

# Metformin augments the levels of molecules that regulate the expression of the insulin-dependent glucose transporter GLUT4 in the endometria of hyperinsulinemic PCOS patients

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**STUDY QUESTION:** Does treatment with the insulin sensitizer metformin modify the levels and activation of proteins related to the expression of the insulin-dependent glucose transporter (GLUT4), such as adenosine monophosphate-activated protein kinase (AMPK) and myocyte enhancer factor 2A (MEF2A), in endometria from hyperinsulinemic hyperandrogenemic polycystic ovary syndrome (PCOS h-Ins) patients?

**SUMMARY ANSWER:** In PCOS h-Ins patients, metformin increases endometrial levels of GLUT4 mRNA and protein levels by normalizing the quantity and activation of molecules that regulate GLUT4 expression to healthy values. These changes could improve endometrial metabolic function.

**WHAT IS ALREADY KNOWN:** PCOS is an endocrine–metabolic disorders closely associated with insulin resistance. In particular, the insulin signaling pathway is impaired in endometria from these patients and the concentration of GLUT4, as well as the molecules involved in its translocation to the cell surface, is decreased. However, there are limited data about the mechanisms that regulate the GLUT4 expression in the endometria and the effect of metformin on them.

**STUDY DESIGN, SIZE AND DURATION:** This is a case–control study in the setting of a research unit, approved by the Ethical Committees of our institution. The groups whose endometria were studied were PCOS h-Ins ( $n = 8$ ); PCOS patients with hyperandrogenemia hyperinsulinemia taking only metformin for at least 3 months (PCOS-MTF,  $n = 8$ ) and healthy fertile women at the time of hysterectomy because of benign pathology as controls (CE,  $n = 8$ ).

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Steroids and sex hormone-binding globulin were measured and glucose and insulin levels were evaluated during an oral glucose tolerance test. Protein levels for  $\alpha$ AMPK (catalytic subunit of AMPK), phosphorylated (p)-AMPK $\alpha$ Thr<sup>172</sup> (activating phosphorylation site), MEF2A, p-MEF2AThr312 (activating phosphorylation site) and GLUT4 were assessed by western blot and immunohistochemistry. In addition, GLUT4 gene expression was evaluated by RT–PCR.

**MAIN RESULTS AND THE ROLE OF CHANCE:** We found significantly lower levels of MEF2A and p-MEF2AThr312 in PCOS h-Ins compared with CE endometria ( $P < 0.05$ ). Also, we detected lower levels of p-AMPK $\alpha$ Thr<sup>172</sup> in PCOS h-Ins endometria compared with the PCOS-MTF group ( $P < 0.05$ ). The ratios of phospho-AMPK/total AMPK and phospho-MEF2A/total MEF2A were significantly increased in the PCOS-MTF compared with the PCOS h-Ins group ( $P < 0.05$ ). The RT–PCR experiments showed lower levels of GLUT4 mRNA transcripts in PCOS h-Ins compared with PCOS-MTF-treated group ( $P < 0.05$ ), the protein levels of GLUT4 were decreased in a similar way.

**LIMITATIONS, REASONS FOR CAUTION:** The limited number of patients included in this study who presented large clinical variability. Therefore, it would be necessary to recruit a greater number of patients to minimize our data dispersion in order to prove the clinical benefits of metformin described by others.

**WIDER IMPLICATIONS OF THE FINDINGS:** Since the insulin sensitizer metformin increases the expression of the GLUT4, it may improve endometrial physiology in PCOS patients and, therefore, promote better reproductive outcomes. These results suggest that in PCOS patients, metformin may act directly at the endometrial level and decrease insulin resistance condition by increasing the expression of GLUT4 and, in this way, indirectly restore endometrial function.

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**Key words:** PCOS / insulin resistance / endometria / metformin / Glut4

## Introduction

Polycystic ovary syndrome (PCOS) is a highly prevalent disease that affects 5–10% of women of childbearing age with important short- and long-term consequences (Diamanti-Kandarakis, 2008). It also has adverse reproductive outcomes like increased incidence of recurrent and spontaneous abortion compared with the normal population (Giudice, 2006). Moreover, recently it has been shown to be associated with alterations in the expression levels of endometrial molecules related to insulin signaling pathway, such as lower expression of IRS-1, IRS-1 Y<sup>612</sup> and decreased ASI60 Thr<sup>642</sup> phosphorylation (Fornés et al., 2010), as well as, impaired expression of the insulin-dependent glucose transporter protein (GLUT4) (Mioni et al., 2004; Mozzanega et al., 2004; Fornés et al., 2010); these findings suggest an endometrial insulin-resistant condition. An adequate expression of molecules involved in glucose uptake is necessary to maintain cellular homeostasis, not only in classical insulin target tissues, but also in those involved in reproductive functions like the endometrium (Von Wolff et al., 2003). It has also been reported that in endometrial cells androgen excess conditions, such as PCOS, influence negatively cellular glucose uptake and the GLUT4 translocation mechanism (Zhang and Liao, 2010; Rosas et al., 2010; Rivero et al., 2012). Even though a large body of evidence has indicated that increased insulin resistance and compensatory hyperinsulinemia play a key role in the pathogenesis of PCOS (Glueck et al., 2002; Nestler, 2008; Diamanti-Kandarakis et al., 2010), there are conflicting reports that metformin may improve pregnancy rates when compared with placebo or in addition to clomiphene (Morin-Papunen et al., 2012; Tang et al., 2012). Likewise, an increase in the mRNA levels of GLUT4 accompanied by a significant improvement of insulin resistance, menstrual pattern and androgen profile was reported in adipocytes from PCOS patients treated with metformin (Jensterle et al., 2008).

One of the molecules that regulate GLUT4 gene expression and that directly participates in the intracellular signaling pathway of metformin is a serine threonine kinase that senses cellular energy homeostasis, the adenosine monophosphate-activated protein kinase (AMPK) (Zhou et al., 2001; Zorzano et al., 2005; McGee and Hargreaves, 2006; Karnieli and Armoni, 2008). AMPK is activated when intracellular ATP concentrations decrease and AMP concentrations increase (Hardie, 2004; Kola et al., 2006). Phosphorylation at threonine 172 (Thr<sup>172</sup> in human AMPK $\alpha$ ) is conserved across species and is required for AMPK activation (Hawley et al., 2002; Hardie, 2004). Several protein kinases responsible for this phosphorylation have been identified and they include LKB1 which is involved in the metformin mechanism of action (Shaw et al., 2005).

One of several AMPK downstream substrates is the myocyte enhancer factor 2A (MEF2A) transcription factor. As has been demonstrated in other insulin-resistant tissues, like muscle and adipocytes, it has been implicated in the regulation of GLUT4 gene expression (Mora and Pessin, 2000; Murgia et al., 2009). MEF2 is a transcription factor with many functions, and at least three isoforms have been reported (Thai et al., 1998; Mora and Pessin, 2000). MEF2A contains a known nuclear localization sequence (amino acids 472–507) (Knight et al., 2003; Holmes et al., 2005), and stimulation of AMPK increases MEF2A expression, its translocation to the nucleus and its binding to the GLUT4 promoter region (Holmes et al., 2005).

Since several reports in the endometria from PCOS patients show an impaired insulin pathway and lower levels of GLUT4 associated with adverse reproductive outcomes, the study of the effects of metformin on the endometria from PCOS patients is of clinical relevance for therapeutic purposes. Therefore, the aim of this study was to evaluate whether the metabolic–endocrine PCOS condition, such as hyperandrogenemia and hyperinsulinemia, is associated with alterations in the molecule levels of proteins that regulate the expression of the GLUT4, specifically AMPK $\alpha$  and MEF2A. Also, we investigated whether these alterations could be pharmacologically reversed at the endometrial level by the insulin sensitizer metformin.

