Effects of metformin on mitochondrial function of leukocytes from polycystic ovary syndrome patients with insulin resistance

Victor M Victor1,2,3, Susana Rovira-Llopis1,2, Celia Bañuls1,2, Noelia Díaz-Morales1, Raquel Castelló1, Rosa Falcón1, Marcelino Gómez1, Milagros Rocha1,2,3 and Antonio Hernández-Mijares1,2,4

1Service of Endocrinology, University Hospital Doctor Peset, Foundation for the Promotion of Health and Biomedical Research in the Valencian Region (FISABIO), Avenida Gaspar Aguilar 90, 46017 Valencia, Spain, 2Institute of Health Research INCLIVA, University of Valencia, Valencia, Spain, 3CIBERehd – Department of Pharmacology and Physiology, University of Valencia, Valencia, Spain and 4Department of Medicine, University of Valencia, Valencia, Spain

Abstract

Objective: Oxidative stress and mitochondrial dysfunction are implicated in polycystic ovary syndrome (PCOS). The present study assesses the effect of metformin treatment on mitochondrial function in polymorphonuclear cells from PCOS subjects. Additionally, we evaluate endocrine parameters and levels of interleukin 6 (IL6) and tumour necrosis factor alpha (TNFα).

Design and methods: Our study population was comprised of 35 women of reproductive age diagnosed with PCOS and treated with metformin for 12 weeks, and their corresponding controls (n=41), adjusted by age and BMI. We evaluated the alteration of endocrinological and anthropometrical parameters and androgen levels. Mitochondrial O2 consumption (using a Clark-type O2 electrode), membrane potential, mitochondrial mass, and levels of reactive oxygen species (ROS) and glutathione (GSH) (by means of fluorescence microscopy) were assessed in polymorphonuclear cells. H2O2 was evaluated with the Amplex Red® H2O2/Peroxidase Assay kit. IL6 and TNFα were measured using the Luminex 200 flow analyser system.

Results: Metformin had beneficial effects on patients by increasing mitochondrial O2 consumption, membrane potential, mitochondrial mass and glutathione levels, and by decreasing levels of reactive oxygen species and H2O2. In addition, metformin reduced glucose, follicle-stimulating hormone, IL6 and TNFα levels and increased dehydroepiandrosterone sulfate levels. HOMA-IR and mitochondrial function biomarkers positively correlated with ROS production (r=0.486, P=0.025), GSH content (r=0.710, P=0.049) and H2O2 (r=0.837, P=0.010), and negatively correlated with membrane potential (r=−0.829, P=0.011) at baseline. These differences disappeared after metformin treatment.

Conclusions: Our results demonstrate the beneficial effects of metformin treatment on mitochondrial function in leukocytes of PCOS patients.
used to treat PCOS due to the way it improves IR (6). It exerts multiple actions on different tissues affected by hyperinsulinemia and/or IR, such as adipose tissue, skeletal muscle, liver, endothelium and ovaries (7), and seems to improve the long-term health outcome of women with PCOS by preventing endometrial cancer, diabetes and cardiovascular disease (8).

It has been postulated that tissue exhibiting IR (e.g. muscle) is characterised by reduced mitochondrial content, protein expression and activity of different enzymes (9, 10), although there is conflicting evidence with respect to this (11, 12). In this sense, the molecular mechanisms exerted by metformin are not altogether clear. One potential mechanism is inhibition of mitochondrial complex I (13, 14, 15), though there are also discrepancies about this (11, 16). Metformin is thought to stimulate AMP kinase by inhibiting mitochondrial complex I (14), though this does not always depend on alterations in the redox state of the cell (17).

Several studies in PCOS patients have demonstrated that acute hyperglycemia and IR induce an increase in reactive oxygen species (ROS) production by peripheral blood leukocytes (18, 19), activate leukocyte–endothelium interactions (20) and proinflammatory transcription factor nuclear kappa B (NF-kB) (21), and increase levels of proinflammatory cytokines (22). In this way, oxidative stress has been implicated in the etiology of IR in leukocytes from PCOS patients. Contrasting results have been obtained in studies investigating the effects of metformin on oxidative stress; for example, Kocer et al. (23) demonstrated that metformin reduced oxidative stress, while Pavlovic et al. (24) showed that it increased it.

