CLINICAL STUDY

Effects of an antiandrogenic oral contraceptive pill compared with metformin on blood coagulation tests and endothelial function in women with the polycystic ovary syndrome: influence of obesity and smoking

Manuel Luque-Ramírez, Covadonga Mendieta-Azcona¹, José M del Rey Sánchez², Milagro Maties² and Héctor F Escobar-Morreale

Department of Endocrinology, Hospital Universitario Ramón y Cajal & Universidad de Alcalá, Carretera de Colmenar km 9¹, E-28034 Madrid, and Centre de Investigación Biomédica en Red Diabetes y Enfeimidades Metabólicas Asociadas CIBERDEM, Spain, ¹Department of Vascular Surgery, Hospital Universitario La Paz, E-28046 Madrid, Spain and ²Department of Clinical Biochemistry, Hospital Universitario Ramón y Cajal, E-28034 Madrid, Spain

(Correspondence should be addressed to H F Escobar-Morreale; Email: hescobarm.hrc@salud.madrid.org)

Abstract

Objective: To study the blood clotting tests and endothelial function of polycystic ovary syndrome (PCOS) patients and non-hyperandrogenic women, and their changes during PCOS treatment, as a function of the presence of obesity and smoking.

Design: Case-control study followed by a randomized clinical trial.

Methods: Blood clotting and endothelial function were analyzed in 40 PCOS patients and 20 non-hyperandrogenic women. Thirty-four PCOS women were randomized to an oral contraceptive containing 35 µg ethinyl-estradiol plus 2 mg cyproterone acetate (Diane³⁵Diario) or metformin (850 mg twice daily), monitoring the changes on these parameters during 24 weeks of treatment. The influence of obesity and smoking was also analyzed.

Results: Blood clotting and endothelial function tests were similar among PCOS patients and controls with the exception of a higher platelet count in the former. Obesity increased circulating fibrinogen levels, prothrombin activity and platelet counts, and reduced prothrombin and activated partial thromboplastin times. Smoking increased fibrinogen levels, platelet counts, and prothrombin activity, and reduced prothrombin time, in relation to the larger waist circumference of smokers. Irrespective of the treatment received, PCOS patients showed a decrease in prothrombin time and an increase in prothrombin activity, with a parallel increase in homocysteine levels in metformin users. The activated partial thromboplastin time decreased markedly in the patients treated with Diane³⁵Diario. Finally, flow-mediated dilation improved in non-smokers irrespective of the drug received, but worsened in smokers.

Conclusions: Oral contraceptives and metformin may exert deleterious effects on blood clotting tests of PCOS women, yet the effects of metformin appear to be milder. Because smoking potentiates some of these effects and deteriorates endothelial function, smoking cessation should be promoted in PCOS patients.

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Introduction

Cardiovascular risk markers (1–3) and early subclinical atherosclerosis (4, 5) cluster in women with the polycystic ovary syndrome (PCOS) as a consequence of their hyperandrogenism and insulin-resistant metabolic milieu. The latter might lead to a prothrombotic state and to endothelial dysfunction (6, 7), especially if amplified by the obesity (8, 9) frequently associated with PCOS (10, 11).

In addition to essential lifestyle modification and weight loss strategies, the current pharmacological treatment of PCOS is based mainly on the administration of two groups of drugs: oral contraceptives and insulin sensitizers. Because oral contraceptives increase the risk for venous thrombosis in the general population (12), an effect that appears to be amplified by obesity (13) and by smoking (14), the possible adverse impact of oral contraceptives on the already unfavorable prothrombotic state of PCOS patients should be considered.

During the past year, we have reported the results of an ample randomized controlled clinical trial (clinicaltrials.gov; NLM Identifier NCT00428311) addressing the effects of an antiandrogenic oral contraceptive pill compared with the insulin sensitizer metformin on a series of classic and non-classic cardiovascular risk factors (3, 15–18). In the present extended report of the aforementioned clinical trial, we show that both oral contraceptives and metformin might exert a detrimental effect on the blood coagulation tests of our young PCOS patients, whereas these drugs have no apparent major impact on their endothelial function.

Subjects and methods

Subjects and experimental design

Forty consecutive hyperandrogenic PCOS patients (age: 26 ± 6 (15–42) year; body mass index (BMI): 29.4 ± 6.3 (18.8–47.5) kg/m²; number of smokers: 17 (43%)) were selected. The diagnosis of PCOS required the presence of clinical and/or biochemical hyperandrogenism together with ovulatory dysfunction, after excluding secondary etiologies (19–21). The specific methods used to establish these criteria have been reported elsewhere (22).

A control group composed by 20 non-hyperandrogenic women (age: 27 ± 7 (13–38) year; BMI: 28.2 ± 6.9 (19.8–40.8) kg/m²; number of smokers: 9 (45%)) was selected as to be similar to the group of patients in terms of age, BMI, and frequency of smokers. PCOS patients and non-hyperandrogenic controls were submitted to a complete evaluation that included among other variables anthropometrical and analytical measurements and Doppler sonography-based exam of endothelial function. A detailed description of the baseline characteristics of patients and non-hyperandrogenic controls has been reported previously elsewhere (23).