## Materials and Methods

### Subjects and design

This is a case–control study in the setting of a research unit, approved by the ethical committees of the Faculty of Medicine and Clinical Hospital of the University of Chile. Twenty-four patients were recruited: eight hyperinsulinemic hyperandrogenemic PCOS patients (PCOS h-Ins;  $n = 8$ ) without any treatment in the last 3 months prior to recruitment into the study; eight PCOS patients previously diagnosed as hyperandrogenemic and hyperinsulinemic who were prescribed only metformin as treatment 850 mg twice a day for at least 12 weeks at the time of the recruitment (PCOS-MTF;  $n = 8$ ) and eight fertile healthy women as controls (CE;  $n = 8$ ) recruited at the time of hysterectomy due to benign uterine pathology. Endometrial and blood samples were obtained from women with PCOS h-Ins, PCOS-MTF and from CEs during the proliferative phase of the menstrual cycle. Sex steroids (testosterone, progesterone and estradiol) and sex hormone-binding globulin (SHBG) concentration were assessed. Glucose and insulin levels were evaluated by an oral glucose tolerance test with 75 g load of glucose (OGTT). In order to determine a hyperinsulinemic condition, we measured plasma glucose and insulin levels 2 h after administration of the glucose.

Human endometria were obtained with a Pipelle suction curette from the corpus of the uteri of women with PCOS. The diagnosis of

hyperinsulinemia was determined when levels of insulin were 2 standard deviations (SDs) of insulin concentration over the mean of the control group, as in previous studies (Maliqueo *et al.*, 2003; Fornés *et al.*, 2010; Kohan *et al.*, 2010; Rosas *et al.*, 2010). All women had normal glycemic values in the OGTT (basal glucose < 110 mg/dl; glucose 120 min < 140 mg/dl). Also, all the PCOS women participants in this investigation had hyperandrogenism and hyperinsulinemia. Control endometria were selected in the proliferative phase because of the similar morphology between proliferative endometrium and PCOS endometria. None of the women, neither controls nor those with PCOS had received hormonal therapy within 3 months prior to the recruitment into the study. The proliferative phase in CE and PCOS endometria was confirmed on the basis of histological dating and classification according to Noyes criteria (Noyes *et al.*, 1950) by an experienced pathologist. The diagnosis of PCOS was made according to the Rotterdam Consensus (The Rötterdam ESHRE/ASRM, 2004) and to the Androgen Excess Society criteria (Azziz *et al.*, 2006) for the definition of PCOS. The exclusion criteria were women who presented hyperprolactinemia (PRL > 35 ng/ml), hypothyroidism (TSH > 5 UI/l), androgen-secreting tumors (total testosterone > 2 ng/ml; DHEAS > 3600 µg/ml), Cushing's syndrome (urine cortisol concentration > 150 µg/24 h and fasting plasma concentration of cortisol between 5 and 25 µg/dl), congenital adrenal hyperplasia (17-OH progesterone > 2.5 ng/ml), women with diabetes or treatment with hormones and/or ovulation induction. The reference values are from the Laboratory of Endocrinology and Reproductive Biology, University of Chile Clinical Hospital.

## Antibodies and reagents

The monoclonal antibodies for phosphorylated AMPK $\alpha$  in Thr<sup>172</sup> and polyclonal antibodies for AMPK $\alpha$  and MEF2A were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies for phosphorylated MEF2A at Thr<sup>312</sup> and monoclonal antibodies for  $\beta$ -actin were purchased from Abcam (Cambridge, MA, USA). Polyclonal antibodies for GLUT4 were obtained from Santa Cruz Biotechnology. Secondary antibodies (mouse monoclonal and rabbit polyclonal) were purchased from Amersham Biosciences (Amersham International, Piscataway, NJ, USA). Protease-inhibitor cocktail was obtained from Roche Mol Biochemicals (Mannheim, Germany), BCA Protein Assay Kit from Pierce (Rockford, IL, USA) and HistostainR SP kit from Zymed Laboratories (San Francisco, CA, USA). TRIzol Reagent and M-MLV Reverse Transcriptase were obtained from Invitrogen (Carlsbad, CA, USA), Taq DNA polymerase from Biotools (Madrid, Spain). Hormone determinations were assayed by commercial kits: serum testosterone, estradiol and progesterone by solid-phase, competitive chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Johnson & Johnson, UK); androstenedione by radioimmunoassay

(Siemens, LA, CA, USA); SHBG and insulin concentrations by Immulite, solid-phase chemiluminescent immunometric assay (Siemens, UK). Glucose measurement was assessed by the glucose oxidase colorimetric assay (Human GmBh Wiesbaden, Germany).

## Immunohistochemistry

Immunostaining was performed on 5-µm sections of formalin-fixed paraffin-embedded endometrial biopsies. Tissue sections were deparaffinized in xylene and hydrated in a series of graded alcohols. The sections were incubated in antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6.0) at 96–98°C for 20 min. Endogenous peroxidase activity was prevented by incubating the samples in 3% (v/v) hydrogen peroxide for 15 min. Non-specific antibody binding was prevented with the specific blocker of the HistostainR SP kit. Specific primary antibody dilution [AMPK $\alpha$  (1/200), p-AMPK $\alpha$ Thr<sup>172</sup> (1/100), MEF2A (1/500),

p-MEF2AThr<sup>312</sup> (1/100) and GLUT4 (1/1800)] was applied to the samples and incubated overnight at 4°C. Negative controls were analyzed on adjacent sections and incubated without primary antibody, as well as, with non-immune species-specific antisera. The secondary antibody was a biotinylated anti-mouse/anti-rabbit immunoglobulin. The reaction was developed by the streptavidin-peroxidase system, and 3,3'-diaminobenzidine was used as the chromogen; counterstaining was carried out with hematoxylin. The slides were evaluated in an optical microscope Olympus BX51 (PA, USA). The immunohistochemical evaluation for each protein was performed by a semi-quantitative method previously described in endometria (Rivero *et al.*, 2012). Using Image-Pro Plus<sup>®</sup> acquisition software,  $\times 400$  images were acquired and processed in TIFF format. Slide analysis was performed by the measurement of positive pixel intensity with the use of the semi-quantitative analysis tool-integrated optical density (IOD) in the Image-Pro Plus 6.2 program. Equally sized areas were taken at random in the stroma and epithelia in different regions of the sample. The data are presented as IOD Arbitrary Units (AU). The mean of these values were obtained per sample and studied group and expressed as the mean  $\pm$  SEM.

## Western blot analysis

The endometrial tissue was homogenized in a lysis buffer (HEPES 20 mmol/l, EDTA 2 mmol/l, EGTA 2 mmol/l, Triton 1%, phenylmethanesulfonyl fluoride 5 µmol/l, Na<sub>3</sub>VO<sub>4</sub> 50 µmol/l) containing protease-inhibitor cocktail (Roche, IN, USA). After centrifugation at 10 000g for 20 min at 4°C, protein concentrations were determined using the bicinchoninic acid assay BCA protein Assay kit (Pierce, IL, USA). Total proteins (50 µg) were denatured and fractionated using 8% one-dimensional-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BioRad, CA, USA). Membranes were blocked for 1 h in TBST (20 mmol/l Tris, pH 7.6; 137 mmol/l NaCl; 0.1% Tween 20) containing 10% (w/v) non-fat dry milk (TBST) (for all markers). Subsequently, the membranes were washed three times for 5 min each in TBST and then incubated with antibodies against AMPK $\alpha$  (1/750), p-AMPK $\alpha$ Thr<sup>172</sup> (1/750), MEF2A (1/750), p-MEF2AThr<sup>312</sup> (1/300) and GLUT4 (1/250) overnight with rocking at 4°C, and with anti- $\beta$ -actin (1:15000) for 1 h at room temperature. The membranes were then washed three times for 5 min each with TBST, followed by incubation for 1/2 h at room temperature with anti-mouse IgG peroxidase-conjugated species specific (1:5000 for  $\beta$ -actin) or with anti-rabbit IgG peroxidase-conjugated species specific [AMPK $\alpha$  (1/10000), p-AMPK $\alpha$ Thr<sup>172</sup> (1/10000), MEF2A (1/1500), p-MEF2AThr<sup>312</sup> (1/1500) and GLUT4 (1/7000)] while rocking. After washing three times for 5 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system (Amersham International) and analyzed by the program UN-SCAN-IT gel 4.1 (Silk Scientific Corporation). The protein levels were normalized by the levels of the protein  $\beta$ -actin and expressed as AU.

## RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from endometrial tissue using Trizol reagent according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (A260:A280), while the integrity of the RNA was determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. The RNA was visualized by adding ethidium bromide (EtBr) to the sample before loading on the gel. The RNA was stored at  $-80^{\circ}\text{C}$  until use. Two micrograms of total RNA was digested with DNase I and transcribed into complementary DNA (cDNA) by reverse transcription with M-MLV Reverse Transcriptase by using random primers in a total volume of 25 µl. The PCR amplifications for GLUT4 were obtained by using gene-specific primers: sense 5'-ATCCTGATGACTGTGGCTCTGCT-3' and antisense

5'-TCGTTCTCATCTGGCCCTAAATAC-3', obtaining a 433 bp amplicon (Kohan et al., 2010). Ribosomal RNA 18s was used as an internal control: sense 5'-GTAACCCGTTGAACCCATT-3' and antisense 5'-CCATCCAATCGGTAGTAGCG-3' with a 200 bp amplicon. Semi-quantitative RT-PCRs were achieved in the exponential linear zone amplification for the studied gene. The PCR conditions for GLUT4 were 2 mmol/l MgCl<sub>2</sub>, 0.20 mmol/l dNTPs, 2 U of Taq DNA polymerase and 30 pmol each primer. The PCR amplification was performed in the thermocycler, model PTC-100 (MJ Research, Inc., Watertown, MA, USA) and Mastercycler Personal (Eppendorf AG, Foster city CA, USA). GLUT4 amplification product was done using 40 cycles at 95°C/1 min, 60°C/1 min, 72°C/1 min followed by a 5-min final extension at 72°C. The PCR products were electrophoretically resolved on 2% agarose gel and stained with EtBr. The bands were evaluated using an image analyzer UN-SCAN-IT gel 4.1 (Silk Scientific Corporation) and normalized relative to the ribosomal mRNA 18s PCR product.

## Statistical evaluation

The number of subjects in this study was calculated assuming  $\alpha = 0.05$  and  $\beta = 0.20$  and a difference between means of 0.25 and SD of 0.16 according to our previous studies (Bacallao et al., 2008; Fornés et al., 2010; Kohan et al., 2010; Rosas et al., 2010). The distribution of the data was analyzed by Kolmogorov–Smirnov test. The results were analyzed by Student's t-test or Mann–Whitney test. For multiple comparisons, ANOVA or Kruskal–Wallis statistical tests were used. *P*-values < 0.05 were considered significant. Multiple regression analyses were done using a backward stepwise method to adjust for dependent variables. Statistical tests were performed using SPSS Statistics® 17.0 for Windows.

## Results

### Clinical and endocrinological characteristics of subjects

Clinical and hormonal characteristics of control and PCOS women are shown in Table I. The women in the CE group were older than the PCOS women because the CE women belonged to a group

**Table I Clinical and endocrine characteristics of studied groups.**

Parameters	Control	PCOS h-Ins	PCOS-MTF
Age (years)	39.7 ± 1.2	26.5 ± 0.7*	28.3 ± 1.1 <sup>†</sup>
BMI (kg/m <sup>2</sup> )	26.7 ± 2.0	34.1 ± 1.2*	32.7 ± 2 <sup>†</sup>
Estradiol (pg/ml) <sup>a</sup>	73.8 ± 22.4	63.1 ± 4.7	58.3 ± 5
Progesterone (ng/ml) <sup>a</sup>	0.7 ± 0.3	1.0 ± 0.25	1.8 ± 1
Androstenedione (ng/ml) <sup>a</sup>	1.8 ± 0.2	4.3 ± 0.2*	3.9 ± 0.2 <sup>†</sup>
Testosterone (ng/ml) <sup>a</sup>	0.47 ± 0.03	0.72 ± 0.02*	0.7 ± 0.02 <sup>†</sup>
SHBG (nmol/l) <sup>a</sup>	45.9 ± 2.5	24 ± 1.7*	27.4 ± 2.3 <sup>†</sup>
FAI <sup>a</sup>	5.2 ± 0.8	12.9 ± 0.6*	11.4 ± 0.6 <sup>†</sup>

Control: women during proliferative phase; PCOS h-Ins, hyperinsulinemic polycystic ovary syndrome (PCOS) patient; PCOS-MTF, hyperinsulinemic PCOS patients treated with metformin; SHBG, sex hormone-binding globulin. The values are mean ± SEM, *n* = 8.

<sup>a</sup>Variables adjusted by age and BMI.

\**P*-value < 0.05 between the PCOS h-Ins and control groups; <sup>†</sup>*P* value < 0.05 between the PCOS-MTF and control groups.

undergoing hysterectomy; the higher body mass index in patients with PCOS is inherent to the syndrome, between 30 and 50% of PCOS patients are obese (Table I) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Since age and BMI may affect the endometrium this was adjusted by multiple regression in all our further statistical analyses. Progesterone levels, in addition to the morphological evaluation by the pathologist, confirmed that all endometrial biopsies were obtained during the proliferative phase. Besides hyperinsulinemia, all PCOS women presented hyperandrogenemia and together with a decreased SHBG blood level leading to a significantly higher free androgen index (FAI) in both PCOS groups compared with controls. Even though testosterone and FAI in PCOS-MTF appeared lower compared with PCOS h-Ins, no statistical difference was obtained (Table I). All the patients with PCOS in this study presented insulin levels 120 min post 75 g load of glucose over the normal value (Table II); therefore, they were diagnosed as women with hyperinsulinemia. Basal glucose levels and those 120 min after the glucose load were within the normal range in all groups of this study. Nevertheless, a significantly higher level of fasting glucose was found in both PCOS groups compared with controls (Table II). However, at 120 min, only in PCOS h-Ins glucose levels were significantly higher than controls, meaning that metformin could be having a systemic effect on insulin resistance in the PCOS-MTF group. When compared fasting insulin levels we found significant higher values in both PCOS groups compared with controls; but as seen for glucose at 120 min, insulin after 120 min was only statistically higher in the PCOS h-Ins group (Table II). A histological study of endometria from PCOS patients shows no differences compared with control women (data not shown), as we have reported recently (Fornés et al., 2010).

