Metformin improves endothelial function in normoinsulinemic PCOS patients: a new prospective

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BACKGROUND: Metformin was reported to improve the alterations of endothelial reactivity in normal-weight subjects with polycystic ovary syndrome (PCOS). The aim of the present study was to investigate the mechanisms of action of this drug on the vascular function of this population. METHODS: Thirteen normal-weight, normoinsulinemic and normolipemic PCOS women were studied before and after 6 months of metformin treatment (1000 mg/day). The endothelial function was assessed by evaluating the flow-mediated dilatation (FMD) of the brachial artery. We correlated this parameter with the endocrine-metabolic features of the patients. RESULTS: Metformin significantly reduced testosterone (1.56 ± 0.52 after 6 months versus 2.98 ± 1.00 at baseline) and 17-hydroxyprogesterone (0.03 ± 0.01 versus 0.06 ± 0.02 nmol/ml) levels, without affecting gluco-insulinemic parameters. Concomitantly, the basal vessel diameter and the FMD significantly increased (4.12 ± 0.68 versus 3.2 ± 0.41 and 5.2 ± 0.6 versus 3.76 ± 0.5 mm, respectively), thus documenting an improved endothelial function. CONCLUSIONS: Our data confirm the positive effects of metformin on the altered vascular reactivity, a precocious marker of cardiovascular risk, in normoinsulinemic PCOS subjects. This improvement seems to be mediated through hormonal changes, thus highlighting the detrimental role of hyperandrogenemia on the endothelial function, even beyond the metabolic factors. However, a direct effect of metformin on the endothelium should not be excluded.

Keywords: polycystic ovary syndrome; cardiovascular disease; endothelial function; androgens; metformin

Introduction

Polycystic ovary syndrome (PCOS), a common and heterogeneous disorder in women of reproductive age, is characterized by chronic anovulation, menstrual cycle disturbances and androgens excess (Dunaif, 1992; Franks, 1995). Besides the reproductive abnormalities, PCOS women show an increased prevalence of insulin resistance, central obesity, dyslipidemia, hypertension, glucose intolerance and frank type 2 diabetes (Wild et al., 1995). These same features, beyond age and smoking, are recognized by the American College of Cardiology as the most important risk factors for cardiovascular disease (CVD) (Smith et al., 2004), which, indeed, appears to be one of the potential major long-term sequelae of PCOS (Conway et al., 1992).

In the last 10 years, several studies have documented, in obese PCOS women, an alteration of endothelium-mediated vessel function (Talbott et al., 1995; Kelly et al., 2002; Lakhani et al., 2004, 2005; Sorensen et al., 2006), which is considered a precocious and sensitive marker for cardiovascular disorders (Steinberg et al., 1996; Bonetti et al., 2003; Meyer et al., 2005; Dokras et al., 2006). However, despite the great amount of data on this issue, the pathogenetic mechanism accounting for the endothelial abnormalities in such women remains controversial: either the association obesity/insulin-resistance or the abnormal androgens levels, or both, has been alternatively ascribed different importance. This disagreement partially arises from the close etio-pathogenic relationship between the metabolic alterations and the hyperandrogenism in PCOS (Legro, 2006; Pasquali et al., 2006).

Central obesity, for instance, is associated with an increased risk of coronary heart disease related to modification in endothelial reactivity, both in relation to the abdominal adiposity per se and to the typical presence of insulin resistance (Brook et al., 2001; Lteif et al., 2007). Actually, circulating insulin, in physiological concentrations, is known to play an important role on the endothelium-mediated vasodilatation by enhancing endothelium-derived nitric oxide (NO) production in insulin-sensitive subjects (Muniyappa et al., 2007). Hence, in conditions characterized by a diminished peripheral insulin sensitivity, the capacity of the endothelium to display a vasodilatory response to even high compensatory insulin plasmatic levels may be impaired (Hsueh et al., 2004; Sharma and Nestler, 2006; Muniyappa and Quon, 2007; Muniyappa et al., 2007).
On the other hand, hyperandrogenemia may exert an independent negative influence on the endothelial function, as suggested by several studies indicating a proatherogenic action for androgens (Adams et al., 1995; Eckardstein and Wu, 2003; Wu and Eckardstein, 2003).

