

1 **Running title:** Metformin lowers BTMs in PCOS

2 **Title: Metformin decreases bone turnover markers in polycystic ovary syndrome: A**
3 **post hoc study**

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20 **Capsule of abstract:** Metformin treatment in premenopausal women with polycystic ovary
21 syndrome for three months was associated with reduced bone turnover as suggested by
22 reduction of bone formation and resorption markers.

23 **Word count: 3636** (excluding title page, abstract, references and figure legend)

24 **Structured abstract**

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

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

30 **Setting:** University clinic

31 **Patient(s):** The study cohort consisted of 74 non-obese women (body mass index [BMI] <27
32 kg/m²) and 44 obese women (BMI ≥27 kg/m²) diagnosed with PCOS, with a mean age of
33 27.6 ± 4.0 (SD) years.

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

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

38 **Result(s):** Serum levels of PINP and CTX were similar between the metformin and placebo
39 groups at baseline in the whole study population. Obese women, when compared with non-
40 obese, had lower baseline levels of PINP and CTX. Levels of PINP and CTX were

41 significantly reduced in the whole study population as well as in both non-obese and obese
42 women after three months of metformin treatment, whereas no significant changes were
43 observed in the placebo group.

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

47 **Clinical Trial Registration Number (primary study):** NCT00994812

48 **Keywords:** polycystic ovary syndrome, metformin, bone turnover markers, procollagen type
49 I amino-terminal propeptide (PINP), carboxy-terminal cross-linking telopeptide of type I
50 collagen (CTX)

51 **Introduction**

52 Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of
53 reproductive age, with a prevalence of 6 to 15%, depending on the diagnostic criteria used (1).
54 Women with PCOS show heterogeneity of characteristics, including oligo/amenorrhea,
55 hyperandrogenism, obesity, insulin resistance and hyperinsulinemia. Peak skeletal mass is
56 attained from late adolescence to the early thirties, and menstrual dysfunction during this
57 period might influence the bone mass accrued. Furthermore, both androgens and estrogens
58 have an independent and possibly additive association with peak bone mass attainment and
59 maintenance (2). It has been postulated that hormonal imbalance in women with PCOS might
60 have a negative effect on bone formation and bone mineral density (BMD) (3), but whether it
61 predisposes to osteoporosis in later life remains elusive. Furthermore, few studies have even
62 reported lower BMD in women with PCOS compared with their healthy counterparts (4, 5).

63 Metformin is one of the widely used drugs for the treatment of PCOS, which acts by
64 inhibiting hepatic glucose production and increasing peripheral tissue sensitivity to insulin.
65 Long-term treatment with metformin has been shown to normalize ovulation, menstrual
66 cyclicity and hyperandrogenism (6). Although the exact mechanism is not fully understood, it
67 is thought that metformin lowers glucose production via activation of 5' adenosine
68 monophosphate-activated protein kinase (AMPK) pathway (7). Further, AMPK subunits are
69 highly expressed in bone tissue, osteoblasts and osteoclasts. Cellular and animal studies have
70 reported that metformin has a direct osteogenic effect and bone loss inhibiting effect (8). Few
71 clinical studies have evaluated the effects of metformin on bone metabolism and bone
72 turnover in diabetics, suggesting a beneficial effect on bone (9). However, there is only
73 limited data as regards its effect on bone metabolism and measures of bone turnover in
74 women with PCOS.

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

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

93 The present study was a post hoc secondary study among a subset of patients
94 who have been described in a previously published prospective multicentre, placebo-
95 controlled, randomized study on the effects of metformin on miscarriage, pregnancy and live-
96 birth rates, which showed that metformin treatment compared with placebo improved
97 pregnancy and live-birth rates in women with PCOS (13). The aim of the present study was to
98 investigate the effects of metformin on bone turnover markers in women with PCOS. In line

99 with the recommendations of the IOF and the IFCC, PINP and CTX were used as reference
100 biochemical markers of bone formation and resorption.

