical markers of bone formation and resorption, respectively [12].

The present study was a post hoc secondary study among a subset of patients who have been described in a previously published prospective multicenter, placebo-controlled, randomized study on the effects of metformin on miscarriage, pregnancy and live-birth rates, which showed that metformin treatment compared with placebo improved pregnancy and live-birth rates in women with PCOS [13]. The aim of the present study was to investigate the effects of metformin on bone turnover markers in women with PCOS. In line with the recommendations of the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine, PINP and CTX were used as reference biochemical markers of bone formation and resorption.

## MATERIALS AND METHODS
### Subjects

The study population consisted of 118 Caucasian women (mean age $27.6 \pm 4.0$ [SD] years, mean body mass index [BMI] $26.5 \pm 6.0$ kg/m$^2$) diagnosed with PCOS according to the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) consensus definition [14]. The present study was a post hoc analysis among a subset of subjects who were selected from a large cohort of subjects in a prospective multicenter, placebo-controlled randomized study on the effects of metformin on miscarriage, pregnancy, and live-birth rates [13]. Only the subjects who were examined at Oulu University Hospital were included in the present study. The primary study was registered under the clinical trial registration number NCT00994812 and was approved by the Ethics Committee of the Northern Ostrobothnia Hospital District (1396/2004) and the National Supervisory Authority for Welfare and Health (D1339/05.01.00.06/2009). The primary study was conducted during 2003–2009, with the first patient enrolment on November 10, 2002.

Women who became pregnant or had a miscarriage before the study period of 3 months were excluded from the present study. All women in the present study were examined and recruited at Oulu University Hospital during 2003–2009. Informed written consent was obtained from all the subjects. Women with diabetes, active liver disease (alanine aminotransferase > 100 IU/L), past or present cardiac failure (New York Heart Association I–IV), and liver or renal failure (s-creatinine > 124 $\mu$mol/L), women who used alcohol and hormone preparations, smokers, and pregnant and lactating women were excluded from the original study. None of the study subjects were using medications known to affect hormonal or metabolic parameters or bone metabolism, and none had a history of fracture in the preceding 6 months. The subjects were not allowed to use calcium, vitamin D, dietary supplements, herbal therapies, or vitamins during the study.

Non-obese women (BMI < 27 kg/m$^2$) received metformin (Diformin; Leiras) at a dose of 500 mg + 1,000 mg daily, or placebo; obese women (BMI $\geq$ 27 kg/m$^2$) received metformin at a dose of 1,000 mg twice daily, or placebo. The limit for BMI was chosen on the basis of earlier studies that indicated increased insulin resistance in women with PCOS at a BMI of 27 kg/m$^2$ [15]. The dose of 1,500 mg of metformin for non-obese women with PCOS and 2,000 mg for obese women was based on earlier studies [16–18], which showed that 1,500 mg and 2,000 mg of metformin was effective enough to restore ovulation in most of the non-obese and obese women with PCOS, respectively, and to improve hyperandrogenism and insulin sensitivity significantly. Furthermore, using a smaller dose in non-obese women was to minimize possible side effects, and thereby dropouts.

Clinical, metabolic, and hormonal parameters were evaluated 1–7 days after spontaneous menstruation in oligomenorrheic subjects or at any other convenient time in amenorrhoeic subjects. A second evaluation was scheduled

## TABLE 1

**Characteristics of the study population.**

| Characteristic | Metformin (n = 57) | Placebo (n = 61) | All subjects |
|---|---|---|---|
| PCO + OA + HA | 21 (36.8) | 16 (26.2) | 37 (31.4) |
| PCO + OA | 35 (61.4) | 44 (72.1) | 79 (66.9) |
| PCO + HA | 1 (1.8) | 1 (1.6) | 2 (1.7) |

*Note.* Values are number (percentage). HA = hyperandrogenism (serum T level > 2.3 nmol/L and/or Ferriman-Gallwey hirsutism score of >7); OA = oligoamenorrhea; PCO = polycystic ovaries on ultrasonography.