A few studies have previously reported an altered endothelial function also in normal-weight PCOS patients who, despite an absence of excessive body weight, show gluco-insulinemic abnormalities and hyperandrogenemia more frequently than the general population (Paradisi et al., 2001; Diamanti-Kandarakis et al., 2005). Metformin, an insulin-sensitizing agent, has been reported to ameliorate the endothelial function of normal-weight PCOS subjects through the improvement of insulin peripheral action (Orio et al., 2005; Meyer et al., 2007).

In the present study, we aimed to verify if metformin can really affect vascular reactivity in a highly selected population of normal-weight and normoinsulinemic patients with PCOS. Furthermore, we sought to get an insight into the mechanisms of action of the drug on the vascular reactivity of this subset of women, in order to indirectly obtain information about the relative contributions of insulin and androgens in the endothelial dysfunction typical of the syndrome.

Materials and Methods

Enrolment and basal study
Thirteen normal-weight women with PCOS were enrolled for this study. All the women had spontaneous onset of puberty and normal sexual development. All the women were euthyroid and none had taken medications known to affect plasma sex steroids for at least 3 months before the study. All subjects were volunteers and gave informed consent to participate in this study which was approved by our local ethics committees (Institutional Review Board of Department of Obstetrics and Gynaecology, Catholic University of Sacred Heart). In accordance with the Rotterdam Consensus Conference (Rotterdam ESHRE/ASRM, 2004), PCOS was diagnosed in the presence of at least two of the following criteria: irregular menstrual cycles (or amenorrhea), clinical and/or biochemical evidence of hyperandrogenism and ultrasound assessment of polycystic ovary [bilateral normal or enlarged ovaries with at least 10 microcysts (2–8 mm diameter) from the inner margin to the outer margin in longitudinal cross-section associated, with an augmented stromal area/total area ratio on ultrasonography] (Fulghesu et al., 2001).

We considered exclusion criteria the following findings:
(i) past or current history of CVD;
(ii) habitual smoking (daily consumption);
(iii) diabetes mellitus (or impaired glucose tolerance as determined by a standard 75 g oral glucose tolerance test);
(iv) hypertension;
(v) intake of hormones/vasoactive medication likely to affect indices of endocrine or endothelial function;
(vi) current hepatic or renal failure;
(vii) other hormonal dysfunction (hypothalamic, pituitary, thyroidal or adrenal causes for the clinical signs);
(viii) neoplasms or unstable mental illness.

Anthropometric measurements included body weight, height, body mass index (BMI) and the determination of waist-to-hip ratio (WHR). A BMI ranging from 19 to 25 kg/m² was considered suggestive of normal-weight. The grade of hirsutism was established using the Ferriman–Gallwey (F–G) score (Ferriman and Gallway, 1961). Patients were asked not to depilate for at least 1 month before each evaluation.

Studies were conducted during the early follicular phase of the menstrual cycles (spontaneous or progestin-induced cycles). The patients were hospitalized to undergo baseline evaluation; after fasting overnight for 10–12 h, blood samples were collected for the following assays: follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), androstenedione (A), 17-hydroxypregesterone (17OHP), sex hormone-binding globulin (SHBG), dehydroepiandrostosterone sulfate (DHEAS) triglycerides, total cholesterol, high and low density lipoproteins (HDL and LDL), very low density lipoprotein (VLDL), non-esterified fatty acids (NEFA) and hepatic and renal chemistries.

All women underwent an oral glucose tolerance test (75 g). Glycemia, insulin and C-peptide were assayed every 30 min for 4 h after glucose ingestion. Glucose and insulin plasma levels were evaluated as fasting values and as area under the curve (AUC) after glucose load, calculated by the trapezoidal rule. A normal insulinnemic response to OGTT was defined by a threshold AUC value of 15 000 μU/ml/240 min, as previously described (Ciampelli et al., 2005).