101 **Material and methods**

102 *Subjects*

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

116 Women who became pregnant or had a miscarriage before the study period of
117 three months were excluded from the present study. All women in the present study were
118 examined and recruited at Oulu University Hospital during 2003–2009. Informed written
119 consent was obtained from all the subjects. Women with diabetes, active liver disease (alanine
120 aminotransferase $>100 \text{ IU/L}$), past or present cardiac failure (New York Heart Association I-
121 IV), liver or renal failure (s-creatinine $>124 \mu\text{mol/L}$), alcohol and hormone preparation users,
122 smokers, pregnancy and lactation were excluded from the original study. None of the study

123 subjects were using medications known to affect hormonal or metabolic parameters, or bone
124 metabolism, and none had a history of fracture in the preceding six months. The subjects were
125 not allowed to use calcium, vitamin D, dietary supplements, herbal therapies or vitamins
126 during the study.

127 Non-obese women (BMI <27 kg/m²) received metformin (Diformin; Leiras) at a
128 dose of 500 mg + 1000 mg daily, or placebo; obese women (BMI ≥27 kg/m²) received
129 metformin at a dose of 1000 mg twice daily, or placebo. The limit for BMI was chosen on the
130 basis of earlier studies that indicated increased insulin resistance in women with PCOS at a
131 BMI of 27 kg/m² (15). The dose of 1500 mg of metformin for non-obese women with PCOS
132 and 2000 mg for obese women was based on earlier studies (16-18) which showed that 1500
133 mg and 2000 mg of metformin was effective enough to restore ovulation in most of the non-
134 obese and obese women with PCOS, respectively, and to improve hyperandrogenism and
135 insulin sensitivity significantly. Furthermore, using a smaller dose in non-obese women was
136 to minimize possible side effects, and thereby dropouts.

137 Clinical, metabolic and hormonal parameters were evaluated 1–7 days after
138 spontaneous menstruation in oligomenorrhic subjects or at any other convenient time in
139 amenorrhoeic subjects. A second evaluation was scheduled three months after the first visit.
140 Fifty-seven women received metformin and 61 received placebo for three months. Blood
141 samples were collected in a fasting state at baseline and at three months of treatment with
142 metformin/placebo and were stored at -20 °C until the time of analysis.

143 All study subjects had polycystic ovaries in ultrasonography according to the
144 ESHRE/ASRM definition (14), the majority of them had oligo-amenorrhea (n=116, 98.3%)
145 and 39 (33.1%) displayed hyperandrogenism (serum testosterone level >2.3 nmol/L,
146 according to the upper limits of our accredited laboratory in fertile-aged women and/or

147 Ferriman-Gallwey hirsutism score of >7) (Table 1). Other patient characteristics at baseline
148 and after three months of treatment are shown in Table 2.

149 *Assays*

150 Serum concentrations of PINP, CTX and 25-hydroxyvitamin D (25OHD) were determined
151 using IDS-iSYS Multi-Discipline Analyser (IDS-iSYS, Immunodiagnosics Systems, Boldon,
152 UK) based on chemiluminescence technology, according to the manufacturer's protocol. In
153 brief, the samples were incubated with specific antibodies, followed by the addition of
154 streptavidin-coated magnetic particles. The magnetic particles were captured, and trigger
155 reagents were added after further incubation. The concentration of analytes in the original
156 sample was directly proportional to the resulting light emitted by the acridinium label. The
157 reportable ranges of the assays for PINP, CTX and 25OHD were 2–230 µg/L, 0.033–6 µg/L
158 and 5–140 µg/L, respectively. The intra- and inter-assay coefficients of variation were 4% and
159 2.2% for PINP, 2.3% and 1.8% for CTX, and 5.1% and 13% for 25OHD.

