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Downregulation of autophagy gene expression in endometria from women with polycystic ovary syndrome

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Abstract

Autophagy, a process of controlled cellular self-digestion, could be involved in cyclic remodeling of the human endometrium. We investigated endometrial mRNA expression of 23 autophagy-related (ATG) genes and transcription factors in healthy controls (n = 12) and anovulatory polycystic ovary syndrome (PCOS) patients (n = 24), as well as in their subgroup (n = 12) before and after metformin treatment. The mRNA levels of transcription factor forkhead box protein O1 (FOXO1) and several molecules involved in autophagosome formation (ATG13, RB1-inducible coiled-coil 1), autophagosome nucleation (ATG14, beclin 1, SH3-domain GRB2-like endophilin B1), autophagosome elongation (ATG3, ATG5, γ-aminobutyric acid receptor-associated protein - GABARAP), and delivery of ubiquitinated proteins to autophagosomes (sequestosome 1), were significantly reduced in anovulatory PCOS compared to healthy endometrium. Free androgen index, but not free estrogen index, insulin levels, or BMI, negatively correlated with the endometrial expression of ATG3, ATG14, and GABARAP in PCOS patients. Treatment of PCOS patients with metformin (2 g/day for 3 months) significantly increased the endometrial mRNA levels of FOXO1, ATG3, and UV radiation resistance-associated gene. These data suggest that increased androgen availability in PCOS is associated with metformin-sensitive transcriptional downregulation of endometrial autophagy.

Keywords: polycystic ovary syndrome; endometrium; autophagy; autophagy-related genes; free androgen index

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine/metabolic disorder in women of reproductive age, characterized by hyperandrogenism, chronic anovulation, and/or polycystic ovaries (Conway et al., 2014). It is frequently accompanied by insulin resistance/hyperinsulinemia and obesity, resulting in higher risk for metabolic syndrome and type II diabetes (Palomba et al., 2015). Prolonged exposure to unopposed estrogen, androgens, and insulin alters endometrial homeostasis by disturbing the ovarian hormonal milieu-dependent cycling process of proliferation, differentiation, and apoptotic cell death, leading to reduced endometrial receptivity and increased risk for endometrial hyperplasia and adenocarcinoma (Shang et al., 2012; Tokmak et al., 2014). While the balance between endometrial cell proliferation and apoptosis in PCOS seems to be shifted towards the former (Avellaira et al., 2006; Gonzalez et al., 2012; Kim et al., 2009; Villavicencio et al., 2007; Yan et al., 2012), the underlying mechanisms, as well as related therapeutic targets, are still insufficiently defined.

Macroautophagy (hereafter autophagy) is a cellular self-digestion process which begins by sequestration of the cytoplasm within a unique compartment termed the phagophore (Yang and Klionsky 2010). After nucleation and elongation, phagophore matures into a double-membrane autophagosome that fuses with the lysosome, allowing degradation of the cargo (Yang and Klionsky 2010). Autophagy is initiated by post-translational modifications of autophagy-related (ATG) proteins, as well as transcriptionally through activation of various transcription factors, including forkhead box protein O (FOXO) family members FOXO1/3 and activating transcription factor 4 (ATF4) (Feng et al., 2015). Autophagy is involved in many physiological and pathological processes (Jiang and Mizushima 2014; Klionsky and Codogno 2013),