In the present study we assess the effects of metformin treatment on endocrine and anthropometrical parameters and on mitochondrial function in PCOS patients by measuring mitochondrial oxygen (O₂) consumption, membrane potential, glutathione (GSH), ROS and H₂O₂ levels, mitochondrial mass, and interleukin 6 (IL6) and tumour necrosis factor alpha (TNFα) in leukocytes from said patients.

**Subjects and methods**

**Subjects**

Forty-one PCOS women of reproductive age (21–33 years old) were enrolled in the study, as were 41 controls adjusted by age and BMI. PCOS subjects were diagnosed according to the Rotterdam criteria (25), and the study was conducted by the Service of Endocrinology, University Hospital Dr Peset, Valencia, Spain. The Rotterdam criteria for the diagnosis of PCOS are as follows: oligoovulation (cycles longer than 35 days or shorter than 26 days) (26); elevated free testosterone levels (>0.5 ng/dl); the cut-off level for free testosterone was the mean ±2S.D. according to normal levels in controls; hirsutism (total Ferriman-Gallwey score >7); and polycystic ovaries (12 or more small (2–9 mm) follicles in each ovary, identified by transvaginal ultrasonography). Ultrasound scans were performed and scored independently by one of two experienced and blinded observers. None of the subjects had galactorrhea or any endocrine or systemic disease that could have affected her reproductive physiology. None of the women reported using any medication that could have interfered with the normal function of the hypothalamic-pituitary-gonadal axis during the previous semester.

Exclusion criteria were organic, malignant, haematological, infectious or inflammatory disease and a history of ischaemic heart disease, stroke or thromboembolism, diabetes mellitus, hyperlipidaemia or hypertension. An anthropometric and analytical evaluation was performed for all subjects, and weight (kg), height (m) and waist (cm) were measured and body mass index (BMI = weight (kg)/height (m)²) calculated.

Blood was collected from the antecubital vein at 0800–1000 h on the second/third day of the menstrual cycle (follicular phase) after 12 h of fasting. Glucose levels were measured using enzymatic techniques and a Dax-72 autoanalyzer (Bayer Diagnostic, Tarrytown, New York, USA). Insulin was measured by an enzymatic luminescence technique. Samples were immediately processed for insulin in order to avoid haemolysis and were frozen until analysis. IR was calculated by homeostasis model assessment (HOMA) using baseline glucose and insulin: HOMA = (fasting insulin (µU/ml) × fasting glucose (mmol/l))/22.5. Luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were measured by specific RIAs. DHEAS-sulfate, sex hormone binding globulin (SHBG), androstenedione and testosterone were measured by specific chemiluminescence techniques in our hospital’s Clinical Analysis Service. High-sensitive C-reactive protein (hsCRP) was quantified by a latex-enhanced immunonephelometric assay (Behring Nephelometer II, Dade Behring, Inc., Newark, DE, USA) with an intra-assay coefficient of variation of 8.7% and sensitivity of 0.01 mg/l.

Total cholesterol and triglycerides were measured by means of enzymatic assays, and HDLc concentrations were recorded with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA) using a direct method. The
intraserial variation coefficient was <3.5% for all determinations. LDLc concentration was calculated using the Friedewald method. Apolipoprotein (Apo) A1 and B was determined by immunonephelometry (Dade Behring BNII, Marburg, Germany) with an intra-assay variation coefficient of <5.5%.

PCOS subjects and controls followed a normocaloric diet. PCOS subjects began treatment with metformin at a dose of 500 mg/day, which was increased to 1000 mg after 2 weeks and to 1500 mg/day after a further 2 weeks. This last dose was maintained for a total of 12 weeks. Subjects returned every 2 weeks for anthropomorphic and endocrine measurements and blood sampling. Compliance was determined by interviewing the patients during these fortnightly visits.

Informed written consent was obtained from all subjects prior to participation. The study was approved by the clinical research ethics committee of the University Hospital Doctor Peset and was performed in accordance with the Helsinki declaration. This trial was registered on clinicaltrials.gov under study number NCT02302326. Four subjects were excluded and two subjects dropped out of the study for personal reasons, because of an inability or unwillingness to comply with the protocol.

**Cells**

Human polymononuclear leukocytes (PMNs) were obtained from citrated blood samples and incubated for 45 min with dextran (3%). The supernatant was centrifuged at 250 \( g \) for 25 min in Fycoll-Hypaque. Lysis buffer was added to the pellet, which was then centrifuged at room temperature (100 \( g \), 5 min). PMNs were evaluated in a Scepter device (Millipore, MA, USA), washed in Hanks’ balanced salt solution (HBSS) and then stored in complete RPMI media.