### Protein expression for phosphorylated and unphosphorylated forms of AMPK in endometria

We evaluated the protein levels of p-AMPKαThr<sup>172</sup>, the catalytic subunit of AMPK by immunohistochemistry, and we found predominant cytoplasm localization in the epithelial and stromal compartments of the endometria in all the studied groups (Fig. 1). However, when analyzed semi-quantitatively by the IOD tool, a significantly lower staining for p-AMPKαThr<sup>172</sup> was detected in PCOS h-Ins in both

**Table II Metabolic parameters of studied groups.**

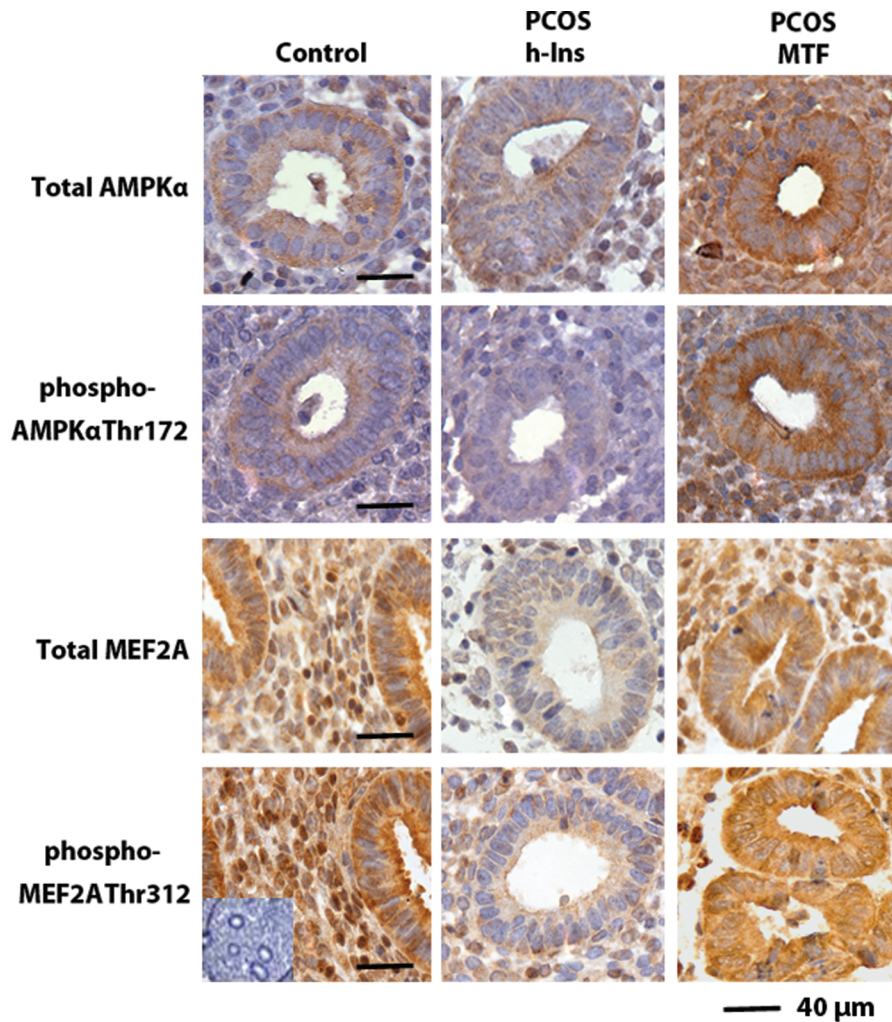
Parameters	Control	PCOS h-Ins	PCOS-MTF
Fasting glucose (mg/dl) <sup>a</sup>	70.8 ± 3	95.3 ± 1.8*	91.2 ± 1.7 <sup>†</sup>
Glucose 120 min (mg/dl) <sup>a</sup>	89.6 ± 5.1	105.4 ± 3*	97.8 ± 3.8
Fasting insulin (μIU/ml) <sup>a</sup>	15.3 ± 1.3	26.9 ± 0.7*	25.0 ± 0.9 <sup>†</sup>
Insulin 120 min (μIU/ml) <sup>a</sup>	53.2 ± 5.8	108 ± 2.7*	93.1 ± 3.1

Control: women during the proliferative phase; PCOS h-Ins, hyperinsulinemic polycystic ovary syndrome (PCOS) patient; PCOS-MTF, hyperinsulinemic PCOS patients treated with metformin. The values are mean ± SEM, *n* = 8.

<sup>a</sup>Variables adjusted by age and BMI.

\**P*-value < 0.05 between the PCOS h-Ins and control groups; <sup>†</sup>*P* value < 0.05 between the PCOS-MTF and control groups.





**Figure 1** Immunohistochemical detection of the  $\alpha$  subunit of AMP protein kinase (AMPK $\alpha$ ), its phosphorylated form (p-AMPK $\alpha$ Thr<sup>172</sup>), MEF2A and its phosphorylated form (p-MEF2AThr<sup>312</sup>) proteins in paraffin wax sections of proliferative endometria obtained from normal women (Control,  $n = 8$ ) (left column), PCOS with hyperinsulinemia (PCOS h-Ins,  $n = 8$ ) (central column) and PCOS treated with metformin (PCOS-MTF,  $n = 8$ ) (right column). Positive staining was detected in epithelial and stromal cells of all studied endometria for all antigens. As a negative control, the primary antibody was omitted (insert in control endometria). Image magnification  $\times 1000$  in all panels. Scale bars represent 40  $\mu\text{m}$ .

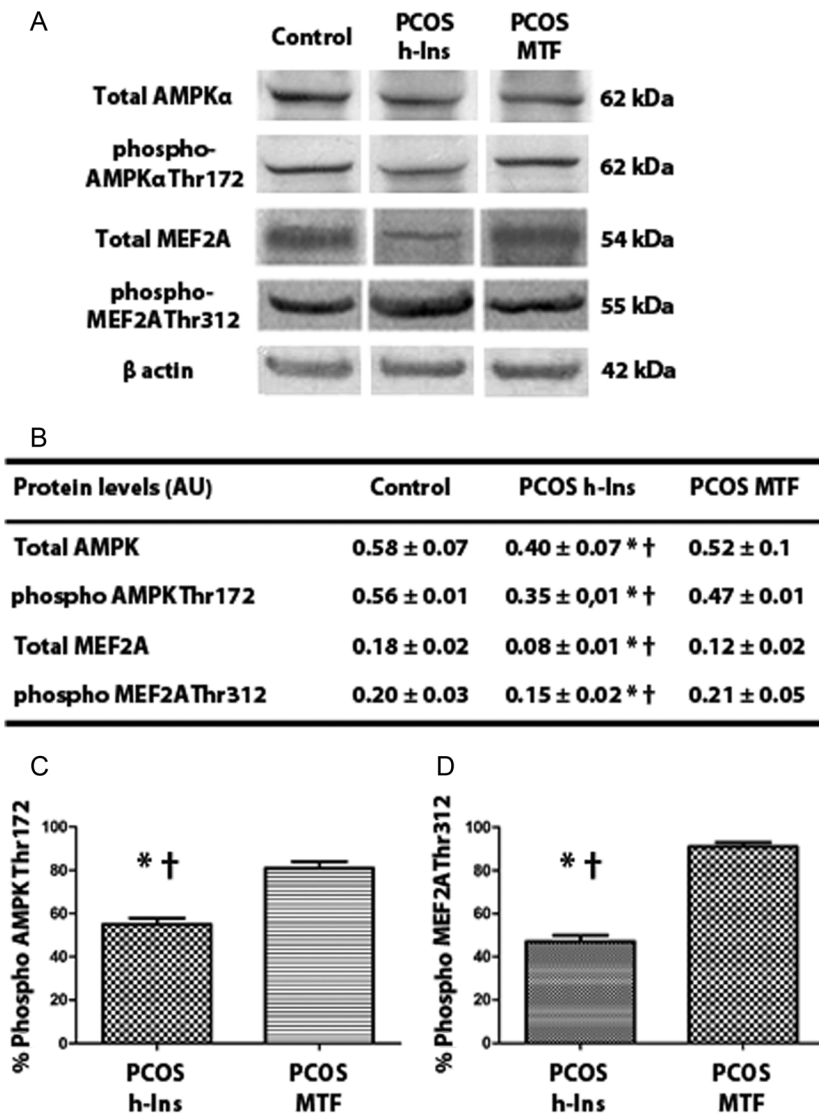
epithelia and stroma compartments ( $34.7 \pm 3.2$ ;  $25.4 \pm 1.2$  IOD AU) compared with control samples ( $57.9 \pm 3.7$ ;  $36.5 \pm 2.1$  IOD AU), respectively ( $P < 0.05$ ). In contrast, when the IOD values of PCOS h-Ins were compared with PCOS-MTF, significantly higher IOD values were found in samples from the metformin-treated group in epithelia ( $118.6 \pm 7$  IOD AU) and stroma ( $78.2 \pm 9$  IOD AU) ( $P < 0.05$ ).

