

**Title:** The increased body iron stores of obese women with polycystic ovary syndrome are a consequence of insulin resistance and hyperinsulinism, and do not result from reduced menstrual losses.

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**Short title:** Iron Stores, Insulin Resistance and PCOS

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## ABSTRACT

**Objective:** Increased serum ferritin levels, indicating increased body iron stores, have been found in overweight and obese PCOS women. This finding might result from the reduced menstrual losses secondary to from oligo- or amenorrhea, or from the hyperinsulinism secondary to insulin resistance because insulin favors the intestinal absorption and the tissue deposition of iron. To explore which of these mechanisms is responsible for the increase in body iron stores in PCOS women, we have monitored the changes in serum ferritin levels during treatment with an antiandrogenic oral contraceptive or with an insulin sensitizer.

**Research Design and Methods:** Thirty-four consecutive PCOS patients were randomized to an oral contraceptive containing 35 µg of ethinyl-estradiol plus 2 mg of cyproterone acetate (Diane<sup>35</sup> Diario) or metformin (850 mg twice daily), and their serum ferritin levels were evaluated at baseline and after 12 and 24 weeks of treatment.

**Results:** Despite the fact that treatment with Diane<sup>35</sup> Diario restored regular menstrual cycles in all the patients, whereas metformin only did so in 50% of them, serum ferritin levels decreased at 12 and 24 weeks of treatment only with metformin, in association with a marked increase in insulin sensitivity. On the contrary, no changes in ferritin and insulin sensitivity were observed with Diane<sup>35</sup> Diario.

**Conclusions:** Our present results suggest that insulin resistance and hyperinsulinism, and not the reduced menstrual losses secondary to from oligo- or amenorrhea, are responsible of the increased ferritin levels and body iron stores found in overweight and obese PCOS women.

**NLM Identifier** NCT00428311

The polycystic ovary syndrome is a predominantly hyperandrogenic disorder (1) that affects 6 to 7% of premenopausal women (2-5). Aside from the hyperandrogenic features and ovarian dysfunction characteristic of the syndrome, obesity and insulin resistance are frequently associated with PCOS (6,7).

We have reported previously (8) that overweight and obese women with PCOS have increased serum ferritin levels that do not relate to chronic inflammation, indicating that body iron stores are increased in these women in agreement with what has been published for other insulin resistant conditions (9). The increase in body iron stores might contribute to the insulin resistance and  $\beta$ -cell dysfunction frequently found in PCOS patients (10), as has been previously proposed for insulin resistance (11), the metabolic syndrome (12), and type 2 diabetes (13,14).

We hypothesized that genetic factors, the absence of a regular menstrual blood loss, or even the hyperinsulinemia resulting from insulin resistance, considering that insulin might stimulate intestinal iron absorption by up-regulating activity of hypoxia-inducible factor-1 alpha and down-regulating hepcidin expression (15,16), may have contributed to the increased body iron stores and serum ferritin levels observed in PCOS patients.

Our recent results suggest that mutations in the hereditary hemochromatosis gene do not play a role in the increased body iron stores of PCOS patients (17). With the aim of evaluating which, of the two remaining etiologic mechanisms we initially suspected (absence of a regular menstrual blood loss or the hyperinsulinemia resulting from insulin resistance), is the major determinant of the increased iron stores found in overweight and obese PCOS patients, we have monitored serum ferritin levels in a group of PCOS patients randomly submitted to treatment with an antiandrogenic oral contraceptive – that regularizes menstrual dysfunction in all cases

but does not improve insulin sensitivity – or to treatment with metformin – that improves insulin sensitivity but does not systematically regularizes menstrual cycles.

As will be seen, our novel results strongly suggest that increased body iron stores in PCOS women are a consequence of insulin resistance and hyperinsulinemia that improves with insulin sensitization.

## **Research Design and Methods**

### *Patients*

The present study derives from a more ample randomized controlled clinical trial addressing the effects of treatment with an antiandrogenic oral contraceptive compared with the insulin sensitizer metformin on classic and non-classic cardiovascular risk factors (ClinicalTrials.gov NLM Identifier NCT00428311).