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3 months after the first visit. Fifty-seven women received metformin and 61 received placebo for 3 months. Blood samples were collected in a fasting state at baseline and at 3 months of treatment with metformin/placebo and were stored at −20 °C until the time of analysis.

All study subjects had polycystic ovaries in ultrasonography according to the ESHRE/ASRM definition (14); the majority of them had oligoamenorrhea (n = 116, 98.3%), and 39 (33.1%) displayed hyperandrogenism (serum T level > 2.3 nmol/L, according to the upper limits of our accredited laboratory in fertile-aged women and/or Ferriman-Gallwey hirsutism score of >7) (Table 1). Other patient characteristics at baseline and after 3 months of treatment are shown in Table 2.

### Assays

Serum concentrations of PINP, CTX, and 25-hydroxyvitamin D (25OHD) were determined using the IDS-iSYS Multi-Discipline Analyser (Immunodiagnostics Systems) based on chemiluminescence technology, according to the manufacturer's protocol. In brief, the samples were incubated with specific antibodies, followed by the addition of streptavidin-coated magnetic particles. The magnetic particles were captured, and trigger reagents were added after further incubation. The concentration of analytes in the original sample was directly proportional to the resulting light emitted by the acridinium label. The reportable ranges of the assays for PINP, CTX, and 25OHD were 2–230 $\mu$g/L, 0.033–6 $\mu$g/L, and 5–140 $\mu$g/L, respectively. The respective intra- and interassay coefficients of variation were 4% and 2.2% for PINP, 2.3% and 1.8% for CTX, and 5.1% and 13% for 25OHD.

Serum concentrations of sex hormone-binding globulin (SHBG), androstenedione (A), dehydroepiandrosterone sulfate (DHEAS), and $E_2$ were analyzed, and oral glucose tolerance tests were carried out after an overnight fast as described earlier (13). Serum T was analyzed using Agilent triple-quadrupole 6410 liquid chromatography/mass spectrometry equipment with an electrospray ionization source operating in positive-ion mode (Agilent Technologies) as detailed earlier (13). Multiple reaction monitoring was used to quantify T by trideuterated T. Intra-assay coefficients of variation of the method were 5.3%, 1.6%, and 1.2% for T at 0.6, 6.6, and 27.7 nmol/L, respectively. Interassay coefficients of variation were 5.3%, 4.2%, and 1.0% for the respective concentrations. The free androgen index (FAI) was calculated using the equa-

tion: 100 × T/SHBG (both as nmol/L). Homeostatic model assessment of insulin resistance (HOMA-IR) and the whole-body insulin sensitivity index (i.e., the Matsuda index) were calculated to quantify the degree of insulin resistance (19, 20).

### Statistical Methods

Statistical analyses were performed using SPSS 25.0 software (IBM). Variables with skewed distribution underwent logarithmic transformation. Independent samples *t* tests were used for comparisons between the metformin and placebo groups, and paired-samples *t* tests were used to evaluate changes between the measurements at baseline and after 3 months of treatment within the groups. General linear modeling was used to evaluate the significant determinant of changes in the levels of BTMs. Analysis of correlation between parameters was performed by using Pearson's correlation coefficient. A value of $P < .05$ was considered statistically significant.

## RESULTS

### Baseline Comparisons and Changes after 3 Months of Metformin/Placebo Treatment in Non-obese and Obese Women

Clinical, hormonal, and metabolic parameters were comparable between metformin and placebo groups at baseline in the non-obese and obese women (Table 2). There was a small but statistically significant decrease in weight ($P = .043$) and BMI ($P = .049$) in the obese group after metformin treatment. In addition, the concentrations of T ($P = .014$) and fasting glucose ($P = .004$) significantly decreased, and the Matsuda index significantly increased ($P = .046$). In the non-obese group treated with metformin, the concentrations of T ($P = .001$), the FAI ($P < .001$), and A ($P = .001$) significantly decreased. No statistically significant changes were observed in any of the clinical, hormonal, and metabolic parameters during placebo treatment in the non-obese and obese groups.