All blood samples were promptly centrifuged and the plasma stored at −20°C until assayed. Levels of LH, FSH, T, 17-OHP, A, DHEAS and SHBG were measured in duplicate by radioimmunoassay methods using a commercial kit (Radim, Pomezia, Italy). Intra-assay and inter-assay coefficients of variation were as follows: LH, 5.6% and 9.1%; FSH, 6.9% and 8.4%; androstenedione and T, 6.1% and 9.3%; insulin, 5.1% and 6.2%; and SHBG, 6.9% and 8.5%. Insulin was dosed by using a recombinant immunoassay. Plasma glucose was determined by the glucose oxidase method. Glucose plasma concentrations were determined by the glucose oxidase technique with a glucose analyzer (Beckam, Fullerton, CA, USA). Total cholesterol and triglyceride concentrations were determined by an enzymatic assay (Bristol, Paris, France). HDL concentrations were determined after precipitation of chylomicrons, VLDL and LDL (Boehringer, Mannheim, Germany); VLDL was separated (as the supernatant) from LDL and HDL by lipoprotein ultracentrifugation. A magnesium chloride/phosphotungstic acid technique was used to precipitate LDL from the bottom fraction after ultracentrifugation. NEFA were determined by an acyl-coenzyme A oxidase-based colorimetric method. All lipids assay were performed according to our standard laboratory procedures, as previously reported (Ciampelli et al., 1999).

Endothelial function assessment
Endothelial function was evaluated between 08.30 and 10.00 h, with subjects having fasted for at least 14 h. The subjects rested supine in a quiet, air-conditioned room (constant temperature, 22–25°C) for 30 min before endothelial function was assessed. Endothelial function was assessed in all women by measurement of flow-mediated dilatation (FMD) of the brachial artery in response to hyperemia of the hand, an NO-mediated process (Benjamin et al., 2004). FMD was measured as previously described, according to the method established by Corretti et al. (2002) and recently published guidelines. All studies were done by the same operator, who was unaware of the hormonal status of the women. Optimal imaging of the right brachial artery was obtained using an Echo-Doppler ultrasound (Esaote AU5 Harmonic, DSM5) and a 7.5 MHz transducer. Brachial artery diameter was measured by a blinded reader, at end-diastole, using electronic calipers from the anterior to the posterior m-line at a fixed distance from an anatomic marker. Images were acquired at baseline (after 30 min supine rest), during hand hyperemia, i.e. 90 s after deflation of a wrist cuff inflated to suprasystolic pressure (to at least 50 mmHg above systolic pressure) for 5 min for measurement
of FMD, and at 4 min after administration of 0.30 mg sublingual trinitrate for measurement of endothelium-independent, nitrate-mediated dilatation (NMD). All hemodynamic measurements were confirmed as having returned to baseline 15 min after release of the wrist cuff before administering the vasoactive drug. Brachial artery blood flow was measured by continuous wave Doppler as the product of the Doppler time–velocity integral, heart rate and brachial artery diameter measured at the time. FMD was calculated as the percentage increase in arterial diameter during hyperemia compared with the diameter at rest, whereas hyperemic flow was measured as the peak flow at 15 s after cuff release. For reproducibility: in our laboratory, the intra- and inter-observer variability for repeated measurements of brachial artery diameter are 0.10–0.11 and 0.09–0.17 mm, respectively. In studies performed on two separate days (5–7 days apart) in eight subjects by a single operator, the within-subject coefficients of variation of the endothelium-dependent and endothelium-independent responses were 4.9 and 3.2%, respectively.

**Interventions**

At the time of first visit, the PCOS patients start a therapy with chlorhydrate metformin 500 mg twice a day. During the study, chronically stabilized therapies not interfering with the parameters under evaluation were permitted. The use of other antidiabetic drugs was not allowed. Patients were recommended not to modify their usual diet and physical activity habits in order not to interfere with the metabolic effect obtained by metformin administration.

Patients returned to the hospital at the 3rd and 6th month of therapy and repeated the basal clinical work-up.

**Statistical analysis**

All results are presented as the mean ± SD and median. Insulin, glucose and C-peptide responses to the glucose load are also expressed as the AUC. The AUC was calculated by the trapezoidal rule method and reported as pmol/l times 240 min for insulin and C-peptide, and mmol/l times 90 min for glucose. The Kolmogorov–Smirnov test revealed that the data were not normally distributed. The significance of differences between the same tests performed before and after treatment was assessed by using the non-parametric Wilcoxon rank-sum test. P < 0.05 was considered statistically significant.