In brief, 34 consecutive PCOS patients were recruited (18). The diagnosis of PCOS was based on the presence of clinical and/or biochemical hyperandrogenism, oligo-ovulation and exclusion of secondary etiologies (19). The methods used to evaluate each particular criterion have been described in detail elsewhere (20).

None of the patients had either a personal history of hypertension, diabetes mellitus or cardiovascular events, or received treatment with oral contraceptives, antiandrogens, insulin sensitizers or drugs that might interfere with blood pressure regulation, lipid profile or carbohydrate metabolism for the previous 6 months. Written informed consent was obtained from all the participants, and the study was approved by the local Ethics Committee and by the Spanish Drug Agency.

After giving informed consent, patients were randomized to receive an antiandrogenic oral contraceptive containing 35  $\mu$ g of ethinyl-estradiol plus 2 mg of cyproterone acetate (Diane<sup>35</sup> Diario, Schering España S.A., Madrid, Spain) or 850 mg of

metformin (Dianben, Merck Farma y Química S.A., Mollet del Vallés, Spain) daily for 24 weeks. Simple randomization was conducted using blocks of 10 sealed opaque envelopes assigning 5 patients to receive Diane<sup>35</sup> Diario and 5 patients to receive metformin. Fifteen patients were allocated to treatment with Diane<sup>35</sup> Diario and 19 were allocated to treatment with metformin.

Treatment was started the first day of a spontaneous menstrual cycle or in women with amenorrhea, after excluding pregnancy by proper testing, and patients were instructed to maintain a diet containing 25-30 Kcal per kg of body weight and day and moderate physical activity throughout the trial. Metformin was started at a 425 mg twice daily dose during the first week to minimize gastrointestinal complaints. Patients were submitted to a complete evaluation at baseline and after 12 and 24 weeks of treatment for this and other studies that included anthropometric and laboratory measurements, a 75g oral glucose tolerance test with measurement of serum insulin and plasma glucose every 30 minutes for 2 hours, and several tests of cardiovascular performance. The 15 patients allocated to Diane<sup>35</sup> Diario completed the study, whereas 7 of the 19 patients allocated to metformin discontinued metformin for different reasons (18).

For the present study, serum ferritin and C-reactive protein (CRP) concentrations were measured by automated immunochemiluminescence (Immulite 2000 Ferritin and High Sensitivity CRP, Diagnostic Products Corporation, Los Angeles, CA) with lower limit of detection of 0.88 pmol/L and 0.1 mg/L respectively, and intra- and inter-assay coefficients of variation below 10%. The technical characteristics of the assays employed for plasma glucose and serum hormone measurements have been described elsewhere (21-23). The composite insulin sensitivity index was calculated from the circulating glucose and insulin concentrations

during the oral glucose tolerance test according to Matsuda and DeFronzo (24).

#### *Statistical analysis*

Data are shown as means  $\pm$  SD unless otherwise stated. The sample size analysis for the clinical trial indicated adequate 0.80 powers with a total of 22 patients, as described in detail in the previous report of the study protocol (18). Normality of continuous variables was assessed by the Kolmogorov-Smirnov test and logarithmic transformation was applied as needed to ensure normality. The baseline characteristics of the patients randomized to receive Diane<sup>35</sup> Diario or metformin were compared by unpaired *t*-test. During the study, the changes in continuous variables were analyzed by using a repeated-measures general lineal model including the arm of treatment as the between-subjects effect and the visit (baseline, 12 and 24 weeks) as the within-subjects effect. To evaluate the differences in the response to each treatment, the interaction among the between-subjects and within-subjects effect was calculated. The results obtained when considering only the patients completing the 3 visits of the protocol were also confirmed by intention to treat analysis assuming no changes in dependent variables at the missing visits with respect to the previous evaluation for patients discontinuing metformin. Categorical variables were analyzed by the  $\chi^2$ , Fisher's exact and McNemar's test as appropriate.  $P < 0.05$  was considered statistically significant. Analyses were performed using SPSS 10 for Macintosh (SPSS Inc, Chicago, Illinois).