None of the patients had either a personal history of hypertension, diabetes mellitus, hyperuricemia, venous thrombosis or cardiovascular events, or received treatment with oral contraceptives, antiandrogens, insulin sensitizers, vitamins or drugs that might interfere with blood pressure regulation, lipid profile, carbohydrates metabolism, blood clotting tests or endothelial function for the previous 6 months. In one patient (allocated to treatment with metformin), presenting with a history of venous thrombosis in a first-degree relative, common causes of hereditary thrombophilia were ruled out by results in assays of plasma homocysteine, antithrombin III, protein C and protein S functional activities, and of resistance to activated protein C, within the normal range.

Thirty-four of the 40 PCOS patients agreed to participate in the randomized clinical trial (clinicaltrials.gov; NLM Identifier NCT00428311). These patients were randomly allocated to receive an oral

contraceptive pill containing 35 µg of ethinyl-estradiol and 2 mg of cyproterone acetate (Diane³⁵Diario, $n = 15$, age: 23 ± 6 year, BMI: 29 ± 6 kg/m², number of smokers: 6 (40%)) or metformin ($n = 19$, age: 25 ± 7 year, BMI: 31 ± 7 kg/m², number of smokers: 8 (42%)), 850 mg twice daily.

A detailed description of the clinical trial and baseline characteristics of the patients has been reported previously (3). The anthropometrical and analytical evaluation performed at baseline was repeated after 12 and 24 weeks of treatment, whereas plasma homocysteine concentrations and endothelial function were evaluated at baseline and after 24 weeks of treatment.

Assays

The blood samples used for the clotting tests were collected in citrate-containing tubes and were immediately mixed by gentle inversion. Fibrinogen levels were measured by the standard Clauss method (24). Blood coagulation tests were conducted using a multiparameter automatic analyzer (Dade Behring BCS, Behringwerke AG, Marburg, Germany). The chronometric and chromogenic assays had reference ranges from 10.7 to 15.5 s for prothrombin time, from 60 to 130% for prothrombin activity and from 25 to 39 s for activated partial thromboplastin time. Platelet counts and mean platelet volume were measured by using an autoanalyzer (Beckman Coulter Inc., Fullerton, CA, USA). In two patients, the initial tests showed entirely abnormal clotting profiles suggesting sample processing and/or assay errors, whereas repeated duplicate analyses conducted before treatment allocation showed results within the normal range ruling out significant coagulopathy.

Plasma homocysteine concentrations were measured by a fluorescence polarization immunoassay (IMx Homocysteine assay, Abbott Laboratories, Abbott Park, IL, USA) with a sensitivity of 0.5 µmol/l and total coefficients of variation below 6%.

Ultrasound evaluation of endothelial function

We used the endothelial-dependent flow mediated vasodilatation and the endothelial-independent vasodilatation on the brachial artery as markers of endothelial function, using the method described by Celermajer (25) and following the guidelines of the American College of Cardiology (26). In brief, vascular reactivity was evaluated in all the women during the luteal phase of menstrual cycle by the same trained operator (C.M.-A.) using a high-resolution 7.5 MHz phased-array transducer (Imagepoint-Hx, Hewlett-Packard). Under light- and temperature-controlled conditions, and after advising the women to avoid smoking and caffeine intake for at least 4 h before starting the procedure, patients and controls were placed in supine with their right arms in a relaxed position.

A longitudinal section of the right brachial artery above the elbow was obtained, and a segment of the artery yielding a clear imaging of the interface between the lumen and the anterior and posterior arterial walls was selected for all further measurements.

Scans were taken at rest, after reactive hyperemia, again at rest, and after nitroglycerin administration. After a 10 min rest, the resting brachial artery diameter was estimated by measuring the distance between the 'm' lines (the interface between media and adventitia) of the anterior and posterior arterial walls at the end of diastole. Reactive hyperemia was then induced by inflating a pneumatic cuff above 250 mmHg for 3 min and the brachial artery diameter was estimated 30, 60, and 90 s after cuff deflation. Fifteen minutes were allowed for vessel recovery, and brachial artery diameter measurements were repeated 4 min after sublingual administration of 0.4 mg of nitroglycerin (Trinispray, Sanofi-Aventis S.A.U., Alcobendas, Madrid, Spain). The results are presented as basal brachial artery diameter, flow-mediated vasodilatation as an index of endothelial-dependent vasodilatation (FMD, $((\text{artery diameter after cuff deflation} - \text{basal artery diameter}) \times 100) / \text{basal artery diameter}$) and endothelial-independent vasodilatation $((\text{artery diameter after nitroglycerin} - \text{basal artery diameter}) \times 100) / \text{basal artery diameter}$). The coefficients of variation for brachial artery diameter before and after reactive hyperemia were 2.6 and 2.4% respectively.

Statistical analysis

Women were grouped as function of the grade of obesity (non-obese, BMI < 30 kg/m²; obese, BMI ≥ 30 kg/m²) and of smoking. The differences in continuous variables between groups were studied by one-way ANOVA or general linear models (GLM) as appropriate. Logarithmic transformation was applied to ensure normality as needed. Treatment effects on blood coagulation tests and ultrasonographic variables were analyzed by separate repeated-measures GLMs that included the arm of treatment, the presence of obesity and smoking as the between-subjects effects, and the visit of evaluation as the within-subjects effect.