Additionally, we evaluated AMPK $\alpha$  to exclude that the differences found were not attributed to lower total proteins contents in the endometria. We found, as seen in Fig. 1, staining in the PCOS-MTF group to be significantly (+30%) higher compared with PCOS h-Ins ( $99.6 \pm 16$  in epithelia and  $77.1 \pm 14$  in stroma, IOD AU), suggesting that metformin could have a local effect in the endometrium of these patients. When the samples were analyzed by western blot, significantly lower levels of p-AMPK $\alpha$ Thr<sup>172</sup> (45%) and AMPK $\alpha$  (28%) were detected in the PCOS h-Ins endometria compared with control ( $P < 0.05$ ) (Fig. 2). In contrast, in the PCOS-MTF samples

higher levels of p-AMPK $\alpha$ Thr<sup>172</sup> (43%) and AMPK $\alpha$  (34%) were observed when compared with PCOS h-Ins ( $P < 0.05$ , respectively) (Fig. 2). To better define if these findings were attributed to differences in the amount of the activated protein versus its total expression, we calculated the percentage of phosphorylation of AMPK and we found a significant increase in the PCOS-MTF group when compared with PCOS h-Ins, suggesting that metformin could increase not only AMPK $\alpha$ , but also its activation (Fig. 2C).

### Protein expression for phosphorylated and unphosphorylated forms of MEF2A in endometria

We assessed the endometrial protein levels of total MEF2A (MEF2At) and MEF2A phosphorylated at Thr<sup>312</sup> (MEF2AThr<sup>312</sup>) in all studied groups. The immunohistochemistry semi-quantification by the IOD



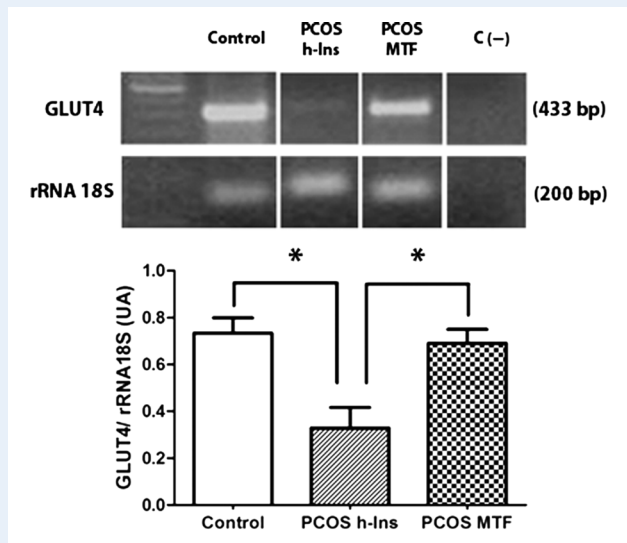
**Figure 2** Western blot analysis of protein levels of  $\alpha$  subunit of AMP protein kinase (AMPK $\alpha$ ), its phosphorylated form (p-AMPK $\alpha$ Thr<sup>172</sup>), MEF2A and its phosphorylated form (p-MEF2AThr<sup>312</sup>) in endometria obtained from normal women (Control,  $n = 8$ ) (left column), PCOS with hyperinsulinemia (PCOS h-Ins,  $n = 8$ ) (central column) and PCOS treated with metformin (PCOS-MTF,  $n = 8$ ) (right column). **(A)** Equal amounts of protein (50  $\mu$ g) were loaded in each lane. **(B)** Band intensities were quantified by scanning densitometry and normalized to intensities observed for  $\beta$ -actin as an internal control. **(C)** Percentage of AMPK $\alpha$  phosphorylation compared with controls (100%). **(D)** Percentage of MEF2A phosphorylation compared with controls (100%). The results are expressed as AU and the values shown are means  $\pm$  SEM. \* $P$ -value  $< 0.05$  PCOS h-Ins compared with the control group; † $P$ -value  $< 0.05$  PCOS h-Ins compared with the PCOS-MTF group.

tool detected significantly lower levels ( $P < 0.05$ ) of MEF2At ( $53.3 \pm 5$  in epithelia and  $57.1 \pm 11$  in stroma IOD AU) and MEF2AThr<sup>312</sup> ( $62.5 \pm 8$  in epithelia and  $48.9 \pm 7$  in stroma IOD AU) in the PCOS h-Ins group compared with CE (MEF2At:  $109.8 \pm 13$  in epithelia and  $125.3 \pm 8$  in stroma IOD AU; MEF2AThr<sup>312</sup>:  $97.7 \pm 2$  in epithelia and  $118.1 \pm 7$  in stroma IOD AU) (Fig. 1). Likewise, a significant lower level was detected in the PCOS h-Ins group when we compared it with PCOS-MTF: MEF2At  $107.9 \pm 12$  in epithelia and  $129.2 \pm 6$  in stroma IOD AU and MEF2AThr<sup>312</sup>:  $103.4 \pm 9$  in epithelia and  $131.2 \pm 8$  in stroma IOD AU ( $P < 0.05$ ) (Fig. 1). These results were

confirmed by western blot studies, where we found a 53% significant reduction in the protein levels of MEF2At in the PCOS h-Ins group compared with CE, interestingly in the PCOS-MTF group these reduction were not found, where MEF2At reaches levels as detected in CE. (Fig. 2). Also, by western blot we found a 43% statistically significant increase in the phosphorylation of MEF2AThr<sup>312</sup> in PCOS-MTF compared with PCOS h-Ins ( $P < 0.05$ ) (Fig. 2). The same was observed if we expressed the data as the percentage of phosphorylation of MEF2A, finding a significant increase in the PCOS-MTF group compared with PCOS h-Ins ( $P < 0.05$ ) (Fig. 2D).

## Protein and gene expression of GLUT4 in endometria

Finally, the RT-PCR analysis showed a significant decreased GLUT4 mRNA transcript levels in endometria from women with PCOS h-Ins compared with CE (50%,  $P < 0.05$ ) (Fig. 3). These results are in agreement with the GLUT4 protein levels detected by immunohistochemistry, which are also diminished (Fig. 4), and western blot (Fig. 5) in PCOS h-Ins compared with CE (75%,  $P < 0.05$ ).



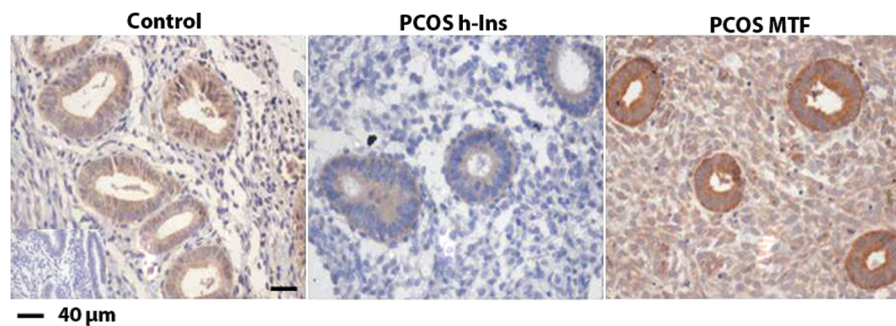
**Figure 3** Analysis of insulin-dependent GLUT4 mRNA levels. Conventional RT-PCR was performed in human endometria obtained from normal women (Control,  $n = 8$ ), PCOS with hyperinsulinemia (PCOS h-Ins,  $n = 8$ ) and PCOS treated with metformin (PCOS-MTF,  $n = 8$ ). (A) Representative gel is shown (2% agarose gel electrophoresis, staining with EtBr). (B) Semi-quantification of mRNA levels of GLUT4 in control, PCOS h-Ins and PCOS-MTF groups. The results are expressed as mean  $\pm$  SEM. RNA ribosomal 18S was used as the internal control. \* $P$ -value  $< 0.05$ .