#### **Results**

The complete baseline clinical, anthropometric, biochemical and hormone profiles of the PCOS patients randomized to treatment with Diane<sup>35</sup> Diario or metformin, showing no differences between both groups, have been reported previously (18). Table 1 shows a selection of these variables, as well

as the circulating ferritin, CRP and hemoglobin concentrations, hematocrit and mean erythrocyte corpuscular volume, and frequency of patients presenting with menstrual dysfunction at baseline, all of which were similar in the patients allocated to each arm of treatment.

Of note, two of the 34 patients, of whom one was allocated to treatment with Diane<sup>35</sup> Diario and another was allocated to metformin, had mean erythrocyte corpuscular volumes below 75 fL in the presence of a hematocrit value above 30%, suggesting a thalassemic trait (25).

During treatment, serum ferritin levels decreased in the whole group of PCOS patients (Wilks'  $\lambda = 0.741$ ,  $F = 4.187$ ,  $P = 0.028$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.741$ ,  $F = 5.412$ ,  $P = 0.010$ ), yet this finding was caused by the decrease observed only in patients treated with metformin, considering the statistically significant interaction of the effect of the visit of evaluation with the arm of treatment (Wilks'  $\lambda = 0.679$ ,  $F = 5.668$ ,  $P = 0.010$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.693$ ,  $F = 6.861$ ,  $P = 0.003$ ).

The changes in serum ferritin levels occurred independently of its possible role as a first-phase inflammatory marker, because no simultaneous changes were observed in serum CRP levels with any of the drugs. Serum CRP concentrations did not change during the study either when considering the whole group of patients (Wilks'  $\lambda = 0.922$ ,  $F = 1.014$ ,  $P = 0.378$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.949$ ,  $F = 0.827$ ,  $P = 0.447$ ), or in response to any of the treatments because there was no significant interaction of the effect of the visit of evaluation with the arm of treatment (Wilks'  $\lambda = 0.909$ ,  $F = 1.200$ ,  $P = 0.319$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.939$ ,  $F = 1.009$ ,  $P = 0.376$ ).

Furthermore, the decrease in serum ferritin levels in the patients treated with metformin was paralleled by a simultaneous

improvement of insulin resistance, as reflected by the increase in the insulin sensitivity index observed in these women. Insulin sensitivity increased in the whole group of PCOS patients (Wilks'  $\lambda = 0.675$ ,  $F = 5.768$ ,  $P = 0.009$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.781$ ,  $F = 4.334$ ,  $P = 0.022$ ), yet this finding was caused by the increase observed in patients treated with metformin, considering the statistically significant interaction of the effect of the visit of evaluation with the arm of treatment (Wilks'  $\lambda = 0.737$ ,  $F = 4.292$ ,  $P = 0.025$ , Figure 1; intention to treat analysis: Wilks'  $\lambda = 0.831$ ,  $F = 3.162$ ,  $P = 0.056$ ).

Of the patients completing the study, menstrual dysfunction (chronic oligomenorrhea or amenorrhea) were present at baseline in 14 patients who received Diane<sup>35</sup> Diario and in 11 patients who received metformin (one patient per group had regular but anovulatory cycles). As expected, menstrual regularity was restored from the first month of treatment in all the patients receiving Diane<sup>35</sup> Diario, whereas regular cycles were restored in only 5 and 6 patients on metformin after 12 and 24 weeks of treatment, respectively (Figure 2).

To evaluate if the decrease in body iron stores observed during treatment with metformin was related to increased menstrual blood losses, even when oligomenorrhea or amenorrhea persisted in about half of these patients, we monitored serum hemoglobin concentrations and mean erythrocyte corpuscular volume during treatment. Serum hemoglobin concentration presented a small yet statistically significant decrease during the study (Wilks'  $\lambda = 0.538$ ,  $F = 9.872$ ,  $P = 0.001$ ; intention to treat analysis: Wilks'  $\lambda = 0.543$ ,  $F = 13.049$ ,  $P < 0.001$ ; Figure 2) that was similar in both arms of treatment considering that there was no statistically significant interaction between the visit of evaluation with the arm of treatment (Wilks'  $\lambda = 0.896$ ,  $F = 1.340$ ,  $P = 0.281$ ; intention to treat analysis: Wilks'  $\lambda = 0.826$ ,  $F = 3.275$ ,  $P = 0.051$ ).