The repeated-measures GLM is a statistical model where changes in a continuous dependent variable, measured more than once in the same individuals (blood coagulation tests and indexes of endothelial function in our study), are explained by a linear combination of functions of multiple independent explanatory variables (arm of treatment, obesity, and smoking in our study). In essence, the repeated-measures GLM is similar to a repeated-measures ANOVA, although the computational background of both tests is entirely different.

A statistically significant result from the statistic Wilks' λ in a between-subject effect indicates differences in the dependent variable between the subjects grouped according to this effect, but without considering the

within-subjects effect. A statistically significant result from the statistic Wilks' λ in the within-subjects effects indicates that there are significant changes in the dependent variables, in all the subjects considered as a whole. And a significant statistic Wilks' λ for the interaction among the between- and within-subjects effects indicates that the changes according to the within-subjects effects are not the same in the different groups of patients or, conversely, that the differences among the groups of patients are not the same at the different levels of the within-subjects effect.

A stepwise multiple regression analysis was used to study the changes in blood coagulation tests and in indexes of endothelial function as a function of the changes in waist circumference, free testosterone, and insulin sensitivity (with all the changes expressed as percentage of baseline values), the presence of obesity (coded 0 for non-obese patients and 1 for obese patients) and the arm of treatment (coded 0 for Diane³⁵Diario and 1 for metformin).

Because seven patients discontinued metformin for different reasons (3), the results obtained when considering only the patients completing the study were confirmed by intention-to-treat analysis assuming, for patients who did not complete the 24 weeks of the study, that the dependent variables had not changed at the missing visit with respect to previous visit. $P < 0.05$ was considered statistically significant.

Results

Case-control study

Anthropometrical and hormonal variables PCOS patients were more hyperandrogenic and insulin resistant compared with the controls (Table 1). Compared with their non-obese counterparts, obese women had higher BMI, waist circumference, waist-to-hip ratio (WHR), and free testosterone concentrations, and a reduced insulin sensitivity that was especially important in obese PCOS patients (Table 1). When smoking was introduced as an independent variable in the GLM, smokers had higher waist circumference (88 ± 17 vs 82 ± 14 cm, $F = 8.165$, $P = 0.006$) and WHR values (0.83 ± 0.10 vs 0.78 ± 0.08 , $F = 11.238$, $P = 0.002$) compared with non-smokers, regardless of the BMI that was similar among smokers and non-smokers (30 ± 7 vs 29 ± 6 kg/m², $F = 1.985$, $P = 0.165$).

Blood coagulation tests and indexes of endothelial function PCOS women showed a higher total platelet count compared with non-hyperandrogenic controls (Table 2). There were no other statistically significant differences either in fibrinogen concentrations or in any test of blood coagulation or endothelial function among PCOS women and non-hyperandrogenic controls

Table 1 Selected anthropometrical variables and hormonal profiles of obese and non-obese polycystic ovary syndrome patients and non-hyperandrogenic control women.

	Non-obese women (BMI <30 kg/m ²)		Obese women (BMI ≥30 kg/m ²)		Effect of PCOS		Effect of obesity		Interaction between PCOS and obesity	
	Controls (n=12)	PCOS (n=24)	Controls (n=8)	PCOS (n=16)	F	P	F	P	F	P
Age (year)	26±8	23±5	29±6	26±7	2.293	0.136	2.293	0.136	0.038	0.846
Body mass index (kg/m ²)	23.3±3.2	25.1±3.3	35.5±3.2	35.8±3.9	1.073	0.305	142.889	<0.001	0.605	0.440
Waist circumference (cm)	74±7	76±10	98±8	100±13	0.422	0.519	75.698	<0.001	0.003	0.960
Waist-to-hip ratio	0.75±0.06	0.76±0.07	0.83±0.08	0.88±0.09	2.392	0.128	22.194	<0.001	1.249	0.268
Free testosterone (ng/dl)	0.5±0.1	1.0±0.4	0.7±0.2	1.6±0.7	31.823	<0.001	10.652	0.002	3.441	0.069
Insulin sensitivity index	11.5±3.8	6.3±4.0	4.5±1.6	2.3±1.0	17.700	<0.001	38.711	<0.001	3.018	0.088

Data are means ±s.d. The differences among obese and non-obese PCOS patients and non-hyperandrogenic controls were analyzed by a two-way general linear model. To convert to SI units, multiply free testosterone by 34.67 (result in pmol/l).

considered as wholes (Table 2). However, there was a statistically significant interaction between PCOS and obesity proving that non-obese PCOS women showed a reduction in prothrombin times and increased prothrombin activities that were comparable to those of obese non-hyperandrogenic women (Table 2). Compared with their non-obese counterparts, obese women had increased fibrinogen levels, prothrombin activity, and platelet counts and reduced prothrombin and activated partial thromboplastin times, although the effect of obesity on prothrombin time and activity was only present in the non-hyperandrogenic population (Table 2). Interestingly, with the exception of platelet count, all the differences between obese and non-obese women lost significance when we explored the role of the waist circumference on these effects, introducing as a covariate in GLM (data not shown).