Interestingly, GLUT4 mRNA transcripts detected in the PCOS-MTF endometria were significantly higher than in the PCOS h-Ins (55%,  $P < 0.05$ ) (Fig. 3), reaching similar levels to those in the control group. Additionally, the GLUT4 protein level evaluated by immunohistochemistry showed significant higher staining in PCOS-MTF endometria ( $51.7 \pm 6.1$  in epithelia and  $41.3 \pm 1.1$  in stroma IOD AU) compared with the PCOS h-Ins group ( $28.1 \pm 8.1$  in epithelia and  $16.0 \pm 6.2$  in stroma IOD AU) ( $P < 0.05$ ) (Fig. 4). Similarly, by western blot lower levels of GLUT4 were found in the PCOS h-Ins group compared with PCOS-MTF ( $P < 0.05$ ) (Fig. 5).

## Discussion

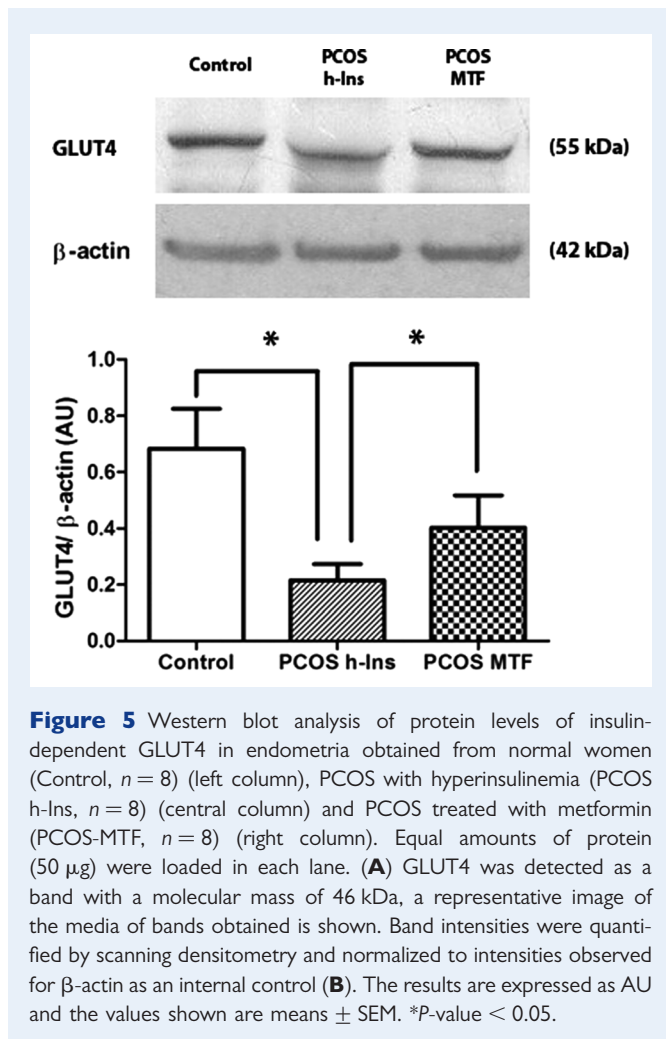
PCOS is a frequent endocrine–metabolic disorder in patients of child-bearing age. The syndrome has a heterogeneous phenotype presentation, obesity and insulin resistance may coexist and the exact mechanism is not well understood. Besides the ovarian dysfunction in PCOS, other tissues of the organism are also affected, including the endometrium. To fulfill its function and properly differentiate toward a receptive state, the endometria need a high supply of energy, particularly from glucose intake for glycolysis or glycogen storage. In this aspect, results from our laboratory and other groups indicate that some molecules involved in the insulin signaling pathway could be altered in the endometrium of women with PCOS. Our group has described alterations in the molecules that participate in insulin signaling pathway in endometria from PCOS hyperinsulinemic patients (Fornés *et al.*, 2010), suggesting that this tissue could be insulin resistant. Other authors supported this notion; in fact Kim *et al.* (2009) evaluated proliferative phase endometria from PCOS patients with laser microdissection and microarrays and found important gene deregulations in pathways of glucose metabolism. In addition, Mioni *et al.* (2004) and Mozzanega *et al.* (2004) described lower GLUT4 gene and protein levels in endometria from this group of patients.

The decreased levels of GLUT4 may be due in part to changes in molecules that regulate the expression of this glucotransporter. It was reported that in PCOS hyperinsulinemic endometria higher levels of PPAR- $\gamma$  transcription could partially account for the lower



**Figure 4** Immunohistochemical detection of insulin-dependent GLUT4 in paraffin wax sections of proliferative endometria obtained from control women (Control,  $n = 8$ ) (left column), PCOS with hyperinsulinemia (PCOS h-Ins,  $n = 8$ ) (central column) and PCOS treated with metformin (PCOS-MTF,  $n = 8$ ) (right column). Positive staining was detected mostly in the cytoplasm of epithelial and stromal cells of all studied endometria. As a negative control, the primary antibody was omitted (insert in control endometria). Image magnification was  $\times 400$  (A–C). Scale bars represent 40  $\mu$ m.





**Figure 5** Western blot analysis of protein levels of insulin-dependent GLUT4 in endometria obtained from normal women (Control,  $n = 8$ ) (left column), PCOS with hyperinsulinemia (PCOS h-Ins,  $n = 8$ ) (central column) and PCOS treated with metformin (PCOS-MTF,  $n = 8$ ) (right column). Equal amounts of protein ( $50 \mu\text{g}$ ) were loaded in each lane. (A) GLUT4 was detected as a band with a molecular mass of 46 kDa, a representative image of the media of bands obtained is shown. Band intensities were quantified by scanning densitometry and normalized to intensities observed for  $\beta$ -actin as an internal control (B). The results are expressed as AU and the values shown are means  $\pm$  SEM. \* $P$ -value  $< 0.05$ .

levels of GLUT4 found in PCOS (Kohan et al., 2010). Moreover, Jensterle et al. (2008) found, in subcutaneous adipose tissue samples from women with PCOS that received metformin or rosiglitazone for 6 months, a significant increase of GLUT4 mRNA expression in both groups. Previously, it was reported that metformin increases serum IGF-I and glycodelin during the luteal phase in addition to enhancing luteal phase uterine vascularity and subendometrial blood flow in PCOS (Jakubowicz et al., 2001; Palomba et al., 2006). These changes may reflect an improved endometrial milieu for the establishment and maintenance of pregnancy. Therefore, the importance of our work resides in the fact that no previous reports are available of the molecular mechanism at the endometrial level that could explain the impaired GLUT4 expression, thus, the implantation process and if these disarrangements could be reversed pharmacologically with metformin treatment.

Taking into account the heterogeneity of phenotypes of the syndrome according to the Rotterdam Consensus diagnostic criteria, all the PCOS patients included in the present study exhibited hyperandrogenism with increased FAI and also with hyperinsulinemia. The patients treated with metformin exhibited diminished levels of testosterone and insulin compared with the untreated group, although not statistically different from the control group. This investigation suggests that in addition to its systemic effects, metformin elicits additional

benefits at the endometrial level. Nevertheless, these results could be restricted to the limited number of patients included in this study who presented large clinical variability. Therefore, it would be necessary to recruit a greater number of patients to confirm the clinical benefits of metformin described by others (Palomba et al., 2009; Diamanti-Kandarakis et al., 2010).