**Measurement of \( O_2 \) consumption**

PMNs were resuspended (5 \( \times \) 10^6 cells/ml) in HBSS medium and placed in a gas-tight chamber. A Clark-type \( O_2 \) electrode (Rank Brothers, Bottisham, UK) was used to measure \( O_2 \) consumption (27). Sodium cyanide (10^{-3} mol/l) was employed to evaluate whether or not \( O_2 \) consumption was mitochondrial (95–99%). Measurements were obtained using the data-device Duo.18 (WPI, Stevenage, UK). The rate of \( O_2 \) consumption (\( V_{O_2,\text{max}} \)) was calculated using the Graph Pad program. Cell viability was shown to be unaltered when evaluated by means of the trypan blue exclusion test and Scepter device (Millipore, MA, USA).

**Membrane potential and mitochondrial mass**

Cells were incubated for 30 min in a fluorometer with the fluorescent probe Tetra-methyl-rhodamine methyl ester, 1 \( \times \) 10^{-7} mol/l (TMRM) and mitochondrial membrane potential (\( \Delta \Psi_m \)) was evaluated using a Synergy Mx plate reader (BioTek Instruments, Winooski, VT, USA). Mitochondrial mass was measured in cells treated with the fluorescent dye 10-N-onyl acridine orange (NAO, 5 \( \times \) 10^{-6} mol/l), which specifically binds to cardiolipin independently of \( \Delta \Psi_m \) (27).

**Measurement of ROS production**

Total ROS production was also assessed by fluorometry using a Synergy Mx plate reader (BioTek Instruments) following incubation (30 min) with the fluorescent probe (5 \( \times \) 10^{-6} mol/l) 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), as described elsewhere (28). Hydrogen peroxide (H\(_2\)O\(_2\)) was assessed with the Amplex Red H\(_2\)O\(_2\)/Peroxidase Assay kit.

**GSH content and proinflammatory cytokines**

Glutathione (GSH) content was evaluated following incubation (30 min) with the fluorochrome 5-Chloro-methylfluorescein Diacetate (CMFDA, 5 \( \times \) 10^{-6} mol/l) (19). In short, cells were seeded on 96-well plates, washed with PBS and then incubated with CMFDA diluted in PBS. After 15 min at 37°C, fluorescence intensities were measured using excitation (492 nm) and emission (517 nm) wavelengths. The levels of intracellular GSH appeared as arbitrary units of fluorescence units. A Luminex 200 flow analyser system was employed to analyse IL6 and TNF\( \alpha \) in serum from patients (Austin, TX, USA).

**Drugs and solutions**

Sodium cyanide, glutathione reductase, N-ethylmaleimide, bathophenanthrolne disulfonic acid, nicotinamide adenine dinucleotide hydroxide NADH, trypan blue, glucose, arginine and HBSS were supplied by Cambrex (Verviers, Belgium). H\(_2\)O\(_2\), haemoglobin and RPMI 1640 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma–Aldrich. Dextran was acquired from Fluka (St Louis, MO, USA). DCFH diacetate was provided by Molecular Probes (Eugene, OR, USA). Ficoll-Paque Plus was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).
**Data analysis**

Statistical analysis was performed with SPSS 17.0 Software (SPSS Statistics, Inc.). Continuous variables were expressed as mean and s.d. or as median and 25th and 75th percentiles for parametric and non-parametric data respectively. The 12-week intervention period following completion of the metformin regimen in PCOS was evaluated using a paired Student’s t-test and control vs PCOS data were evaluated using an unpaired Student’s t-test. All the tests used a CI of 95% and differences were considered significant when \( P < 0.05 \). Correlations were calculated using Pearson’s correlation coefficient. Significant differences were considered when \( P < 0.05 \).

**Results**

**Clinical and metabolic characteristics**

The clinical and metabolic characteristics of the subjects are presented in Table 1. No differences were obtained between values among controls at baseline and after 3 months (data not shown). In PCOS subjects at baseline there was a significant increase \( (P < 0.05) \) with respect to controls in waist circumference, systolic blood pressure, androstenedione and a significant decrease in HDLc and SHBG levels. Moreover, after treatment with metformin there was a decrease in glucose and FSH levels \( (P < 0.05) \) and an increase in DHEA-S \( (P < 0.05) \) when compared to baseline values.