Furthermore, the higher free testosterone levels of the obese women compared with their non-obese counterparts did not explain the effects of obesity on blood clotting tests (data not shown).

Compared with non-smokers, smokers had higher fibrinogen levels, platelet counts and prothrombin activity, and reduced prothrombin time, (Fig. 1), yet the effect on the platelet count was not observed in obese women (Fig. 1). It must be noted, however, that with the exception of the effect on the platelet count, all the undesirable effects of smoking on blood coagulation tests lost statistical significance when the waist circumference or the WHR were introduced as covariates in the GLM (data not shown).

Finally, neither PCOS nor obesity or smoking influenced any of the indexes of endothelial function (Table 2).

Table 2 Blood clotting tests, flow-mediated dilatation, and endothelial-independent vasodilation of the brachial artery in polycystic ovary syndrome patients compared with non-hyperandrogenic controls, as a function of the presence or absence of obesity.

	Non-obese women (BMI <30 kg/m ²)		Obese women (BMI ≥30 kg/m ²)		Effects of PCOS		Effects of obesity		Interaction between PCOS and obesity	
	Controls (n=12)	PCOS (n=24)	Controls (n=8)	PCOS (n=16)	F	P	F	P	F	P
Fibrinogen (mg/dl)	254±35	280±43	337±70	367±77	3.172	0.080	29.339	<0.001	0.020	0.888
Prothrombin time (s)	13±2	12±1	12±1	12±1	3.433	0.069	8.301	0.006	5.705	0.020
Prothrombin activity (%)	89±19	100±9	106±12	101±15	0.738	0.394	5.787	0.019	4.582	0.037
Activated partial thromboplastin time (s)	33±3	32±4	30±3	30±2	0.006	0.937	7.157	0.010	0.822	0.369
Mean platelet volume (fl)	9.3±0.9	9.1±0.8	9.2±1.1	8.8±1.1	1.565	0.216	0.986	0.325	0.218	0.642
Platelet count (×10 ⁹ /μl)	223±40	251±44	267±60	297±64	4.169	0.046	9.804	0.003	0.002	0.961
Baseline brachial artery diameter (cm)	0.36±0.04	0.35±0.04	0.39±0.04	0.34±0.06	3.549	0.065	0.820	0.369	2.392	0.128
Flow-mediated dilatation (%)	5.6±9.3	7.1±10.7	5.3±5.4	10.3±9.6	1.480	0.229	0.305	0.583	0.419	0.520
Endothelial-independent vasodilatation (%)	22.4±9.8	23.5±10.4	15.3±10.2	25.0±7.4	1.620	0.208	0.122	0.728	2.081	0.155

Data are means ±s.d. The differences among non-obese and obese PCOS women and non-hyperandrogenic controls were analyzed by two-way general linear model. To convert to SI units, multiply fibrinogen by 0.0294 (result in μmol/l).

Figure 1 Influence of PCOS, smoking, and obesity on plasma fibrinogen concentrations, prothrombin time and activity, and platelet counts. Data were submitted to a GLM that included PCOS, smoking, and obesity as independent variables. The white boxes represent non-smokers and the patterned boxes represent smokers. The box-plot includes the median (horizontal line) and the interquartile range (box), and the whiskers indicate the minimum and maximum data values, unless outliers are present in which case the whiskers extend to a maximum of 1.5 times the interquartile range. The figures below the X-axis indicate the number of subjects in each subgroup. * $P < 0.05$ for the differences between women smokers and non-smokers. † $P < 0.05$ for the differences between obese and non-obese women. ‡ $P < 0.05$ for the interaction between PCOS status and the presence of obesity. § $P < 0.05$ for the interaction between PCOS status and smoking. || $P < 0.05$ for the interaction between smoking and the presence of obesity. ¶ $P < 0.05$ for the differences between PCOS patients and non-hyperandrogenic women.

Randomized clinical trial

The baseline characteristics of the patients allocated initially to Diane³⁵Diario or metformin, and those of the women on metformin who completed the study, are summarized in Table 3. There were no differences at baseline between the women allocated to Diane³⁵Diario, the women allocated to metformin who started the randomized trial, and the women allocated to metformin who completed the study (Table 3). There was no difference in the frequencies of smokers either among the women allocated to receive metformin who started the trial or those who completed it (42 vs 42%, $\chi^2 = 0.001$, $P = 0.981$) or among the women who started the trial in the Diane³⁵Diario arm of treatment and those who completed the treatment with metformin (40 vs 42%, $\chi^2 = 0.008$, $P = 0.999$) and PCOS patients did not change their smoking habits throughout the study.

Fibrinogen Plasma fibrinogen concentrations changed differently in the PCOS patients treated with Diane³⁵Diario; these levels decreased in obese patients reaching normal values, but increased in the non-obese ones (Fig. 2) reaching fibrinogen levels that were higher than those of the non-hyperandrogenic controls (Fig. 2). On the contrary, metformin did not induce any change in fibrinogen levels (Fig. 2).

These changes in plasma fibrinogen concentrations were associated only with the changes in waist circumference (stepwise multiple regression model: $R^2 = 0.181$, $\beta = 0.461$, $P = 0.015$; by intention-to-treat analysis: $R^2 = 0.172$, $\beta = 0.443$, $P = 0.009$). The changes in plasma fibrinogen with treatment throughout the study were similar in smokers and in non-smokers (data not shown).