**Results**

At the beginning of the study, the age of the PCOS patients was of 24.69 ± 4.44 years.

No patient dropped out of the study. The metformin treatment was tolerated very well by all the patients and only three reported the appearance of gastrointestinal disorders in the first month.

Table I shows the clinical, hormonal and metabolic characteristics of PCOS at baseline and after 3 and 6 months of therapy with metformin. During the study, no significant variations were observed in either body weight or body fat distribution. Women affected by PCOS presented a mild to moderate hirsutism as showed by an augmented F–G score. Metformin treatment induced a significant decrease in the F–G score after 6 months of treatment. Moreover, the majority of the studied patients presented an improvement in menstrual abnormalities already in the first 3 months of metformin administration and a restoration of regular cycles was reached in ~75% of patients by the end of the treatment.

Our data revealed a normoinsulinemic response to a glucose load in the PCOS patients and, furthermore, no evidence of significant modifications in the glycemic and insulimemic responses to glucose load was documented in these PCOS

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<th>Table 1. Clinical, metabolic and hormonal features of PCOS patients at baseline and after 3 and 6 months of metformin treatment.</th>
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<td>Clinical and metabolic feature</td>
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<td>Age (years)</td>
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<td>BMI (kg/m²)</td>
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<td>WHR</td>
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<td>Menstrual abnormalities</td>
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<td>Ferriman Score</td>
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<td>Fasting insulin (pmol/l)</td>
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<td>AUC-glucose (mmol/l/240 min)</td>
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<td>AUC-insulin (pmol/l/240 min)</td>
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<td>AUC-C-peptide (pmol/l/240 min)</td>
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<td>Total cholesterol (mmol/l)</td>
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<td>LDL-cholesterol (mmol/l)</td>
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<td>FSH (mIU/ml)</td>
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<td>LH (mIU/ml)</td>
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Data are expressed as mean ± SD and median.

*BMI < 0.05 versus PCOS patients at baseline; **BMI < 0.01 versus PCOS patients at baseline.

BMI, body mass index; WHR, waist-to-hip ratio; HDL, high density lipoprotein; LDL, low density lipoproteins; AUC, area under the curve; T, testosterone; A, androstenedione; 17OHP, 17-hydroxyprogesterone; SHBG, sex hormone-binding globulin; DHEAS, dehydroepiandrosterone sulfate; FSH: follicle-stimulating hormone; LH, luteinizing hormone.
women after either 3 or 6 months of therapy. The mean serum levels of total cholesterol, HDL-, LDL-cholesterol and triglycerides were normal and metformin administration was unable to induce significant variations of these parameters in PCOS patients.

At baseline, serum LH, testosterone, 17OHP and androstenedione levels were in the upper limit of the normal range, whereas SHBG levels were at the lower limit of the normal range.

A significant improvement in plasma T levels was observed during metformin administration in PCOS women. We also observed a statistically significant decrease in 17OHP levels after 6 months of treatment. Gonadotrophins levels and the plasmatic concentrations of A, DHEAS and SHBG remained unchanged during metformin therapy.

The changes in endothelial function parameters in PCOS women during the study protocol are illustrated in Table II and Fig. 1. Metformin treatment induced a modification in endothelial performance, as evidenced by the significant increase in brachial artery diameter at rest, which underwent a 30.31% increase in the mean value after 3 months and a 37.23% increase after 6 months when compared with baseline. Moreover, the brachial artery diameter after the FMD (with median value of 4.6 and 5.02 mm, respectively, at 3 and 6 months versus 3.76 mm at baseline) and the difference between basal diameter of vessel and the diameter after stimulus were significantly raised during metformin treatment, achieving a statistically significant difference after 6 months of treatment. In our PCOS subjects, metformin did not affect NMD, which is an index of the endothelium-independent vascular reactivity (median of 4.69 and 5.35 mm, respectively, at 3 and 6 months versus 4.2 mm at baseline).