160 Serum concentrations of sex hormone-binding globulin (SHBG),
161 androstenedione, dehydroepiandrosterone sulfate (DHEAS) and estradiol (E2) were analyzed,
162 and oral glucose tolerance tests were carried out after an overnight fast as described earlier
163 (13). Serum testosterone (T) was analyzed using Agilent triple-quadrupole 6410 liquid
164 chromatography/mass spectrometry equipment with an electrospray ionization source
165 operating in positive-ion mode (Agilent Technologies, Wilmington, DE) as detailed earlier
166 (13). Multiple reaction monitoring was used to quantify T by trideuterated testosterone. Intra-
167 assay coefficients of variation of the method were 5.3, 1.6, and 1.2% for T at 0.6, 6.6, and
168 27.7 nmol/liter, respectively. Inter-assay coefficients of variation were 5.3, 4.2, and 1.0% for
169 the respective concentrations. The free androgen index (FAI) was calculated using the
170 equation: $100 \times T/\text{SHBG}$ (both as nmol/L). Homeostatic model assessment of insulin

171 resistance (HOMA-IR) and the whole-body insulin sensitivity index, *i.e.*, the Matsuda index
172 were calculated to quantify the degree of insulin resistance (19, 20).

173 ***Statistical methods***

174 Statistical analyses were performed using SPSS 25.0 software (IBM, Armonk, NY). Variables
175 with skewed distribution underwent logarithmic transformation. Independent samples *t*-tests
176 were used for comparisons between the metformin and placebo groups and paired-samples *t*-
177 tests were used to evaluate changes between the measurements at baseline and after three
178 months of treatment within the groups. General linear modeling was used to evaluate the
179 significant determinant of changes in the levels of BTMs. Analysis of correlation between
180 parameters was performed by using Pearson's correlation coefficient. A value of $P < 0.05$ was
181 considered statistically significant.

182 **Results**

183 ***Baseline comparisons and changes after three months of metformin/placebo treatment in*** 184 ***non-obese and obese women***

185 Clinical, hormonal and metabolic parameters were comparable between metformin and
186 placebo groups at baseline in the non-obese and obese women (Table 2). There was a small
187 but statistically significant decrease in weight ($P=0.043$) and BMI ($P=0.049$) in the obese
188 group after metformin treatment. In addition, the concentrations of testosterone ($P=0.014$) and
189 fasting glucose ($P=0.004$) significantly decreased, and the Matsuda index significantly
190 increased ($P=0.046$). In the non-obese group treated with metformin, the concentrations of
191 testosterone ($P<0.001$), the FAI ($P<0.001$) and androstenedione ($P=0.001$) significantly
192 decreased. No statistically significant changes were observed in any of the clinical, hormonal
193 and metabolic parameters during placebo treatment in the non-obese and obese groups.

194 ***Baseline comparisons of BTMs and 25OHD***

195 The baseline levels of PINP ($P=0.307$), CTX ($P=0.980$) and 25OHD ($P=0.281$) did not differ
196 between the metformin and placebo groups in the whole study population. However, obese
197 women when compared with non-obese women had significantly lower levels of PINP (39.6
198 ± 15.9 [mean \pm SD] $\mu\text{g/L}$ vs. 50.0 ± 21.6 , $P=0.003$) and CTX (0.32 ± 0.14 vs. 0.46 ± 0.21 ,
199 $P<0.001$), and similar levels of 25OHD (22.0 ± 8.0 vs. 19.5 ± 6.4 , $P=0.076$). Furthermore, in
200 both metformin and placebo groups, obese women had lower levels of PINP and CTX
201 compared with non-obese women though the difference in PINP levels did not reach
202 statistical significance in the metformin group (Table 3).