Finally, the mean erythrocyte corpuscular volume remained unchanged during the study (Wilks'  $\lambda = 0.979$ ,  $F = 0.241$ ,  $P = 0.788$ ; intention to treat analysis: Wilks'  $\lambda = 0.986$ ,  $F = 0.224$ ,  $P = 0.801$ ; Figure 2) without any interaction between the visit of evaluation with the arm of treatment (Wilks'  $\lambda = 0.816$ ,  $F = 2.598$ ,  $P = 0.096$ ; intention to treat analysis: Wilks'  $\lambda = 0.844$ ,  $F = 2.865$ ,  $P = 0.072$ ).

## Conclusions

Two years ago we hypothesized that the increased body iron stores found in overweight and obese PCOS women could have a multifactorial origin (8) in which the absence of a regular menstrual blood loss in PCOS patients, the presence of increased oxidative stress in these women (26,27), the hyperinsulinemia resulting from the insulin resistance frequently associated with the disorder, and genetic factors such as mutations and polymorphisms in the classic hemochromatosis gene and in the genes encoding other molecules involved in iron metabolism, may play a role.

As a first step, we recently ruled out any association of mutations in the classic hemochromatosis gene with serum ferritin levels in PCOS women (17). Pursuing into our initial hypotheses, in the present study we provide substantial evidence to suggest that hyperinsulinemia and insulin resistance play a major role on the increased body iron stores of overweight and obese women with PCOS patients and that, on the contrary, the reduced menstrual losses secondary to oligo- or amenorrhea do not contribute importantly to this increased iron stores. On the one hand, treatment with metformin reduced serum ferritin levels in parallel with an evident increase in insulin sensitivity, even when menstrual regularity was restored only in half of the patients treated with this drug. On the other hand, although Diane<sup>35</sup> Diario restored regular menstrual cycles in all the patients with no concurrent change in insulin

sensitivity, serum ferritin levels did not change as a result of this treatment.

Combining the finding of a reduction in serum ferritin levels only in the patients treated with metformin with the lack of changes observed during treatment with Diane<sup>35</sup> Diario, our interpretation is that hyperinsulinemia and insulin resistance are among the major factors influencing the increase in serum ferritin levels observed in PCOS patients, as has been demonstrated in other insulin-resistant disorders such as type 2 diabetes (9). On the contrary, the changes in serum ferritin levels were not paralleled by changes in serum CRP levels, as would have been expected if inflammation was the actual responsible for the increased serum ferritin concentrations in PCOS patients.

Moreover, metformin was not specifically related to the decrease in circulating hemoglobin observed during the study, which occurred similarly with both treatments. The reduction in serum ferritin levels observed only in response to metformin, and not after treatment with Diane<sup>35</sup> Diario, was apparently not related to the increased menstrual losses in these women as serum ferritin levels remained unchanged in the women treated with the oral contraceptive, despite the restoration of regular menses from the first month in all of them.

Of note, the fact that the decrease in hemoglobin concentrations observed in PCOS patients throughout the study was independent from the drug applied might suggest a role of the amelioration of androgen excess on this finding, considering the well-known stimulatory effect of androgens on erythropoiesis (28). Also, the possibility that our present results could have been influenced by a carrier status for  $\beta$ -thalassemia mutations (a frequent condition in the Mediterranean area that may associate increased iron stores (25)) seems unlikely, especially when the only two patients presenting with hematological profiles suggestive of such a

trait were allocated one to each arm of treatment, and both completed the study.

Therefore, and considering that insulin might stimulate intestinal iron absorption by up-regulating activity of hypoxia-inducible factor-1 alpha and down-regulating hepcidin expression (15,16), the amelioration of insulin resistance and hyperinsulinemia by metformin may explain the reduction in serum ferritin levels and iron stores found in the subset of PCOS patients treated with this insulin sensitizer, especially when, to our best knowledge, no direct interaction of metformin with the intestinal absorption of iron has been described to date.