Prothrombin time and activity When considered as a whole, PCOS patients suffered a decrease in prothrombin time and an increase in prothrombin activity during the clinical trial (Fig. 2), which was caused by the changes observed in the non-obese patients irrespective of the treatment received, and in the obese women treated with metformin (Fig. 2). On the contrary, no changes were observed in obese PCOS patients on Diane³⁵Diario (Fig. 2).

Compared with non-hyperandrogenic controls, the prothrombin time was reduced and the prothrombin activity was increased throughout the study in the non-obese subgroup of patients irrespective of the treatment applied (Fig. 2). In obese patients, however, these variables were comparable with the controls at baseline, but became abnormal during treatment only in the subgroup of obese women allocated to metformin (Fig. 2). Of note, only one obese patient allocated to metformin presented with mildly decreased prothrombin time and mildly increased prothrombin activity at the end of the study, whereas these

Table 3 Baseline characteristics of polycystic ovary syndrome patients randomly allocated to receive Diane³⁵Diario or metformin.

	Diane ³⁵ Diario (n=15)	Metformin (inten- tion-to-treat analysis) (n=19)	Metformin (patients completing the study) (n=12)	F	P
Age (year)	23±6	25±7	25±7	0.351	0.706
Body mass index (kg/m ²)	29.2±5.7	30.5±6.9	28.4±6.0	0.441	0.646
Waist circumference (cm)	83±12	89±18	85±16	0.543	0.585
Waist-to-hip ratio	0.79±0.06	0.82±0.11	0.80±0.09	0.646	0.529
Free testosterone (ng/dl)	1.1±0.4	1.3±0.6	1.2±0.6	0.781	0.464
Insulin sensitivity index	4.4±3.5	3.8±2.4	4.1±2.8	0.179	0.837
Fibrinogen (mg/dl)	311±81	326±75	299±41	0.578	0.566
Prothrombin time (s)	12±5	12±1	12±0.8	0.072	0.931
Prothrombin activity (%)	103±7	99±14	101±11	0.409	0.667
Activated partial thromboplastin time (s)	30±2	32±4	33±5	2.220	0.121
Mean platelet volume (fl)	9.0±1.0	9.0±1.0	8.8±0.9	0.157	0.856
Platelet count (×10 ³ /μl)	273±58	268±56	251±46	0.601	0.553
Baseline brachial artery diameter (cm)	0.36±0.04	0.35±0.05	0.36±0.05	0.558	0.576
Flow-mediated dilatation (%)	7.5±9.4	7.2±10.0	4.5±10.7	0.341	0.713
Endothelial-independent vasodilatation (%)	22.5±8.1	25.7±10.9	25.3±12.3	0.422	0.658

Data are means ± s.d. The differences among groups of treatment were analyzed by one-way ANOVA. The *F* and *P* values showed no differences among any of the groups in the variables presented here. To convert to SI units, multiply fibrinogen by 0.0294 (result in μmol/l) and free testosterone by 34.67 (result in pmol/l).

parameters remained within the normal range in all the other patients throughout the study.

The stepwise multiple regression analysis did not show any statistically significant association between the changes observed in prothrombin time and activity with the changes in waist circumference, free testosterone, and insulin sensitivity (data not shown). Smoking showed no statistically significant effect on prothrombin time or activity (data not shown).

Because the effect of metformin on prothrombin time and activity might be mediated by a decrease in the intestinal absorption of vitamin B₁₂, with a subsequent increase in homocysteine levels which in turn increases tissue factor and activates the extrinsic coagulation pathway (27), we measured homocysteine levels at baseline and at the end of the study. Plasma homocysteine concentrations increased in the PCOS patients treated with metformin, irrespective of smoking and obesity, and in the smokers allocated to Diane³⁵Diario, whereas these levels actually decreased with the oral contraceptive in non-smokers (Fig. 3). However, the influence of smoking on circulating homocysteine did not reach statistical significance when waist circumference was introduced as a covariate in the GLM.

Activated partial thromboplastin time The activated partial thromboplastin time decreased throughout the study in the patients treated with Diane³⁵Diario, irrespective of the presence or absence of obesity and smoking (Fig. 2). As a consequence of this change, at the end of the study, the activated partial thromboplastin time of the patients treated with Diane³⁵Diario was shorter than that of the patients treated with metformin and than that of the non-hyperandrogenic controls, which were similar (Fig. 2). Of note, one obese patient and one non-obese woman on Diane³⁵Diario

presented with mildly decreased activated partial thromboplastin times at the end of the study, whereas this variable remained within the normal range in all the other patients throughout the study.

In the stepwise multiple regression analysis, the changes observed in activated partial thromboplastin time throughout the trial were associated only with the arm of treatment in favor of the administration of metformin (stepwise multiple regression model: $R^2 = 0.329$, $\beta = -0.596$, $P = 0.001$; by intention-to-treat analysis: $R^2 = 0.403$, $\beta = -0.649$, $p < 0.001$), whereas no other association was observed either in the obese or non-obese women.