203 ***Changes in BTMs and 25OHD after three months of metformin/placebo treatment***

204 The levels of PINP and CTX were significantly decreased after three months of metformin
205 treatment in both non-obese and obese women, whereas no significant differences were
206 observed in the placebo group (Table 3, Figure 1). The average declines from the baseline
207 values were 25.7% for PINP and 31.1% for CTX in the non-obese ($P<0.001$ for both) and
208 32% for PINP ($P<0.001$) and 24.1% for CTX ($P=0.022$) in the obese women. Concentrations
209 of 25OHD increased in both non-obese and obese women in the metformin and placebo
210 groups, although statistically significant differences were observed only in non-obese women
211 in the metformin group and obese women in the placebo group.

212 ***Baseline comparisons of BTMs and 25OHD, and changes after three months of*** 213 ***metformin/placebo treatment in NA and HA women***

214 The subjects were further divided into normoandrogenic (NA) and hyperandrogenic (HA)
215 (serum testosterone level >2.3 nmol/L and/or Ferriman-Gallwey hirsutism score of >7). The
216 baseline concentrations of PINP, CTX and 25OHD were comparable between NA and HA
217 women in metformin and placebo groups. The levels of PINP and CTX were significantly
218 decreased after three months of metformin treatment in both NA ($P<0.001$ for PINP and

219 CTX) and HA women ($P=0.002$ for PINP, $P=0.001$ for CTX), whereas no significant
220 differences were observed in the placebo group (Supplemental Table 1). The average declines
221 from the baseline values were 30% for PINP and 31% for CTX in NA women, and 22.8% for
222 PINP and 27% for CTX in HA women. The levels of 25OHD were increased in NA women
223 in both metformin ($P=0.013$) and placebo groups ($P=0.006$).

224 *Correlation analyses and general linear modeling*

225 Changes in PINP and CTX levels did not show any statistically significant correlations when
226 compared with changes in the levels of estradiol, testosterone, SHBG, the FAI,
227 androstenedione, DHEAS, fasting glucose, fasting insulin, HOMA-IR or Matsuda index
228 during metformin treatment. In general linear modeling, only metformin treatment, not BMI
229 group or androgenic status, showed statistically significant interaction with the changes in the
230 levels of PINP ($P<0.001$) and CTX ($P=0.001$). As regards the changes in 25OHD levels,
231 metformin treatment ($P=0.772$) and BMI ($P=0.442$) did not show any significant interactions.

232 **Discussion**

233 The present study showed that serum levels of the bone formation marker PINP and the bone
234 resorption marker CTX significantly decreased during treatment with metformin in women
235 with PCOS compared with those treated with placebo. During three months of metformin
236 treatment, the average declines of PINP and CTX levels from baseline values were 27.4% and
237 30% respectively in the whole population. Furthermore, the significant decreases in the levels
238 of PINP and CTX were observed in both non-obese and obese women with PCOS in the
239 metformin group.

240 Bone turnover depends on bone formation and resorption through crosstalk between
241 osteoblasts and osteoclasts. Reduced levels of markers of bone formation and resorption are
242 associated with low bone turnover and a slower rate of bone loss. Studies have shown that

243 low bone turnover could slow bone loss and give rise to a bone density exceeding that
244 expected for age. Conversely, increased bone turnover is associated with accelerated bone
245 loss and potential deterioration in bone quality (8, 21). Bone turnover markers reflect whole-
246 body bone turnover, underlying changes in bone mass and bone histomorphometric
247 parameters and are thus predictive of total-body bone loss. Furthermore, there is a moderate
248 association between baseline levels of BTMs and subsequent changes in BMD (22).

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

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

263 In the present study, the baseline levels of BTMs in obese women with PCOS were
264 already decreased when compared with non-obese. Similar results have been observed in
265 healthy premenopausal women with higher BMI (33). It has been postulated that higher BMI
266 may be associated with increased secretion of various hormones from adipocytes (including
267 estrogen) influencing osteoblast and osteoclast activity (34). In the present study, however,

268 the estradiol levels in obese women with PCOS were not increased when compared with those
269 of non-obese women. Furthermore, the concentrations of estradiol remained unchanged
270 throughout the treatment period in both groups suggesting that the decrease in BTMs may not
271 be related to estradiol effect.