**Mitochondrial \( \Delta \psi_m \) and mitochondrial mass**

The Fluoroskan plate reader revealed a significantly lower \( (P < 0.001) \) TMRM fluorescence in PCOS subjects in basal

| Table 1 Anthropometric parameters, lipoprotein profile, hydrocarbonated metabolism parameters and circulating androgens of 41 control and 35 PCOS women treated with metformin during a 12-week period. |
|---------------------------------|---------------------------------|---------------------------------|
|                                  | Controls (n=41)                 | PCOS baseline (n=35)            | PCOS metformin (n=35)          |
| Age (years)                     | 26.2 ± 4.4                      | 25.2 ± 7.3                      | –                               |
| Body weight (kg)                | 78.5 ± 17.1                     | 83.7 ± 23.6                     | 83.4 ± 23.7                     |
| BMI (Kg/m²)                     | 29.6 ± 6.5                      | 30.9 ± 8.9                      | 30.8 ± 8.8                      |
| Waist circumference (cm)        | 87.0 ± 16.6*                    | 95.2 ± 17.1                     | 95.4 ± 15.8                     |
| Systolic BP (mmHg)              | 110 ± 11*                       | 120 ± 16                        | 116 ± 16                        |
| Diastolic BP (mmHg)             | 69 ± 10                         | 73 ± 13                         | 72 ± 11                         |
| Total cholesterol (mg/dl)       | 175.0 ± 32.6                    | 177.9 ± 33.4                    | 173.7 ± 33.2                    |
| LDLc (mg/dl)                    | 107.0 ± 27.6                    | 114.1 ± 27.5                    | 109.4 ± 27.8                    |
| HDLc (mg/dl)                    | 51.3 ± 13.4*                    | 45.3 ± 10.4                     | 44.6 ± 7.7                      |
| Triglycerides (mg/dl)           | 68.5 (52.0;97.3)                | 82.5 (49.8;133.3)               | 75.0 (58.8;143.0)               |
| Apolipoprotein A (mg/dl)        | 142.8 ± 30.6                    | 132.6 ± 19.4                    | 131.2 ± 14.5                    |
| Apolipoprotein B (mg/dl)        | 80.7 ± 22.1                     | 85.3 ± 24.2                     | 84.9 ± 27.0                     |
| hsCRP (mg/l)                    | 2.76 ± 2.20*                    | 4.15 ± 3.07                     | 3.69 ± 3.02                     |
| Glucose (mg/dl)                 | 86.3 ± 10.2                     | 85.5 ± 10.5                     | 81.5 ± 10.5†                    |
| Insulin (μIU/ml)                | 9.8 ± 5.6*                      | 14.7 ± 8.9                      | 13.4 ± 8.6                      |
| HOMA-IR                         | 2.13 ± 1.28*                    | 3.16 ± 2.12                     | 2.78 ± 2.19                     |
| FSH (mIU/ml)                    | 4.38 ± 2.44                     | 4.94 ± 1.50                     | 4.25 ± 1.74†                    |
| LH (mIU/ml)                     | 5.55 ± 5.27                     | 6.40 ± 5.04                     | 5.53 ± 3.38†                    |
| Testosterone (ng/ml)            | 0.44 ± 0.21*                    | 0.77 ± 0.38                     | 0.70 ± 0.36                     |
| DHEA-S (µg/dl)                  | 220.5 ± 99.3*                   | 353.2 ± 175.7                   | 398.9 ± 206.8†                  |
| Androstenedione (ng/ml)         | 2.42 ± 0.97*                    | 4.09 ± 1.72                     | 3.56 ± 1.63                     |
| SHBG (nmol/l)                   | 102.2 ± 70.4*                   | 53.7 ± 37.9                     | 43.4 ± 22.3                     |