Previous reports have indicated the active role of AMPK heterotrimeric complex in GLUT4 expression and in the mechanism of action of metformin in PCOS (Kola et al., 2006; Diamanti-Kandarakis et al., 2010). In addition, it has been established that  $\alpha$  is the catalytic subunit of AMPK (AMPK $\alpha$ ) and that metformin administration augments its phosphorylation levels resulting in its activation and enhanced gene transcription (Hawley et al., 2002). It is important to note that this is the first report to address AMPK expression in PCOS endometria. The finding that protein levels of AMPK $\alpha$  were lower in the PCOS h-Ins group compared with control MTF is consistent with the impaired expression of GLUT4 reported previously in PCOS endometria (Fornés et al., 2010; Mioni et al., 2004). It is possible that metformin exerts a direct local effect in PCOS endometrium augmenting AMPK $\alpha$  protein content and consequently GLUT4 transcription levels. Metformin treatment enhances not only the AMPK total protein contents in PCOS h-Ins but also p-AMPK $\alpha$ Thr<sup>172</sup>, reported as an activating phosphorylation site in other insulin-resistant tissues (Long et al., 2006). Therefore, the higher rate p-Thr<sup>172</sup>AMPK $\alpha$ /AMPK $\alpha$  found in PCOS-MTF endometria compared with PCOS h-Ins may indicate that metformin could increase not only the levels of AMPK $\alpha$  but also its activation, suggesting that AMPK $\alpha$  could be a therapeutic target for the metabolic homeostasis in PCOS endometria.

Insulin, fundamentally through the PI3K/Akt pathway induces the phosphorylation of the AS160 protein (Akt substrate of 160 kDa) in the threonine 642 site (pAS160T<sup>642</sup>) promoting GLUT4 translocation to the cell surface; an alteration in the levels of these molecules has been reported in PCOS endometria (Fornés et al., 2010). The same occurs in muscle cells where metformin favors AS160 phosphorylation levels and consequently GLUT4 translocation (Karlsson et al., 2005). The molecular insulin signal also activates atypical PKCs, such as PKC Zeta (PKC $\zeta$ ), that participate in actin remodelling allowing the translocation of GLUT4 to the plasma membrane. Rivero et al. (2012) revealed lower phospho-PKC $\zeta$  in PCOS hyperinsulinemic endometria suggesting an impaired mechanism of GLUT4 vesicles translocation to the plasma membrane. Furthermore, Rosas et al. (2010) described altered the expression of the WAVE family proteins, involved in the cortical actin remodelling, compromising the cell surface GLUT4 exposure in hyperinsulinemic PCOS endometria. Therefore, the data obtained in the present investigation increase our knowledge on the effects of metformin in PCOS endometria and on the benefits in the functionality and increased expression of GLUT4.

Another molecule involved in GLUT4 expression is the transcription factor MEF2A (Mora and Pessin, 2000; Murgia et al., 2009). The significantly lower levels of MEF2A and its activation found in PCOS h-Ins could be compensated with metformin and it seems that the most important mechanism of action is the activation based on the significant higher rate of phospho-MEF2A/total MEF2A detected in PCOS-MTF. These results suggest an alteration in the expression and phosphorylation levels of MEF2A in endometria of hyperandrogenic and hyperinsulinemic PCOS women, as already mentioned



for AMPK. Additionally, the beneficial effects of metformin treatment in PCOS-MTF might be by direct effects over the PCOS h-Ins endometria, since MEF2A is a known substrate of activated AMPK in other insulin-resistant tissues (Holmes *et al.*, 2005). Furthermore, it has been shown that MEF2A possesses a nuclear localization sequence and also a binding site on the promoter region of GLUT4 gene, which indicates a regulatory action on GLUT4 transcription (Thai *et al.*, 1998; Mora and Pessin, 2000). In addition, in the skeletal muscle, Holmes *et al.* (2005) proved that AMPK activation produces a rise of the nuclear localization of MEF2 and the GLUT4 enhancer factor (GEF) with a parallel increase in the mRNA GLUT4 transcripts. Therefore, both MEF2 and GEF protein binding are necessary for normal GLUT4 mRNA expression in this experimental model.

Therefore, if we consider that AMPK and MEF2A and its phosphorylated forms have an important role in GLUT4 expression and that the alterations found in PCOS h-Ins endometria were reverted in PCOS-MTF, we should accordingly find an increase in GLUT4 expression in PCOS-MTF, as reported in other tissues (Al-Khalili *et al.*, 2005; Jensterle *et al.*, 2008). In fact, as we expected, the PCOS-MTF endometria exhibited significant more GLUT4 transcripts than in PCOS h-Ins. Zhang and Liao (2010) reported in primary endometrial epithelial cell cultures obtained from hysterectomies, that metformin increases GLUT4 mRNA. Recently, Zhai *et al.* (2012) reported in PCOS patients an increased endometrial content of GLUT4 protein and mRNA after metformin treatment, similar to the data obtained in the present investigation, although a potential molecular mechanism of metformin action was not addressed. Importantly, the present data reveal that the increase in GLUT4 mRNA in endometria of patients treated with metformin is also translated to a higher level of GLUT4 protein. This is in agreement with a direct local effect of metformin in PCOS endometrium over the molecules described before that regulates GLUT4 gene expression, like AMPK and MEF2A.

In conclusion, in hyperinsulinemic PCOS patients, metformin acts locally by increasing endometrial gene and protein GLUT4 abundance through the normalization of the expression and activation of molecules that regulate GLUT4 expression. Accordingly, the insulin sensitizer metformin, by increasing the expression of the GLUT4, may improve the endometrial physiology in PCOS patients and, therefore, promote better reproductive outcomes. These results suggest that metformin in PCOS patients may act directly at the endometrial level improving the insulin resistance condition by increasing the expression of GLUT4 and, in this way, indirectly recover the endometrial function in PCOS patients.

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## Authors' roles

R.C. contributed to the conception and design of the study, acquisition of data, analysis and interpretation, drafting the article and the final approval of the version to be published. C.R. contributed substantially to the acquisition and analysis of data and approved the final draft for publication. K.K. contributed in the analysis of data, article revision and of final version. F.G. contributed in the interpretation of data,

revised the article critically for important intellectual content. D.V. contributed to conception of the study, critical revision of article and approved the final draft for publication. C.R. contributed in the conception and design of the study, the critical revision and final version. M.V. conception and design, analysis and interpretation of data, revised the article critically for important intellectual content and approved the final draft for publication.

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## Conflict of interest

None declared.

## References

- Al-Khalili L, Forsgren M, Kannisto K, Zierath JR, Lönnqvist F, Krook A. Enhanced insulin-stimulated glycogen synthesis in response to insulin, metformin or rosiglitazone is associated with increased mRNA expression of GLUT4 and peroxisomal proliferator activator receptor gamma co-activator 1. *Diabetologia* 2005;**48**:1173–1179.
- Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE *et al.* Position statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab* 2006;**91**:4237–4245.
- Bacallao K, Leon L, Gabler F, Soto E, Romero C, Valladares L, Vega M. In situ estrogen metabolism in proliferative endometria from untreated women with polycystic ovarian syndrome with and without endometrial hyperplasia. *J Steroid Biochem Mol Biol* 2008;**110**:163–169.
- Diamanti-Kandarakis E. Polycystic ovarian syndrome: pathophysiology, molecular aspects and clinical implications. *Expert Rev Mol Med* 2008;**10**:e3.
- Diamanti-Kandarakis E, Charikleia DC, Kandaraki E, Economou FN. Metformin: an old medication of new fashion: evolving new molecular mechanisms and clinical implications in polycystic ovary syndrome. *Eur J Endocrinol* 2010;**162**:193–212.
- Fornés R, Ormazabal P, Rosas C, Gabler F, Vantman D, Romero C, Vega M. Changes in the expression of insulin signaling pathway molecules in endometria from polycystic ovary syndrome women with or without hyperinsulinemia. *Mol Med* 2010;**16**:129–136.
- Giudice LC. Endometrium in PCOS: implantation and predisposition to endocrine cancer. *Best Pract Res Clin Endocrinol Metab* 2006;**20**:235–244.
- Glueck CJ, Ping W, Goldenberg N, Sieve-Smith L. Pregnancy outcomes among women with polycystic ovary syndrome treated with metformin. *Hum Reprod* 2002;**17**:2858–2864.
- Hardie DG. The AMP-activated protein kinase pathway—new players upstream and downstream. *J Cell Sci* 2004;**117**:5479–5487.
- Hawley S, Gadalla A, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 2002;**51**:2420–2425.
- Holmes BF, Sparling DP, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 2005;**289**:E1071–E10716.