272 Androgens have been shown to influence bone metabolism directly through their
273 action on osteoblasts by promoting bone formation, and also indirectly by inhibition of bone
274 resorption (2). Furthermore, SHBG plays a crucial role in bone metabolism and remodeling as
275 it binds to testosterone and 17β -estradiol, thereby regulating their bioavailability and access to
276 target cells (35) and may play a role in the determination of bone mass in premenopausal
277 women. In the present study, the concentrations of testosterone decreased in both non-obese
278 and obese women with PCOS and those of FAI decreased in non-obese women treated with
279 metformin, which might be associated with decreased levels of BTMs. However, the changes
280 in testosterone, SHBG and FAI levels during metformin treatment did not correlate with the
281 changes in the levels of BTMs. Furthermore, the decrease in the levels of BTMs was not
282 dependent on the androgen status of the women, as both NA and HA women with PCOS
283 showed similar declines.

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

291 The important prohormone 25OHD influences BMD by regulating calcium
292 metabolism, but 25OHD per se may not have any significant influence on BTMs (37). Even

293 though 25OHD levels showed an increasing trend in non-obese and obese subjects in both
294 treatment groups, significant increases were observed only in the non-obese subjects treated
295 with metformin and obese treated with placebo. It must be noted that the seasonal variation of
296 25OHD levels was not taken into account in the present study, which could be one
297 explanation for the differences observed between the two treatment groups. Furthermore,
298 studies on the effect of metformin on 25OHD levels are sparse, with conflicting results. It has
299 been reported that treatment with metformin in T2DM has no effect on 25OHD levels (38,
300 39), while one study revealed that metformin improves 25OHD levels (40).

301 According to the Endocrine Society Clinical Practice Guideline (41), almost half (51
302 %) of our study population were vitamin D deficient [25(OH)D below 20 µg/L] and 34% had
303 vitamin D insufficiency [25(OH)D of 21–29 µg/L]. This is in line with studies reporting low
304 levels of 25OHD in women with PCOS (42). BTMs decreased significantly in the metformin
305 group compared with the placebo group in women with both deficient and insufficient
306 vitamin D levels (Supplemental Table 2), suggesting that the decrease in BTMs was not
307 dependent on vitamin D levels in our study population.

308 There are several strengths as well as limitations in our study. A potential limitation
309 may be the selection of the study subjects as this is a post hoc analysis of a previously
310 conducted study. However, the subjects who participated in the present study did not differ
311 from the subjects of the primary study as regards PCOS phenotypes or anthropometric,
312 hormonal and metabolic parameters (data not shown). The duration of the treatment was three
313 months which may be a limiting factor. There is an interplay between resorption and
314 formation locally in bone, meaning that when resorption increases formation increases and
315 vice versa. However, resorption is a faster process (2-3 weeks) when compared with the
316 formation (three months) (43, 44). Thus, the present study period of three months should have
317 been sufficient to depict changes in bone formation and resorption reflected by BTMs. The

318 factors leading to biological variability in BTMs were minimized, as all blood samples were
319 collected in a fasting state. This is particularly important as regards CTX levels, as they
320 decrease by about 20% after food intake (11). Since none of the study subjects had any active
321 liver disease or history of renal failure, the effect of clearance of PINP and CTX from the
322 circulation by hepatic endothelium and the kidneys were controlled. The timing of samples
323 was not scheduled according to the season, but earlier studies have shown that there is no
324 significant seasonal variation in the levels of BTMs (45). The samples were taken during the
325 follicular phase in oligomenorrhoeic women, and at any time in amenorrhoeic women.
326 Previous studies have shown that variations in the levels of BTMs over the menstrual cycle
327 are so small that the effect of the menstrual cycle can be considered to be insignificant (10,
328 11). The effect of oligo-amenorrhea compared with regular menstrual cycles on BTMs could
329 not be analyzed in the present study, as the number of women with regular cycles were few.
330 Even though the study period of three months was sufficient to show the changes in the levels
331 of BTMs, the effect of treatment with metformin should be assessed during a longer study
332 period to account for its effect on various hormonal and metabolic parameters.