### Baseline Comparisons of BTMs and 25OHD

The baseline levels of PINP ($P = .307$), CTX ($P = .980$), and 25OHD ($P = .281$) did not differ between the metformin and placebo groups in the whole study population. However, obese women when compared with non-obese women had significantly lower levels of PINP (39.6 ± 15.9 [mean ± SD] $\mu$g/L vs. 50.0 ± 21.6 $\mu$g/L, $P = .003$) and CTX (0.32 ± 0.14 $\mu$g/L vs. 0.46 ± 0.21 $\mu$g/L, $P < .001$), and similar levels of 25OHD (22.0 ± 8.0 $\mu$g/L vs. 19.5 ± 6.4 $\mu$g/L, $P = .076$). Furthermore, in both metformin and placebo groups, obese women had lower levels of PINP and CTX compared with non-obese women, though the difference in PINP levels did not reach statistical significance in the metformin group (Table 3).

### Changes in BTMs and 25OHD after 3 Months of Metformin/Placebo Treatment

The levels of PINP and CTX were significantly decreased after 3 months of metformin treatment in both non-obese and

ORIGINAL ARTICLE: REPRODUCTIVE ENDOCRINOLOGY

## TABLE 2

Clinical, hormonal, and metabolic parameters at baseline and after 3 months of treatment with metformin/placebo in the study population.

| | Non-obese (BMI < 27 kg/m²) (n = 74) | | | | Obese (BMI ≥ 27 kg/m²) (n = 44) | | | |
| | Metformin (n = 40) | | Placebo (n = 34) | | Metformin (n = 17) | | Placebo (n = 27) | |
| Parameter | Baseline | 3 mo | Baseline | 3 mo | Baseline | 3 mo | Baseline | 3 mo |
|---|---|---|---|---|---|---|---|---|
| Age (y) | 27.1 (3.1) | | 27.9 (4.2) | | 28.8 (3.8) | | 27.3 (5.0) | |
| Weight (kg) | 61.0 (7.8) | 60.4 (7.5) | 62.3 (8.7) | 62.3 (8.7) | 89.7 (11.7) | 88.4 (11.8)[a] | 90.0 (14.1) | 90.1 (14.0) |
| BMI (kg/m²) | 22.5 (2.2) | 22.3 (2.2) | 22.7 (2.6) | 22.7 (2.5) | 33.4 (4.3) | 32.9 (4.4)[b] | 33.3 (4.4) | 33.3 (4.5) |
| WHR | 0.76 (0.05) | 0.76 (0.06) | 0.78 (0.06) | 0.78 (0.07) | 0.83 (0.06) | 0.83 (0.05) | 0.85 (0.05) | 0.84 (0.05) |
| Hirsutism score | 4.8 (3.1) | 5.1 (3.3) | 4.9 (3.2) | 4.6 (2.8) | 7.3 (3.7) | 7.0 (3.9) | 6.7 (4.9) | 6.0 (4.7) |
| $E_2$ (pmol/L) | 209.9 (93.8) | 204.9 (158.8) | 231.2 (98.4) | 236.5 (133.4) | 204.3 (53.6) | 207.2 (98.1) | 197.4 (108.4) | 200.2 (95.1) |
| T (nmol/L) | 1.6 (0.7) | 1.2 (0.6)[c] | 1.7 (0.7) | 1.6 (0.6) | 1.6 (0.7) | 1.3 (0.5)[d] | 1.4 (0.6) | 1.5 (1.0) |
| SHBG (nmol/L) | 59.0 (20.2) | 70.0 (41.3) | 56.6 (18.1) | 60.9 (27.0) | 43.1 (13.7) | 41.9 (16.1) | 35.1 (13.7) | 36.6 (30.4) |
| FAI | 3.0 (1.9) | 2.1 (1.3)[c] | 3.3 (2.2) | 3.1 (1.9) | 4.2 (2.1) | 3.6 (2.1) | 4.8 (3.5) | 5.0 (3.4) |
| DHEAS (µmol/L) | 5.3 (2.3) | 5.6 (2.5) | 6.2 (3.3) | 6.0 (2.7) | 4.9 (2.1) | 5.3 (2.2) | 5.4 (2.3) | 5.3 (1.9) |
| A (nmol/L) | 17.8 (9.5) | 14.6 (5.7)[e] | 21.4 (7.7) | 20.0 (8.1) | 15.7 (5.4) | 14.5 (5.7) | 17.1 (6.7) | 17.9 (8.0) |
| Fasting glucose (mmol/L) | 5.0 (0.5) | 4.9 (0.4) | 5.1 (0.4) | 5.0 (0.4) | 5.3 (0.4) | 5.1 (0.3)[f] | 5.3 (0.3) | 5.3 (0.3) |
| Fasting insulin (mU/L) | 5.6 (3.1) | 5.8 (2.8) | 6.4 (2.8) | 7.7 (6.5) | 17.2 (17.0) | 12.1 (5.9) | 14.5 (6.5) | 15.0 (7.9) |
| HOMA-IR | 1.3 (0.7) | 1.3 (0.6) | 1.5 (0.7) | 1.8 (1.7) | 4.2 (3.8) | 2.8 (1.4) | 3.4 (1.6) | 3.6 (1.9) |
| Matsuda index | 8.3 (3.7) | 9.0 (4.7) | 7.3 (3.6) | 6.6 (3.4) | 3.5 (2.3) | 4.2 (2.0)[g] | 3.4 (2.1) | 3.7 (3.3) |