Data are expressed as mean ± s.d. for parametric data or as median (25th and 75th percentiles) for non-parametric data. *Statistical significance \( (P < 0.05) \) was determined by an unpaired Student’s t-test (control vs PCOS). †Statistical significance \( (P < 0.05) \) was determined by a paired Student’s t-test (baseline vs 12 weeks).
ROS production was enhanced in PCOS in basal conditions ($P<0.01$) and ($P<0.05$) in the PCOS group with respect to controls. Metformin treatment induced a decrease in DCFH-DA fluorescence, demonstrating a decline in ROS production (Fig. 2A, $P<0.05$) in relation to baseline. $\text{H}_2\text{O}_2$ production was higher in PCOS subjects ($P<0.05$) than in controls under basal conditions, and was lower among the former patients in treatment ($P<0.05$) than at baseline (Fig. 2B). In contrast, CMFDA fluorescence was lower ($P<0.01$) in the PCOS group than in controls.
under basal conditions, indicating a decrease in GSH levels (Fig. 2C), and was higher after treatment with PCOS (P<0.05) than before treatment.

**IL6 and TNFα levels**

IL6 and TNFα levels (pg/ml) (P<0.01) were higher in the PCOS group in basal conditions, and (P<0.05) in the PCOS-treated group with respect to the control group. After metformin treatment there was a decrease in IL6 and TNFα levels (P<0.05) in comparison to basal conditions (Fig. 3A and B respectively).

**Correlation studies**

When we explored potential correlations among HOMA-IR and mitochondrial function biomarkers, we found that the former was positively correlated with ROS production (r=0.486, P=0.025), GSH content (r=0.710, P=0.049) and H2O2 (r=0.837, P=0.010), and negatively correlated with membrane potential (r=-0.829, P=0.011) at baseline. These differences were not present after metformin treatment (Table 2).

**Discussion**

The aim of the present study was to throw light on the effects of treatment with metformin on mitochondrial function in PCOS patients. Specifically, we set out to demonstrate how this drug exerts beneficial effects on the leukocytes of these patients by evaluating endocrine and anthropometrical parameters and mitochondrial function. In comparison to controls, PCOS subjects exhibited higher values in waist circumference, systolic blood pressure, hsCRP, testosterone, DHEA-S and androstenedione, and lower levels of HDLc, SHBG, insulin and HOMA-IR, thereby suggesting the appearance of insulin resistance. We have observed how metformin reduces glucose and FSH in PCOS subjects, increases mitochondrial O2 consumption, membrane potential, mitochondrial mass and GSH levels, and decreases production of ROS and H2O2. Moreover, we have also seen how metformin induces a decrease in IL6 and TNFα levels in leukocytes from these patients. In addition, we demonstrate that HOMA-IR and mitochondrial function biomarkers are positively correlated with ROS production, GSH content and H2O2, and negatively correlated with membrane potential at baseline. These differences are not present after metformin treatment.

It has been shown that the leukocytes of women with PCOS are in a proinflammatory state due in part to high levels of glucose and plasma hsCRP (30). We have previously reported an inhibition of mitochondrial O2, membrane potential and antioxidant levels, an impairment of complex I activity and an increase in ROS production in leukocytes from these women (19). In the present work, we demonstrate that metformin exerts beneficial effects by increasing mitochondrial O2 consumption, membrane potential and mitochondrial mass in PCOS patients. In line with the present results, it has recently been shown that 2-week treatment with metformin enhances mitochondrial respiration in mitochondrial-deficient AMP kinase mice (31).

We have previously demonstrated that insulin resistance in women with PCOS can stimulate ROS production by leukocytes (19). Our present results show that ROS production decreases after a 12-week treatment with

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**Table 2** Correlation coefficients between HOMA-IR and mitochondrial function biomarkers in PCOS women.

<table>
<thead>
<tr>
<th>HOMA-IR</th>
<th>PCOS baseline</th>
<th>PCOS metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPrP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial O2 consumption</td>
<td>0.545 1.163</td>
<td>0.510 0.197</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>0.829 0.011</td>
<td>0.406 0.318</td>
</tr>
<tr>
<td>Mitochondrial mass</td>
<td>0.108 0.799</td>
<td>0.262 0.531</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>0.486 0.025</td>
<td>0.212 0.614</td>
</tr>
<tr>
<td>GSH content</td>
<td>0.710 0.049</td>
<td>0.409 0.314</td>
</tr>
<tr>
<td>H2O2</td>
<td>0.837 0.010</td>
<td>0.094 0.825</td>
</tr>
</tbody>
</table>

Correlation coefficients were estimated by Pearson’s correlation for all parameters. Data are shown as r correlation coefficient and P value.