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

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344 **Conflicts of interest:** None

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474 **Figure Legend 1.** Concentrations of bone turnover markers at baseline and after three months
475 of metformin/placebo treatment in women with PCOS. The *bars* represent means and the
476 *error bars* standard deviation. BMI = body mass index in kg/m², PINP = procollagen type I

477 amino-terminal propeptide, CTX = carboxy-terminal cross-linking telopeptide of type I
478 collagen. ** $P < 0.001$, * $P = 0.022$

Table 1. Characteristics of the study population

	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: PCO = Polycystic ovaries in ultrasonography; OA = oligo-amenorrhea; HA = hyperandrogenism (serum testosterone level >2.3 nmol/L and/or Ferriman-Gallwey hirsutism score of >7); n = number of subjects

Table 2. Clinical, hormonal and metabolic parameters at baseline and after three months of treatment with metformin/placebo in the study population

	Non-obese (BMI <27 kg/m ²) (n=74)				Obese (BMI ≥27 kg/m ²) (n=44)			
	Metformin (n=40)		Placebo (n=34)		Metformin (n=17)		Placebo (n=27)	
	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months
Age (years)	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) ^c	90.0 (14.1)	90.1 (14.0)
BMI (kg/m ²)	22.5 (2.2)	22.3 (2.2)	22.7 (2.6)	22.7 (2.5)	33.4 (4.3)	32.9 (4.4) ^d	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)
E2 (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) ^a	1.7 (0.7)	1.6 (0.6)	1.6 (0.7)	1.3 (0.5) ^e	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) ^a	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) ^b	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: Data shown as mean (SD)

BMI = body mass index; WHR = waist-hip ratio; E2 = estradiol; T = testosterone; SHBG = sex hormone-binding globulin; FAI = free androgen index; DHEAS = dehydroepiandrosterone sulphate; A = androstenedione; HOMA-IR = homeostatic model assessment of insulin resistance; n = number of subjects.

Hirsutism score according to Ferriman-Gallwey criteria. Conversion factor to SI-units: insulin, 6.945 (pmol/L)

Paired-samples *t*-test: ^a *P*<0.001, ^b *P*=0.001, ^c *P*=0.043, ^d *P*=0.049, ^e *P*=0.014, ^f *P*=0.004, ^g *P*=0.046 compared with baseline

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

Treatment	PINP (µg/L) at baseline	PINP (µg/L) after 3 months	<i>P</i> value ^a	CTX (µg/L) at baseline	CTX (µg/L) after 3 months	<i>P</i> value ^a	25OHD (µg/L) at baseline	25OHD (µg/L) after 3 months	<i>P</i> value ^a
Metformin									
All women	44.2 (19.1)	32.1 (13.0)	<0.001	0.40 (0.20)	0.28 (0.15)	<0.001	20.3 (7.3)	23.2 (8.7)	0.003
BMI <27	47.0 (19.4) ^b	34.9 (13.0)	<0.001	0.45 (0.20) ^d	0.31 (0.16)	<0.001	21.6 (7.9)	24.3 (8.8)	0.017
BMI ≥27	37.5 (16.9) ^b	25.5 (10.8)	<0.001	0.29 (0.14) ^d	0.22 (0.10)	0.022	17.4 (4.9)	20.7 (8.1)	0.106
Placebo									
All women	48.0 (21.3)	47.1 (21.4)	0.576	0.40 (0.20)	0.38 (0.20)	0.147	21.8 (7.7)	24.8 (9.9)	0.007
BMI <27	53.6 (23.7) ^c	53.8 (24.6)	0.907	0.47 (0.22) ^e	0.45 (0.22)	0.433	22.6 (8.2)	24.8 (11.1)	0.101
BMI ≥27	41.0 (15.4) ^c	38.7 (12.5)	0.361	0.33 (0.14) ^e	0.30 (0.15)	0.188	20.8 (7.0)	24.8 (8.3)	0.036

Note: Data shown as mean (SD)

PINP = procollagen type I amino-terminal propeptide; CTX = carboxy-terminal cross-linking telopeptide of type I collagen; 25OHD = 25-hydroxyvitamin D; BMI = body mass index in kg/m².