Platelet markers The mean platelet volume increased slightly during the trial in PCOS patients irrespective of the treatment applied, obesity and smoking, yet the increase only reached statistical significance in the intention-to-treat analysis, and the mean platelet volume attained was not different when compared with non-hyperandrogenic controls (Fig. 4). These changes in platelet volume were associated only with the change in abdominal circumference (stepwise multiple regression model: $R^2 = 0.122$, $\beta = -0.394$, $P = 0.042$; by intention-to-treat analysis: $R^2 = 0.142$, $\beta = -0.410$, $P = 0.016$).

The platelet count showed a small decrease in the obese women treated with Diane³⁵Diario that reached statistical significance only in the intention-to-treat analysis (Fig. 3), whereas no changes were observed in non-obese patients on Diane³⁵Diario or in women treated with metformin (Fig. 4).

Also according to the intention-to-treat analysis, smoking resulted in an increase in the platelet count that was independent of the arm of treatment and of obesity (Wilks' λ : 0.756, F : 3.875, $P = 0.035$), yielding

Figure 2 Changes in the blood clotting tests of PCOS patients submitted to treatment with Diane³⁵Diario or metformin for 24 weeks, as a function of obesity. Black circles represent the data from the patients allocated to Diane³⁵Diario, whereas white circles represent the data from the patients allocated to metformin. The small figures under the X-axis indicate the number of patients at each visit. Data are means $\pm$ S.E.M., and were submitted to a repeated-measures general lineal model introducing the arm of treatment and the presence or absence of obesity and smoking as between-subjects effects, and the visit of evaluation (baseline, 12 and 24 weeks) as the within-subjects effect. The shaded areas represent the 95% confidence intervals of the control group of non-hyperandrogenic women. The results were evaluated according to the analysis of the patients who completed the study and also according to intention-to-treat analysis. * $P < 0.05$ for the changes observed throughout the trial compared with baseline values considering all patients as a whole irrespective of the arm of treatment (in the analysis of the patients who completed the study and by intention-to-treat analysis). † $P < 0.05$ for the differences observed in the changes of each variable depending on the arm of treatment (in the analysis of the patients who completed the study). ‡ $P < 0.05$ for the differences between the values of PCOS patients at the end of the study, irrespective of the arm of treatment, and those of the control group of non-hyperandrogenic women (in the analysis of the patients who completed the study and by intention-to-treat analysis). § $P < 0.05$ for the differences observed in the changes of each variable depending on the arm of treatment (by analysis of the patients who completed the study and by intention-to-treat analysis). || $P < 0.05$ only for PCOS women on Diane³⁵Diario compared with PCOS women on metformin and with the control group of non-hyperandrogenic women (in the analysis of the patients who completed the study and by intention-to-treat analysis). ¶ $P < 0.05$ for the changes observed throughout the trial compared with baseline values among obese and non-obese women, irrespective of the arm of treatment (only by intention-to-treat analysis). ** $P < 0.05$ for the differences observed in the changes of each variable with respect to baseline depending on the arm of treatment and of obesity (in the analysis of the patients who completed the study and by intention-to-treat analysis). †† $P < 0.05$ for the differences observed in the changes of each variable with respect to baseline depending on the arm of treatment and of obesity (in the analysis of the patients who completed the study). ††† $P < 0.05$ only for obese PCOS women on metformin compared with the control group of obese non-hyperandrogenic women (in the analysis of the patients who completed the study). §§ $P < 0.05$ only for non-obese PCOS women on Diane³⁵Diario at the end of the study compared with the non-obese control subgroup (in the analysis of the patients who completed the study and by intention-to-treat analysis). |||| $P < 0.05$ for non-obese PCOS patients at the end of the study compared with the non-obese control subgroup, irrespective of the arm of treatment (in the analysis of the patients who completed the study and by intention-to-treat analysis). ¶¶ $P < 0.05$ only for non-obese PCOS women on Diane³⁵Diario compared with non-obese PCOS women on metformin at the end of the study and with the non-obese control subgroup (in the analysis of the patients who completed the study and by intention-to-treat analysis).

values that were nevertheless not different compared with those of the controls (Fig. 4). These small changes did not show any significant association in the multiple regression analysis (data not shown).

Indexes of endothelial function None of the indexes of endothelial function changed during the study as a function of the drug used for treatment and of the presence or absence of obesity (Fig. 4). FMD improved with both

Figure 3 Changes in homocysteine levels of PCOS patients submitted to treatment with Diane³⁵Diario or metformin for 24 weeks as a function of smoking. Black circles represent the data from PCOS women on Diane³⁵Diario, whereas white circles represent the data from the PCOS women on metformin. The upper panel represents the data of smokers and the lower panel represents the data of non-smokers PCOS women. The small figures under the X-axis indicate the number of patients at each visit. Data are means $\pm$ S.E.M., and were submitted to a repeated-measures general linear model introducing the arm of treatment, the presence or absence of obesity and smoking as between-subjects effects, and the visit of evaluation (baseline and 24 weeks) as the within-subjects effect. The results were evaluated according to the analysis of patients who completed the study and to the intention-to-treat analysis. * $P < 0.05$ for the differences observed in the changes of each variable with respect to baseline depending on the arm of treatment and of smoking (in the analysis of the patients who completed the study and by intention-to-treat analysis).

drugs in non-smokers and worsened in smokers, irrespective of obesity (Fig. 5). On the contrary, neither the arm of treatment nor obesity or smoking influenced endothelium-independent vasodilatation (Fig. 4).