**Discussion**

The undamaged endothelium plays a crucial role in multiple mechanisms essential for the maintenance of vascular homeostasis, mostly through the activities of NO: by modulation of the tone of the underlying vascular smooth muscle; by inhibition of the lipid breakdown, inflammatory processes and vessel growth and by anticoagulant and antiplatelet, and thus antiatherogenic, activities (Lüscher and Vanhoutte, 1990; Vane et al., 1990; Voetsch et al., 2004).

For these major reasons, endothelial dysfunction is a key event in the pathophysiology of CVDs and appears as a strong independent predictor of CVD. In order to obtain a specific evaluation of arteries flow-resistance, some functional tests based on pharmacological stimuli have been validated in past experimental trials. In particular, the brachial artery FMD is an important marker of healthy endothelium: previous studies, indeed, have suggested that an impaired FMD represents a strong independent predictor of CVD in patients with peripheral arterial disease (Gokce et al., 2003).

The use of this diagnostic procedure has allowed several investigators to document a defective FMD in women with PCOS. Out of the multiple factors that may account for such dysfunction, most of the attention has been focused on the metabolic impairment typical of the syndrome. Dokras et al. (2006) hypothesized that insulin resistance and obesity may both give a determinant contribution to the endothelium damage in PCOS, whereas several other studies suggested that insulin resistance, but not obesity per se, could influence FMD (Paradisi et al., 2001, Meyer et al., 2005; Brinkworth et al., 2006). Furthermore, some authors interestingly have reported that the endothelium-mediated vasodilatory response is reduced also in normal-weight PCOS women compared with age- and BMI-matched control groups, thus

**Table II.** Endothelial function parameters in PCOS patients at baseline and after 3 and 6 months of metformin treatment.

<table>
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<th>Brachial artery flow</th>
<th>PCOS patients (13)</th>
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<td></td>
<td>Baseline</td>
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<tr>
<td>Basal diameter (mm)</td>
<td>3.20 ± 0.43 (3.14)</td>
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<tr>
<td>Diameter after FMD (mm)</td>
<td>3.76 ± 0.53 (3.76)</td>
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<tr>
<td>Diameter after NMD (mm)</td>
<td>4.25 ± 0.41 (4.20)</td>
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<tr>
<td>Δ after FMD (mm)</td>
<td>0.57 ± 0.33 (0.44)</td>
</tr>
<tr>
<td>Δ after NMD (mm)</td>
<td>1.05 ± 0.56 (0.99)</td>
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</tbody>
</table>

Data are expressed as mean ± SD, with median in parentheses.

*P < 0.05 versus PCOS patients at baseline; **P < 0.01 versus PCOS patients at baseline.

FMD, flow-mediated dilatation; NMD, nitrate-mediated dilatation.

**Figure 1:** Endothelial function parameters in PCOS patients at baseline and after 3 and 6 months of metformin treatment. FMD, flow-mediated dilatation; NMD, nitrate-mediated dilatation; Difference FMD, difference between basal diameter and the diameter after FMD; Difference NMD, difference between basal diameter and the diameter after NMD. **P < 0.01 versus baseline; *P < 0.05 versus baseline.
suggesting that factors other than obesity may be involved in such derangement (Paradisi et al., 2001; Orio et al., 2004; Tarkun et al., 2004; Kravariti et al., 2005).

In this regard, other investigators, while confirming the presence of endothelial dysfunction in PCOS independently of the presence of excessive body weight, have nevertheless laid emphasis on the key role of insulin-resistance in the development of the endothelial damage (Dokras et al., 2006; Sorensen et al., 2006; Nácul et al., 2007). In particular, Orio et al. (2005) treated a group of normal-weight, normoinsulinemic young PCOS women with metformin and observed an improvement in the endothelial function which was related to an improvement of insulin sensitivity.

The results from our study are not in agreement with this conclusion. We examined a group of highly selected PCOS women with normal BMI and normal insulinemic response to glucose load. In accordance with previous reports in literature, metformin did not affect the already normal insulin levels in our patients. Notwithstanding the lack of any metabolic change, we observed a significant increase of the vessel basal diameter and FMD after 3 and 6 months of metformin treatment. The finding of an increment in FMD in our relatively small population gains further relevance in the light of the evidence that such changes could be attenuated by the increment in baseline diameter, which could mask the percentage change in the diameter after stimulus. In this regard, a modification of 10% in width of brachial artery, as observed in our patients, might cause a decrease of absolute FMD value that is a result of the change in resting tone (Corretti et al., 2002).