^a *P* values according to paired-samples *t*-test.

^{b,c,d,e} *P* values according to independent samples *t*-tests for baseline comparisons in the same treatment group.

^b *P*=0.083, ^c *P*=0.001, ^d *P*=0.015, ^e *P*=0.004.

Supplemental Table 1. Bone turnover markers and 25OHD at baseline and after three months of treatment with metformin/placebo in normoandrogenic and hyperandrogenic study population

Treatment	PINP (µg/L) at baseline	PINP (µg/L) after 3 months	<i>P</i> value ^a	CTX (µg/L) at baseline	CTX (µg/L) after 3 months	<i>P</i> value ^a	25OHD (µg/L) at baseline	25OHD (µg/L) after 3 months	<i>P</i> value ^a
Metformin									
NA (n=35)	46.0 (19.2)	32.3 (13.2)	<0.001	0.42 (0.21)	0.29 (0.16)	<0.001	20.8 (8.1)	24.2 (9.1)	0.013
HA (n=22)	41.3 (19.0)	31.9 (13.0)	0.002	0.37 (0.17)	0.27 (0.13)	0.001	19.5 (6.1)	21.7 (8.0)	0.129
Placebo									
NA (n=44)	49.9 (20.7)	48.1 (20.4)	0.377	0.43 (0.21)	0.40 (0.20)	0.081	22.4 (6.5)	25.9 (9.8)	0.006
HA (n=17)	43.2 (22.6)	44.6 (24.4)	0.572	0.33 (0.15)	0.34 (0.19)	0.817	20.3 (10.2)	21.7 (9.7)	0.497

Note: Data shown as mean (SD)

PINP = procollagen type I amino-terminal propeptide; CTX = carboxy-terminal cross-linking telopeptide of type I collagen;

25OHD = 25-hydroxyvitamin D; NA = normoandrogenic; HA = hyperandrogenic (serum testosterone level >2.3 nmol/L and/or Ferriman-Gallwey hirsutism score of >7).

^a*P* values according to paired-samples *t*-test.

Supplemental Table 2. Bone turnover markers at baseline and after three months of treatment with metformin/placebo in vitamin D deficient and vitamin D insufficient women with PCOS

Treatment	PINP (µg/L) at baseline	PINP (µg/L) after 3 months	<i>P</i> value^a	CTX (µg/L) at baseline	CTX (µg/L) after 3 months	<i>P</i> value^a
Metformin						
Vit D deficient (n=32)	41.2 (19.4)	28.8 (13.3)	<0.001	0.37 (0.18)	0.25 (0.13)	<0.001
Vit D insufficient (n=18)	48.2 (18.8)	36.2 (12.3)	0.001	0.45 (0.18)	0.32 (0.15)	0.001
Placebo						
Vit D deficient (n=28)	49.2 (23.0)	47.4 (21.8)	0.528	0.40 (0.23)	0.38 (0.22)	0.355
Vit D insufficient (n=22)	49.5 (21.7)	48.6 (22.8)	0.696	0.41 (0.18)	0.41 (0.20)	0.984

Note: Data shown as mean (SD)

PINP = procollagen type I amino-terminal propeptide; CTX = carboxy-terminal cross-linking telopeptide of type I collagen;

Vit D deficient = 25(OH)D below 20 µg/L; Vit D insufficient = 25(OH)D 21–29 µg/L.

^a*P* values according to paired-samples *t*-test.

Figure(s)