*Note.* Values are mean (standard deviation). Hirsutism score according to Ferriman-Gallwey criteria. Conversion factor to SI units: insulin, 6.945 (pmol/L). A = androstenedione; BMI = body mass index; DHEAS = dehydroepiandrosterone sulfate; FAI = free androgen index; HOMA-IR = homeostatic model assessment of insulin resistance; SHBG = sex hormone-binding globulin; WHR = waist-hip ratio.

[a]–[g] Paired-samples *t* test: [a]$P=.043$; [b]$P=.049$; [c]$P<.001$; [d]$P=.014$; [e]$P=.001$; [f]$P=.004$; [g]$P=.046$ vs. baseline.

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obese women, whereas no significant differences were observed in the placebo group (Fig. 1, Table 3). The average declines from the baseline values were 25.7% for PINP and 31.1% for CTX in the non-obese women ($P<.001$ for both) and 32% for PINP ($P<.001$) and 24.1% for CTX ($P=.022$) in the obese women. Concentrations of 25OHD increased in both non-obese and obese women in the metformin and placebo groups, although statistically significant differences were observed only in non-obese women in the metformin group and obese women in the placebo group.

### Baseline Comparisons of BTMs and 25OHD, and Changes after 3 Months of Metformin/Placebo Treatment in Normoandrogenic and Hyperandrogenic Women

The subjects were further divided into normoandrogenic (NA) and hyperandrogenic (HA) (serum T level > 2.3 nmol/L and/or Ferriman-Gallwey hirsutism score of >7). The baseline concentrations of PINP, CTX, and 25OHD were comparable between NA and HA women in the metformin and placebo groups. The levels of PINP and CTX were significantly decreased after 3 months of metformin treatment in both NA ($P<.001$ for PINP and CTX) and HA women ($P=.002$ for PINP, $P=.001$ for CTX), whereas no significant differences were observed in the placebo group (Supplemental Table 1, available online). The average declines from the baseline values were 30% for PINP and 31% for CTX in NA women, and 22.8% for PINP and 27% for CTX in HA women. The levels of 25OHD were increased in NA women in both metformin ($P=.013$) and placebo groups ($P=.006$).

### Correlation Analyses and General Linear Modeling

Changes in PINP and CTX levels did not show any statistically significant correlations when compared with changes in the levels of $E_2$, T, SHBG, the FAI, A, DHEAS, fasting glucose, fasting insulin, HOMA-IR, or Matsuda index during metformin treatment. In general linear modeling, only metformin treatment, not BMI group or androgenic status, showed a statistically significant interaction with the changes in the levels of PINP ($P<.001$) and CTX ($P=.001$). As regards the changes in 25OHD levels, metformin treatment ($P=.772$) and BMI ($P=.442$) did not show any significant interactions.