- Jakubowicz DJ, Seppälä M, Jakubowicz S, Rodriguez-Armas O, Rivas-Santiago A, Koistinen H, Koistinen R, Nestler JE. Insulin reduction with metformin increases luteal phase serum glycodelin and insulin-like growth factor-binding protein 1 concentrations and enhances uterine vascularity and blood flow in the polycystic ovary syndrome. *J Clin Endocrinol Metab* 2001;**86**:1126–1133.
- Jensterle M, Janez A, Mlinar B, Marc J, Prezelj J, Pfeifer M. Impact of metformin and rosiglitazone treatment on glucose transporter 4 mRNA expression in women with polycystic ovary syndrome. *Eur J Endocrinol* 2008;**158**:793–801.
- Karlsson HK, Hällsten K, Björnholm M, Tsuchida H, Chibalin AV, Virtanen KA, Heinonen OJ, Lönnqvist F, Nuutila P, Zierath JR. Effects of metformin and rosiglitazone treatment on insulin signaling and glucose uptake in patients with newly diagnosed type 2 diabetes: a randomized controlled study. *Diabetes* 2005;**54**:1459–1467.
- Karnieli E, Armoni M. Transcriptional regulation of the insulin-responsive glucose transporter GLUT4 gene: from physiology to pathology. *Am J Physiol Endocrinol Metab* 2008;**295**:E38–E45.
- Kim JY, Song H, Kim H, Kang HJ, Jun JH, Hong SR, Koong MK, Kim IS. Transcriptional profiling with a pathway-oriented analysis identifies dysregulated molecular phenotypes in the endometrium of patients with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2009;**94**:1416–1426.
- Knight JB, Eyster CA, Griesel BA, Olson AL. Regulation of the human GLUT4 gene promoter: interaction between a transcriptional activator and myocyte enhancer factor 2A. *Proc Natl Acad Sci USA* 2003;**100**:14725–14730.
- Kohan K, Carvajal R, Gabler F, Vantman D, Romero C, Vega M. Role of the transcriptional factors FOXO1 and PPARG on gene expression of SLC2A4 in endometrial tissue from women with polycystic ovary syndrome. *Reproduction* 2010;**140**:123–131.
- Kola B, Boscaro M, Rutter GA, Grossman AB, Korbonits M. Expanding role of AMPK in endocrinology. *Trends Endocrinol Metab* 2006;**17**:205–215.
- Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. *J Clin Invest* 2006;**116**:1776–1783.
- Maliqueo M, Clementi M, Gabler F, Johnson M, Palomino A, Sir Peterman T, Vega M. Expression of steroid receptors and proteins related to apoptosis in endometria of women with polycystic ovary syndrome. *Fertil Steril* 2003;**80**:812–819.
- McGee SL, Hargreaves M. Transporter isoform gene. Proceedings of the Australian Physiological Society Symposium: Integrative Aspects of Human Muscle Performance Exercise and skeletal muscle glucose transporter 4 expression: molecular mechanism. *Clin Exp Pharmacol Physiol* 2006;**33**:395–399.
- Mioni R, Chiarelli S, Xamin N, Zuliani L, Granzotto M, Mozzanega B, Maffei P, Martini C, Blandamura S, Siculo N et al. Evidence for the presence of glucose transporter 4 in the endometrium and its regulation in polycystic ovary syndrome patients. *J Clin Endocrinol Metab* 2004;**59**:4089–4096.
- Mora S, Pessin JE. The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter. *J Biol Chem* 2000;**275**:16323–16328.
- Morin-Papunen L, Rantala AS, Unkila-Kallio L, Tiitinen A, Hippelainen M, Perheentupa A, Tinkanen H, Bloigu R, Puukka K, Ruokonen A et al. Metformin improves pregnancy and live-birth rates in women with polycystic ovary syndrome (PCOS): a multicenter, double-blind, placebo-controlled randomized trial. *J Clin Endocrinol Metab* 2012;**97**:1492–1500.
- Mozzanega B, Mioni R, Granzotto M, Chiarelli S, Xamin N, Zuliani L, Siculo N, Marchesoni D, Vettor R. Obesity reduces the expression of GLUT4 in the endometrium of normoinsulinemic women affected by the polycystic ovary syndrome. *Ann N Y Acad Sci* 2004;**1034**:364–374.
- Murgia M, Jensen TE, Cusinato M, Garcia M, Richter E, Schiaffino S. Multiple signalling pathways redundantly control glucose transporter GLUT4 gene transcription in skeletal muscle. *J Physiol* 2009;**587**:4319–4327.
- Nestler JE. Metformin for the treatment of the polycystic ovary syndrome. *N Engl J Med* 2008;**358**:47–54.
- Noyes R, Hertig A, Rock J. Dating the edometrial biopsy. *Fertil Steril* 1950;**1**:3–5.
- Palomba S, Russo T, Orio F Jr, Falbo A, Manguso F, Cascella T, Tolino A, Carmina E, Colao A, Zullo F. Uterine effects of metformin administration in anovulatory women with polycystic ovary syndrome. *Hum Reprod* 2006;**21**:457–465.
- Palomba S, Falbo A, Zullo F, Orio F. Evidence-based and potential benefits of metformin in the polycystic ovary syndrome: a comprehensive review. *Endocr Rev* 2009;**30**:1–50.
- Rivero R, Garin C, Ormazabal P, Silva A, Carvajal R, Gabler F, Romero C, Vega M. Protein expression of PKCZ (protein kinase C Zeta), Munc18c, and Syntaxin-4 in the insulin pathway in endometria of patients with polycystic ovary syndrome (PCOS). *Reprod Biol Endocrinol* 2012;**10**:17.
- Rosas C, Gabler F, Vantman D, Romero C, Vega M. Levels of Rabs and WAVE family proteins associated with translocation of GLUT4 to the cell surface in endometria from hyperinsulinemic PCOS women. *Hum Reprod* 2010;**25**:2870–2877.
- Shaw RJ, Lamia K, Vasquez D, Seung-Hoi Koo, Bardeesy N, Depinho R, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 2005;**310**:1642–1646.
- Tang T, Lord JM, Norman RJ, Yasmin E, Balen AH. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, Dchiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Database Syst Rev* 2012:CD003053. doi:10.1002/14651858.CD003053.pub5.
- Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL. Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem* 1998;**273**:14285–14292.
- The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Hum Reprod* 2004;**19**:41–47.
- Von Wolff M, Ursel S, Hahn U, Steldinger R, Strowitzki T. glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy. *J Clin Endocrinol Metab* 2003;**88**:3885–3892.
- Zhai J, Liu CX, Tian ZR, Jiang QH, Sun YP. Effects of metformin on the expression of GLUT4 in endometrium of obese women with polycystic ovary syndrome. *Biol Reprod* 2012;**87**:29.
- Zhang L, Liao Q. Effects of testosterone and metformin on glucose metabolism in endometrium. *Fertil Steril* 2010;**93**:2295–2298.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001;**108**:1167–1174.
- Zorzano A, Palacín M, Gumà A. Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. *Acta Physiol Scand* 2005;**183**:43–58.