The absence of significant changes of both fasting insulin plasma levels and insulin resistance evaluated by AUC-insulin (Paradisi et al., 2001, Ciampelli et al., 2005) allows us to transcend the correlation between glycoinsulinemic status and endothelial function.

In fact, our data seem to strengthen the contention that hyperandrogenism may play a pivotal role in the endothelial dysfunction in this typology of patients. The main modification observed in our PCOS women regarded the testosterone levels that underwent a significant decrease during the treatment with the insulin-sensitizer, metformin, and such biochemical change was clinically mirrored by an improvement of hirsutism score, which was statistically significant after 6 months of treatment. These data are in keeping with previous reports documenting an amelioration in clinical and hormonal features in normal-weight PCOS following a minimal change in the metabolic status (Morin Papunen et al., 1998).

The evidence that the major change concomitant to that observed in vascular diameter is represented by a decrease in testosterone levels supports the contention that not only insulin resistance, but furthermore hyperandrogenism, could be a contributing factor to CVD in PCOS (Paradisi et al., 2001; Apridonidze et al., 2005). Our hypothesis seems to rest on a solid physiopathologic basis. Previous studies have demonstrated the presence of androgen receptors on the vessel wall (Fujimoto et al., 1994), suggesting a role for androgens in vessel remodeling. In particular, androgens are believed to exert either a direct effect on the vasculature or an indirect effect through the amplification of vascular smooth muscle cell proliferation and proteoglycan biosynthesis, which are two critical contributors to the development of atherosclerosis (O’Brien et al., 1998; Skälen et al., 2002; Hashimura et al., 2005; Sudhir et al., 2005). Dihydrotestosterone regulates DNA synthesis in human vascular cells (Somjen et al., 1998) and testosterone worsens endothelial dysfunction in experimental atherosclerosis (Hutchison et al., 1997), suggesting that androgens might operate as proatherogenic factors. Different studies show that T, but not DHEA or E2, at physiological concentrations enhances apoptosis-related damage in human vascular endothelium (Ling et al., 2002). These mechanisms should support the proatherogenic effect of androgens on human endothelium and may represent a possible pathway of action for sex hormones in CVD.

Metformin treatment not only significantly decreased ovarian androgenic production, expressed as T plasma levels, but also induced a significant reduction in the adrenal production rate of steroids, expressed as 17OHP plasma levels. No previous studies have analyzed the relationship between 17OHP and endothelial function, which means that the possible association between this finding and the increment in vascular diameter is difficult to interpret.

Finally, we observed a return to a menstrual regularity in >75% of treated subjects, confirming the ability of metformin to reduce plasma androgen concentrations in PCOS women, influencing the steroidogenesis sites, and to restore the menstrual cyclicity (Glueck et al., 2001; Vrbíková et al., 2002; Ben-Haroush et al., 2004).

However, as a consequence of the absence of a close correlation between drug-induced metabolic and hormonal modifications, we cannot exclude the hypothesis that metformin could directly affect the endothelial function. Previous studies have, indeed, suggested a direct vasoactive effect of metformin, independently of its action on insulin, weight and inflammation mediators (De Jager et al., 2005; Jadhav et al., 2006); this drug is able to stimulate intracellular AMP-activated protein kinase (Cleasby et al., 2004; Shaw et al., 2005) and was reported to phosphorylate the endothelial isoform of NO synthases in human aortic endothelial cells (Morrow et al., 2003; Davis et al., 2006).

In conclusion, the present study suggests the importance of the hyperandrogenic status on the endothelial structure and function. Our data, though to be interpreted with caution given the small sample size and the lack of a control group, may provide an indication to reduce the cardiovascular risk in PCOS women by intervening on the androgen levels. Moreover, the results further enforce the prominent role of metformin in the management of PCOS: this drug could be able to prevent the CVD in the long term in women with PCOS either by decreasing insulin resistance or by influencing the androgen secretion, or possibly through a direct action on the endothelium.

Conflict of interest: All authors have nothing to declare.

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