## DISCUSSION

The present study showed that serum levels of the bone formation marker PINP and the bone resorption marker CTX significantly decreased during treatment with metformin in women with PCOS compared with those treated with placebo. During 3 months of metformin treatment, the average declines of PINP and CTX levels from baseline values were 27.4% and 30%, respectively, in the whole population. Furthermore, the significant decreases in the levels of PINP and CTX were observed in both non-obese and obese women with PCOS in the metformin group.

Bone turnover depends on bone formation and resorption through cross-talk between osteoblasts and osteoclasts. Reduced levels of markers of bone formation and resorption are associated with low bone turnover and a slower rate of bone loss. Studies have shown that low bone turnover could slow bone loss and give rise to a bone density exceeding that expected for age. Conversely, increased bone turnover

## TABLE 3

Bone turnover markers and 25OHD at baseline and after 3 months of treatment with metformin/placebo in the study population.

| Treatment | PINP ($\mu$g/L) at baseline | PINP ($\mu$g/L) after 3 mo | P value[a] | CTX ($\mu$g/L) at baseline | CTX ($\mu$g/L) after 3 mo | P value[a] | 25OHD ($\mu$g/L) at baseline | 25OHD ($\mu$g/L) after 3 mo | P value[a] |
|---|---|---|---|---|---|---|---|---|---|
| Metformin | | | | | | | | | |
| All women | 44.2 (19.1) | 32.1 (13.0) | <.001 | 0.40 (0.20) | 0.28 (0.15) | <.001 | 20.3 (7.3) | 23.2 (8.7) | .003 |
| BMI < 27 kg/m$^2$ | 47.0 (19.4)[b] | 34.9 (13.0) | <.001 | 0.45 (0.20)[c] | 0.31 (0.16) | <.001 | 21.6 (7.9) | 24.3 (8.8) | .017 |
| BMI ≥ 27 kg/m$^2$ | 37.5 (16.9)[b] | 25.5 (10.8) | <.001 | 0.29 (0.14)[c] | 0.22 (0.10) | .022 | 17.4 (4.9) | 20.7 (8.1) | .106 |
| Placebo | | | | | | | | | |
| All women | 48.0 (21.3) | 47.1 (21.4) | .576 | 0.40 (0.20) | 0.38 (0.20) | .147 | 21.8 (7.7) | 24.8 (9.9) | .007 |
| BMI < 27 kg/m$^2$ | 53.6 (23.7)[c] | 53.8 (24.6) | .907 | 0.47 (0.22)[e] | 0.45 (0.22) | .433 | 22.6 (8.2) | 24.8 (11.1) | .101 |
| BMI ≥ 27 kg/m$^2$ | 41.0 (15.4)[d] | 38.7 (12.5) | .361 | 0.33 (0.14)[e] | 0.30 (0.15) | .188 | 20.8 (7.0) | 24.8 (8.3) | .036 |

Note. Values are mean (standard deviation). 25OHD = 25-hydroxyvitamin D; BMI = body mass index; CTX = carboxy-terminal cross-linking telopeptide of type I collagen; PINP = procollagen type I amino-terminal propeptide.
[a]P values according to paired-samples t test.
[b–e]P values according to independent samples t tests for baseline comparisons in the same treatment group: [b]P=.083; [c]P=.015; [d]P=.001; [e]P=.004.

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is associated with accelerated bone loss and potential deterioration in bone quality (8, 21). Bone turnover markers reflect whole-body bone turnover, underlying changes in bone mass and bone histomorphometric parameters, and are thus predictive of total-body bone loss. Furthermore, there is a moderate association between baseline levels of BTMs and subsequent changes in BMD (22).

Cellular studies have shown that metformin is a potent stimulator of AMPK activation in osteoblasts, resulting in their differentiation and mineralization, and stimulates type 1 collagen production in osteoblast-like cell lines, suggesting a direct osteogenic effect (23–25), whereas few studies have not shown such an effect (26, 27). It has been reported that treatment with metformin prevents bone loss in ovariectomized rats, suggesting protective effects of metformin against bone loss (28, 29). In contrast, one study showed that metformin has no effect on bone mass in rodents (30).