The reduction in body iron stores with metformin, as reflected by the decrease in serum ferritin levels, may be especially favorable in overweight and obese women with PCOS considering that increased iron stores contribute to insulin resistance and hyperinsulinemia by reducing hepatic insulin extraction and metabolism (29), and by decreasing glucose uptake in muscle (30). In conceptual agreement with an important causative role for increased iron stores in insulin resistance (9), repeated phlebotomies in diabetic patients with iron overload (31), and even in healthy volunteers (32), result in an amelioration of insulin resistance, and a similar effect is observed with vegetarian diets that improve insulin sensitivity by lowering iron stores (33). Moreover,  $\beta$ -cell dysfunction may result from oxidative damage and apoptosis induced by iron deposits in pancreatic islets, as has been described in animal models of hemochromatosis (34), facilitating the

development of abnormalities in glucose tolerance, including type 2 diabetes, that are frequently associated with PCOS (35).

For the reasons discussed above, our present preliminary results suggest that metformin might be useful to break the vicious cycle of insulin resistance, increased iron absorption and deposition in tissues, and further worsening of insulin resistance in PCOS patients. However, longer studies are needed in order to delineate with precision the mechanisms underlying the decrease in body iron stores observed in PCOS patients treated with metformin, and the clinical consequences of such a decrease.

In summary, our present results suggest that insulin resistance and hyperinsulinism, and not the reduced menstrual losses secondary to from oligo- or amenorrhea, are responsible for the increased ferritin levels and body iron stores found in overweight and obese PCOS women.

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**Table 1.** Baseline characteristics of the patients randomized to receive Diane<sup>35</sup> Diario or metformin.

	<b>Diane<sup>35</sup> Diario (n = 15)</b>	<b>Metformin (n = 19)</b>	<b>P value</b>
<i>Age (yr)</i>	23.4 ± 5.6	25.1 ± 6.6	0.445
<i>Body mass index (kg/m<sup>2</sup>)</i>	29.2 ± 5.7	30.5 ± 6.9	0.563
<i>Waist circumference (cm)</i>	83 ± 12	89 ± 18	0.306
<i>Waist to hip ratio</i>	0.79 ± 0.06	0.82 ± 0.11	0.292
<i>Free testosterone (pmol/l)</i>	38 ± 14	45 ± 21	0.210
<i>Insulin sensitivity index</i>	4.4 ± 3.5	3.8 ± 2.4	0.564
<i>Ferritin (pmol/l)</i>	61 ± 35	54 ± 50	0.628
<i>C-reactive protein (mg/l)</i>	2.1 ± 2.8	4.2 ± 6.1	0.199
<i>Hemoglobin (g/l)</i>	131 ± 7	130 ± 10	0.600
<i>Hematocrit (%)</i>	38 ± 2	38 ± 3	0.444
<i>Mean erythrocyte corpuscular volume (fl)</i>	87 ± 4	85 ± 8	0.339
<i>Menstrual dysfunction, n (%)</i>	1 (93)	3 (84)	0.397

Data are means ± SD, or raw numbers (%). Data were submitted to unpaired *t* test or to Fisher's exact test, as appropriate. Reproduced in part from Luque-Ramírez et al. (18), Copyright 2007, *The Endocrine Society*, with permission.

## Legends to Figures

**Figure 1.** Changes in serum ferritin and C-reactive protein levels, and in the insulin sensitivity index, during treatment of PCOS with Diane<sup>35</sup> Diario or metformin in the patients who completed the study. Data are means  $\pm$  SEM.

\*  $P < 0.05$  compared with baseline values when considering the whole group of patients.

†  $P < 0.05$  for the differences in the changes of each variable depending on the arm of treatment.

**Figure 2.** Changes in menstrual dysfunction, serum hemoglobin concentration and mean erythrocyte corpuscular volume, during treatment of PCOS with Diane<sup>35</sup> Diario or metformin in the patients who completed the study.