Finally, the change in FMD was associated only with the changes observed in waist circumference (stepwise multiple regression model: $R^2 = 0.118$, $\beta = -0.390$, $P = 0.044$; by intention-to-treat analysis: $R^2 = 0.100$, $\beta = -0.358$, $P = 0.041$).

Discussion

Our present results indicate that obesity and abdominal adiposity influence the abnormalities in blood coagulation found in PCOS patients, as has been also

demonstrated for many other cardiovascular risk factors frequently associated with this prevalent disorder (10, 23).

The young PCOS patients studied here presented a procoagulant profile consisting of increased plasma fibrinogen levels and platelet counts, and decreased prothrombin times. Non-obese PCOS patients had prothrombin times and activities that were comparable to those of obese non-hyperandrogenic women, and clearly abnormal when compared with the non-obese controls. These findings suggest that PCOS is not only associated with abnormalities in fibrinolysis (6), but also with thrombophilia in conceptual agreement with the effect of insulin resistance on coagulation previously reported in the general population (8).

Furthermore, our results indicate that smoking amplifies the blood coagulation abnormalities found here, and that this deleterious effect appears to be mediated, at least partly, by the abdominal adiposity of smokers. In this regard, abdominal adiposity is not only an intrinsic characteristic of PCOS patients (10) but also very common in smokers in studies conducted in the general population (28–30). The fact that the effect of smoking on the blood coagulation abnormalities found in our series of young women was no longer statistically significant when correcting for the influence of abdominal adiposity strongly suggests that the synergistic effect of PCOS and smoking on abdominal adiposity may worsen the prothrombotic profile of these women (31, 32).

To our best knowledge, our present study is the first evaluating the differential effects that oral contraceptive pills and metformin might exert on the blood clotting tests of PCOS women, considering also the impact of obesity and smoking.

In conceptual agreement with the increase in the risk of venous thromboembolism reported with the use of oral contraceptives in the general population – which derives from the facilitation of the liver synthesis of procoagulant factors and the induction of resistance to activated protein C (33) – Diane³⁵Diario also exerted a detrimental effect on the clotting tests evaluating extrinsic and intrinsic coagulation pathways in our series of young PCOS patients. However, although Diane³⁵Diario is still considered a safe drug for the treatment of PCOS (34) and it has been suggested that the risk of thrombotic events in women taking oral contraceptives containing ethinyl-estradiol plus cyproterone acetate (the components of Diane³⁵Diario) does not appear to be higher than that observed with other third generation oral contraceptive pills, this issue is still a matter of debate (35). Therefore, although the overall effect of oral contraceptives on the classic and non-classic cardiovascular risk markers associated with PCOS is beneficial in our experience (3, 15–18), it must be noted that others have reported an undesirable impact of contraceptive pills on the cardiovascular risk profile of these women (36–38).

Figure 4 Changes in platelet markers and endothelial function of PCOS patients submitted to treatment with Diane³⁵Diario or metformin for 24 weeks, as a function of obesity. Black circles represent the data from the patients allocated to Diane³⁵Diario, whereas white circles represent the data from the patients allocated to metformin. The small figures under the X-axis indicate the number of patients at each visit. Data are means $\pm$ S.E.M., and were submitted to a repeated-measures general lineal model introducing the arm of treatment and the presence or absence of obesity and smoking as between-subjects effects, and the visit of evaluation (baseline, 12 and 24 weeks) as the within-subjects effect. The shaded areas represent the 95% confidence intervals of the control group of non-hyperandrogenic women. The results were evaluated according to the analysis of patients who completed the study and to the intention-to-treat analysis. * $P < 0.05$ compared with baseline values in the whole group of patients, irrespective of the arm of treatment (by intention-to-treat analysis). † $P < 0.05$ for the differences observed in the changes of each variable with respect to baseline depending on the arm of treatment and of obesity (by intention-to-treat analysis).

Of interest, some of the effects of Diane³⁵Diario on blood coagulation parameters are modulated by obesity: while this contraceptive pill increases fibrinogen levels in non-obese PCOS patients, the opposite effect was observed in the obese subgroup of patients treated with this drug, because fibrinogen concentrations decreased in parallel with the reduction in the waist circumference observed in these women (18). Together with our previous finding of an increase in serum adiponectin levels only in the obese women treated with Diane³⁵Diario, the present finding of a reduction of fibrinogen levels in these women may suggest that, in sharp contrast to what has been published (38), oral contraceptives could have beneficial effects in obese PCOS patients that are not present in the non-obese subgroup.

But more surprising is the finding of a worsening in blood clotting tests, consisting of a decrease in prothrombin time and an increase in prothrombin activity, in the PCOS patients allocated to treatment with metformin in our study. Despite the beneficial impact of

metformin on some aspects of the cardiovascular risk profile of PCOS women (15, 16), metformin might reduce vitamin B₁₂ absorption thereby increasing homocysteine levels (39). Considering that homocysteine increases the rate of synthesis of tissue factor, which in turn activates the extrinsic coagulation pathway (27), the finding of an increase in plasma homocysteine concentrations during treatment with metformin suggests that this mechanism may contribute to the parallel changes in prothrombin time and activity observed in our patients. In conceptual agreement, an increase in circulating homocysteine levels during metformin administration to PCOS patients has been previously reported (40, 41), although this finding is not universal (42).