## FIGURE 1
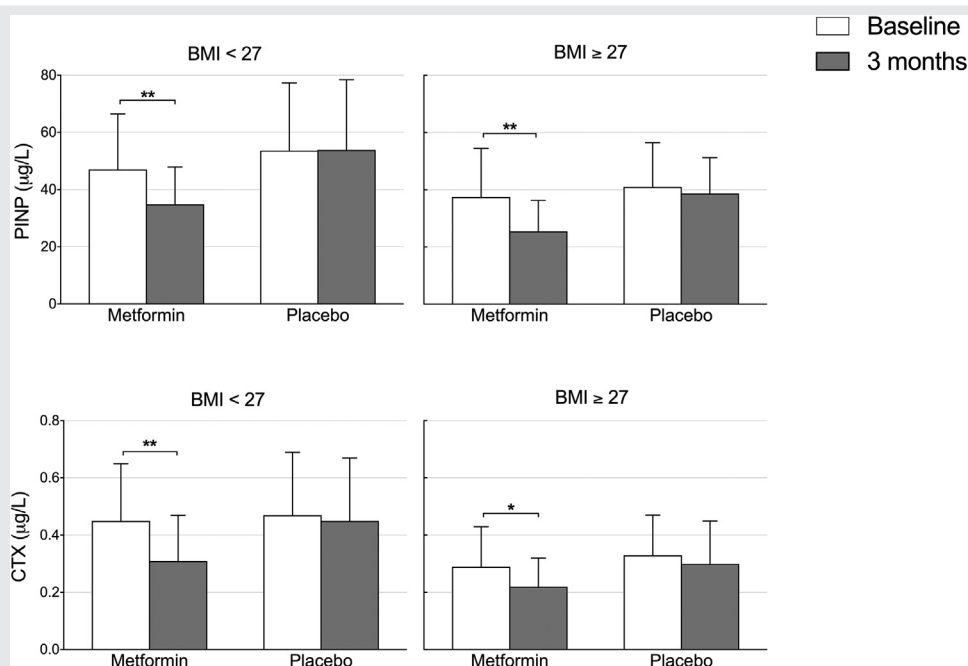


Concentrations of bone turnover markers at baseline and after 3 months of metformin/placebo treatment in women with polycystic ovary syndrome. The bars represent means and the error bars standard deviations. CTX = carboxy-terminal cross-linking telopeptide of type I collagen; PINP = procollagen type I amino-terminal propeptide. *P=.022; **P<.001.

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Studies on the effects of metformin on bone turnover in PCOS are still lacking, even though metformin is widely used in the treatment of the condition. In clinical studies, the effect of metformin on bone has been investigated mainly in diabetics. It has been reported that metformin reduces fracture risk in patients with type 2 diabetes mellitus (T2DM) (9), whereas one study found no association between metformin and fracture incidents (31). Furthermore, it has also been reported that metformin decreases the markers of bone formation and resorption and bone remodeling in T2DM (32).

In the present study, the baseline levels of BTMs in obese women with PCOS were already decreased when compared with non-obese women. Similar results have been observed in healthy premenopausal women with higher BMI (33). It has been postulated that higher BMI may be associated with increased secretion of various hormones from adipocytes (including estrogen), influencing osteoblast and osteoclast activity (34). In the present study, however, the $E_2$ levels in obese women with PCOS were not increased when compared with those of non-obese women. Furthermore, the concentrations of $E_2$ remained unchanged throughout the treatment period in both groups, suggesting that the decrease in BTMs may not be related to $E_2$ effect.

Androgens have been shown to influence bone metabolism directly through their action on osteoblasts by promoting bone formation, and also indirectly by inhibition of bone resorption (2). Furthermore, SHBG plays a crucial role in bone metabolism and remodeling because it binds to T and $17\beta$-$E_2$, thereby regulating their bioavailability and access to target cells (35), and may play a role in the determination of bone mass in premenopausal women. In the present study, the concentrations of T decreased in both non-obese and obese women with PCOS, and FAI decreased in non-obese women treated with metformin, which might be associated with decreased levels of BTMs. However, the changes in T, SHBG, and FAI levels during metformin treatment did not correlate with the changes in the levels of BTMs. Furthermore, the decrease in the levels of BTMs was not dependent on the androgen status of the women, because both NA and HA women with PCOS showed similar declines.