Data are percentages (upper panel) or means  $\pm$  SEM (lower panels).

\*  $P < 0.05$  compared with baseline values when considering the whole group of patients.

†  $P < 0.05$  for the differences in the changes of each variable depending on the arm of treatment.

Figure 1

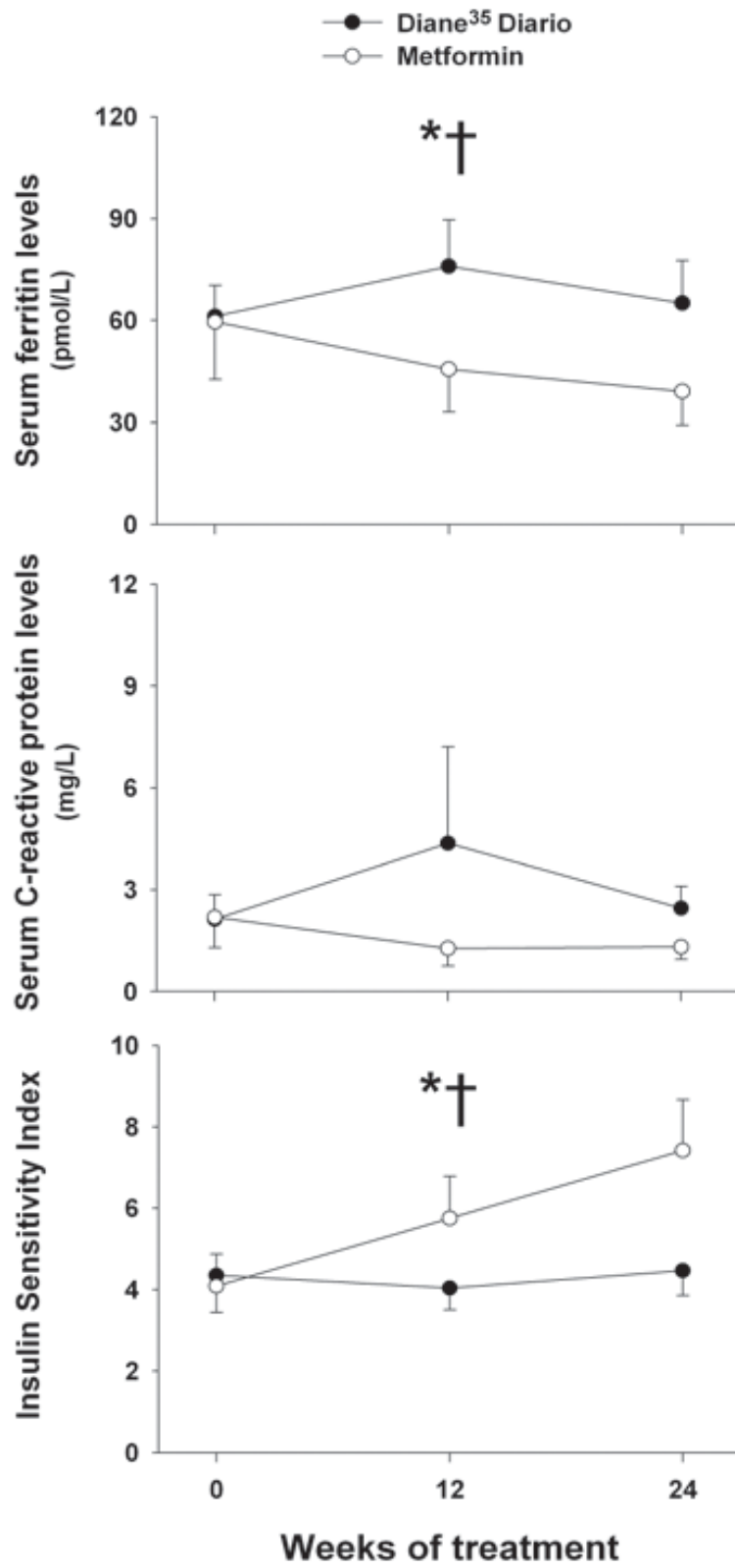


Figure 2