The fact that the undesirable effects of metformin on prothrombin time and activity were especially important in the subgroup of obese PCOS patients may be explained by the fact that obesity may be associated *per se* with a decrease in vitamin B₁₂ levels (43), and

Figure 5 Changes in blood flow-mediated dilatation of the whole of PCOS patients submitted to treatment with Diane³⁵Diario or with metformin for 24 weeks, as a function of smoking. Black squares represent the data from the smokers irrespective of the arm of treatment, whereas white squares represent the data from the non-smokers patients irrespective of the arm of treatment. The small figures under the X-axis indicate the number of patients at each visit. Data are means $\pm$ S.E.M., and were submitted to a repeated-measures general lineal model introducing the arm of treatment, the presence or absence of obesity, and smoking as between-subjects effects, and the visit of evaluation (baseline and 24 weeks) as the within-subjects effect. The results were evaluated according to the analysis of patients who completed the study and to the intention-to-treat analysis. * $P < 0.05$ for the effect of smoking on FMD throughout the study considering patients as a whole and irrespective of the arm of treatment and of obesity (in the analysis of the patients who completed the study and by intention-to-treat analysis).

this relative deficiency might be amplified by metformin administration leading to the increase in homocysteine concentrations mentioned above. However, because we did not find any effect of obesity on baseline homocysteine levels, this hypothesis remains merely speculative. Nevertheless, considering that increased homocysteine concentrations appear to mediate the effects of metformin on prothrombin time and activity, this undesirable effect could be prevented by the concurrent administration of calcium, vitamin B or folic acid supplements (41, 44), whereas the undesirable effects of oral contraceptives on coagulation cannot be avoided. Furthermore, despite the small magnitude of the changes in blood clotting tests observed here as a result of PCOS treatment that, with a few individual exceptions, occurred within the normal range, it must be highlighted that even mild reductions of activated partial thromboplastin time increased the risk for thrombotic events in large population studies (45).

In our series of young PCOS patients, there were no obvious abnormalities in the indexes of endothelial function evaluated, despite the fact that the patients were insulin resistant when compared with non-hyperandrogenic controls matched for age and BMI. Although insulin resistance is related with the appearance of endothelial dysfunction in subjects with type 2 diabetes, it has been postulated that variations in the

mechanism of insulin resistance in PCOS patients may affect endothelial function differently (46). The insulin resistance of PCOS patients has been proposed to involve a post-receptor defect in the phosphatidylinositol 3-kinase insulin-signaling pathway (47) that may reduce the vasodilatory action of insulin (48), thereby favoring the vasoconstrictory action through the MAP kinase pathway (49). Yet insulin resistance is not universal in PCOS patients (50) and even in insulin-resistant PCOS patients, this defect may be tissue-specific (51, 52).

Therefore, in the hypothetical case that endothelial cells from PCOS patients are not more insulin resistant than that of non-hyperandrogenic women (53), the detrimental effect on endothelium exerted by oxidative stress, hypoadiponectinemia, and the proinflammatory milieu characteristics of PCOS women (10) may be counteracted by the anti-inflammatory and vasodilatory actions of their frequently increased insulin levels, and by a putative vasodilatory effect of androgens (54, 55), preserving endothelial function until extreme obesity develops. Nevertheless, the presence of endothelial dysfunction in PCOS, especially when evaluated on the brachial artery by sonographic methods, is still a matter of intense debate (46), and, the small sample size of our study limits severely its power in detecting differences among PCOS women and non-hyperandrogenic controls (56).

The deleterious effect of smoking on endothelial function merits a specific consideration. In non-smoker PCOS patients, treatment with either Diane³⁵Diario or metformin improved endothelial function in parallel with a decrease in waist circumference, but smoking prevented the occurrence of this beneficial effect inducing a further deterioration of FMD in these women. Furthermore, the inclusion of smokers in our series may contribute to explain the differences with previous studies showing beneficial effects of metformin on endothelial function (57, 58), because in these earlier reports only non-smokers were included (57) or smoking was strongly discouraged (58). Finally, the intrinsic limitations of the ultrasonographic methods used here to evaluate endothelial function and the small sample size of our clinical trial – a hindrance further exaggerated by the subgrouping of the patients according to obesity and smoking – may have prevented us from finding differential effects of oral contraceptives and metformin on these indexes.

In summary, both oral contraceptives and metformin may exert deleterious effects on blood clotting tests of PCOS women, although the effects of metformin appear to be milder and potentially preventable by the co-administration of vitamin supplements. Because smoking potentiates some of these effects and deteriorates endothelial function, smoking cessation should be aggressively promoted in PCOS patients. Finally, most if not all the abnormalities in blood coagulation tests and indexes of endothelial function are influenced

by abdominal adiposity, highlighting the contribution of adipose tissue dysfunction to the cardiovascular risk of PCOS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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