Increased mechanical loading secondary to increased body weight stimulates bone formation through stimulation of osteoblast activity (36). Body weight, covering fat mass and lean mass, has an impact on both bone turnover and bone density. In the present study, there was a decrease in BTMs in both obese and non-obese subjects in the metformin group, suggesting that metformin, not body weight, was the influencing factor in bone turnover. It is possible that the effect of metformin on BTMs is mediated via other mechanisms at the cellular level, which has to be investigated in future studies.

The important prohormone 25OHD influences BMD by regulating calcium metabolism, but 25OHD per se may not have any significant influence on BTMs (37). Even though 25OHD levels showed an increasing trend in non-obese and obese subjects in both treatment groups, significant increases were observed only in the non-obese subjects treated with metformin and obese subjects treated with placebo. It must be noted that the seasonal variation of 25OHD levels was not taken into account in the present study, which could be one explanation for the differences observed between the two treatment groups. Furthermore, studies on the effect of metformin on 25OHD levels are sparse, with conflicting results. It has been reported that treatment with metformin in T2DM has no effect on 25OHD levels (38, 39), whereas one study revealed that metformin improves 25OHD levels (40).

According to the Endocrine Society Clinical Practice Guideline (41), almost half (51%) of our study population was vitamin D deficient [25(OH)D below 20 $\mu$g/L], and 34% had vitamin D insufficiency [25(OH)D of 21–29 $\mu$g/L]. This is in line with studies reporting low levels of 25OHD in women with PCOS (42). Bone turnover markers decreased significantly in the metformin group compared with the placebo group in women with both deficient and insufficient vitamin D levels (Supplemental Table 2), suggesting that the decrease in BTMs was not dependent on vitamin D levels in our study population.

There are several strengths as well as limitations in our study. A potential limitation may be the selection of the study subjects: this is a post hoc analysis of a previously conducted study. However, the subjects who participated in the present study did not differ from the subjects of the primary study as regards PCOS phenotypes or anthropometric, hormonal, and metabolic parameters (data not shown). The duration of the treatment was 3 months, which may be a limiting factor. There is an interplay between resorption and formation locally in bone, meaning that when resorption increases formation increases, and vice versa. However, resorption is a faster process (2 to 3 weeks) when compared with formation (3 months) (43, 44). Thus, the present study period of 3 months should have been sufficient to depict changes in bone formation and resorption reflected by BTMs. The factors leading to biological variability in BTMs were minimized, because all blood samples were collected in a fasting state. This is particularly important as regards CTX levels, because they decrease by approximately 20% after food intake (11). Because none of the study subjects had any active liver disease or history of renal failure, the effects of clearance of PINP and CTX from the circulation by hepatic endothelium and the kidneys were controlled. The timing of samples was not scheduled according to the season, but earlier studies have shown that there is no significant seasonal variation in the levels of BTMs (45). The samples were taken during the follicular phase in oligomenorrheic women and at any time in amenorrhoeic women. Previous studies have shown that variations in the levels of BTMs over the menstrual cycle are so small that the effect of the menstrual cycle can be considered to be insignificant (10, 11). The effect of oligo-amenorrhea compared with regular menstrual cycles on BTMs could not be analyzed in the present study, because there were few women with regular cycles. Even though the study period of 3 months was sufficient to show the changes in the levels

of BTMs, the effect of treatment with metformin should be assessed during a longer study period to account for its effect on various hormonal and metabolic parameters.

In conclusion, metformin treatment of premenopausal women with PCOS for 3 months was associated with reduced bone turnover, as suggested by reductions in markers of bone formation and resorption, leading to slower bone remodeling preventing bone loss. However, long-term intervention studies with BMD measurements and fracture assessment are necessary to demonstrate the effects of metformin on bone turnover and remodeling in PCOS conclusively.

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