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**Figure 3**

Effect of metformin treatment on levels of IL6 (A) and TNFα (B); both were evaluated with the Luminex 200 flow analyser system. *P<0.005, **P<0.01 vs control group. *P<0.05 vs PCOS in basal conditions.
metformin. In this sense, there are several studies that show that the beneficial effect of metformin on mitochondrial oxidative stress occurs through a decline in ROS production (16, 32), or, according to a previous in vitro study, through activation of AMP kinase and mitochondrial biogenesis (33). This evidence may explain, in part, the beneficial effects on mitochondrial membrane potential and mitochondrial mass that we have observed after treatment with metformin. In line with this idea, other studies have demonstrated that metformin enhances the expression of PGC1α, a key regulator of mitochondrial biogenesis (34), and the activity of mitochondrial enzymes (35). Furthermore, it has recently been described that metformin suppresses glutoneogenesis by inhibiting mitochondrial glycerolphosphate dehydrogenase, which identifies it as a novel therapeutic target for insulin resistance treatment (36).

Leukocytes are highly sensitive to the oxidative damage mediated by ROS released from different types of cells and sources (37). Paradoxically, they are implicated in the generation of ROS, high levels of which are harmful to cells, as they trigger lipid peroxidation and cell death (38). These deleterious effects of ROS are counteracted by the organism’s complex antioxidant system, including GSH, which plays a key role in protecting cells from oxidative stress. Therefore, impaired ROS production, GSH levels and mitochondrial membrane potential – all characteristic of PCOS – represent dysfunction within the respiratory chain, which compromises the function of the mitochondria as a source of energy. This aspect of PCOS has been the focus of little attention until now, but our findings provide evidence that the beneficial effects of metformin are a result of an improvement in the different mitochondrial parameters that we have evaluated. In this context, other studies have demonstrated a protective effect of metformin against oxidative stress – for example, that induced by doxorubicin in cardiomyocytes – through its modulation of the ferritin heavy chain (FHC), the main iron-storage protein (39). In addition, metformin has demonstrated beneficial effects on mitochondrial function in both the heart and the brain (40, 41).

Finally, we explored potential correlations between HOMA-IR and mitochondrial function biomarkers in PCOS and found that HOMA-IR was positively correlated with ROS production, GSH content and H2O2, and was negatively correlated with membrane potential at baseline. These results suggest that HOMA-IR is related to mitochondrial function in PCOS and these differences disappeared after metformin treatment.

Leukocytes can also release proinflammatory cytokines such as TNFα and IL6, which play an important role in the development of IR in PCOS patients (19). The present study demonstrates that metformin decreases TNFα and IL6 levels, which would represent a beneficial effect, as an augmented release of proinflammatory cytokines following activation by ROS-induced oxidative stress is thought to inhibit insulin signalling and impair glucose uptake (18). It has been suggested that metformin inhibits the activation of NF-κB in HUVEC exposed to inflammatory cytokines, thereby reducing the expression of genes that encode adhesion and proinflammatory molecules (42). In line with this idea, Huang et al. (43) have demonstrated that metformin inhibits IL6 production and activation of the TNFα-induced NF-κB pathway.

The present study has some limitations, including the size of the study population. In addition, our results do not throw light on the mechanism by which metformin can suppress mitochondrial glycerol-3-phosphate dehydrogenase in leukocytes from PCOS patients, which needs to be explored in future research.

In conclusion, the present study demonstrates that metformin can exert beneficial effects on mitochondrial function in PCOS patients and helps to control levels of proinflammatory cytokines. Future studies of mitochondria in these circumstances may uncover the molecular mechanism underlying metformin’s efficacy in ameliorating IR in PCOS patients, thereby helping to develop strategies to reduce the risk of the onset of type 2 diabetes in women with this syndrome.

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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Author contribution statement
The authors’ responsibilities were as follows: A Hernández-Mijares conducted the study. M Gómez provided overall supervision and patient...
follow-up. S Rovira-Llopis, C Bañuls, N Diaz-Morales, R Castelló and R Falcón performed the laboratory analyses and collected data. M Rocha assisted in the design of the experiment and provided support throughout the course of the study and analysis. V M Víctor, A Hernández-Mijares, S Rovira-Llopis, C Bañuls and M Rocha performed statistical analyses, interpreted the data and prepared the manuscript. A Hernández-Mijares and V M Víctor were responsible for its final content.

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