contributing to cellular maintenance and acting as a survival response to nutrient deprivation and other forms of cellular stress (Parzych and Klionsky 2014). On the other hand, autophagy can also promote cell death via excessive self-digestion and degradation of essential cellular constituents, as well as through the induction of apoptosis (Fitzwalter and Thorburn 2015; Scarlatti et al., 2009). The ATG gene beclin 1 (*BECN1*) is monoallelically deleted in 40-75% of cases of human ovarian, breast, and prostate cancer (Futreal et al., 1992; Gao et al., 1995; Russell et al., 1990), and its heterozygous disruption increases the frequency of spontaneous malignancies in mice (Qu et al., 2003), thus suggesting a tumor suppressor role of autophagy. Interestingly, recent studies also implicate autophagy in the regulation of ovarian and endometrial physiology. Autophagy has been linked to apoptotic cell death induction in ovarian granulosa and luteal cells during follicular atresia and corpus luteum regression in rats (Choi et al., 2011; Choi et al., 2010). Moreover, it has been reported that autophagy is induced in human endometrial glandular cells during the secretory phase of the menstrual cycle, possibly contributing to caspase-dependent apoptotic cell death via an increase in the BAX/BCL2 ratio (Choi et al., 2012). Autophagy genes γ-aminobutyric acid receptor-associated protein (GABARAP)-like 1 (*GABARAPL1*) and *GABARAPL3* are downregulated during the late-secretory phase of the menstrual cycle, in which the implantation window is closed and the endometrial tissue returns to the non-receptive phenotype (Tseng et al., 2010). Interestingly, a recent study demonstrated that the levels of DNA damage-regulated autophagy modulator protein 2 during controlled ovarian hyperstimulation were significantly decreased in ovarian granulosa cells of PCOS patients (Dai and Lu 2012), indicating an autophagic dysfunction in this disorder. In addition, dysregulation of autophagy was observed in the endometrial cells of patients and mice with endometriosis

(Allavena et al., 2015; Mei et al., 2015; Ruiz et al., 2016), another condition characterized by estrogen-dependent hyperproliferation of endometrial cells (Aznaurova et al., 2014).

Metformin is an insulin-sensitizing agent that has been increasingly used in the treatment of PCOS for regulating menstrual cycles, improving clinical signs of hyperandrogenism, inducing ovulation, improving pregnancy rates/outcomes, ameliorating metabolic syndrome, and reversing endometrial hyperplasia (Nathan and Sullivan 2014; Shafiee et al., 2014). The beneficial metabolic effects of metformin are mainly mediated by the activation of the intracellular energy sensor adenosine monophosphate-activated protein kinase (AMPK), a heterotrimeric enzyme that regulates cellular energy homeostasis by increasing glucose uptake and inhibiting lipid synthesis (Hardie et al., 2012). AMPK activation by metformin also causes inhibition of the main autophagy suppressor mammalian target of rapamycin (mTOR) (Kim and Guan 2015), leading to autophagy induction-dependent proliferation block and cell death in different cancer cell lines (Sesen et al., 2015; Shi et al., 2012; Tomic et al., 2011; Wang et al., 2015), including endometrial adenocarcinoma cells (Takahashi et al., 2014). The in vitro treatment with metformin has recently been found to reduce mTOR activity in the endometrial tissue from PCOS patients (Li et al., 2015a), thus suggesting the drug's potential to trigger autophagic response in PCOS endometrium.

Since the induction of autophagy is apparently involved in cyclic remodeling of normal endometrium, we hypothesized that endometrial autophagy might be disturbed during anovulatory cycles in PCOS, as well as possibly influenced by metformin treatment. To test these assumptions, we examined the endometrial expression of various autophagy-regulating genes in

anovulatory PCOS patients and normally ovulating non-PCOS controls, as well as in PCOS patients before and after metformin therapy.

2. Material and methods

2.1 Subjects and ethics statement

All subjects were recruited prospectively from infertile women who attended the IVF unit of the Clinic for Gynecology and Obstetrics, Clinical Center of Serbia (Belgrade, Serbia). Control group consisted of 12 women with tubal factor or male infertility, with regular menstrual cycles, normal hormonal status, no clinical or biochemical signs of hyperandrogenism, and normal ovarian morphology on ultrasound, who underwent endometrial biopsy to exclude luteal phase defect. The PCOS group included 24 women in which the diagnosis of PCOS was established according to Rotterdam consensus (Rotterdam 2004), requiring two of the following three criteria: oligo/anovulation, clinical or biochemical hyperandrogenism, and polycystic ovaries on ultrasound (at least 12 follicles of 2-9 mm in diameter in one ovary or an ovarian volume >10 cm^3). Inclusion criteria for both groups were age between 20 and 40 yrs, and body mass index (BMI) between 18 and 40. Exclusion criteria were any acute or chronic illness (including PCOS-unrelated causes of androgen excess such as congenital adrenal hyperplasia, Cushing's syndrome, or an androgen-secreting tumor), abnormal FSH, smoking, alcohol consumption, taking oral contraceptives or other medications, undergoing caloric restriction, or taking regular physical exercise. Caloric restriction and physical exercise were considered as exclusion criteria due to their possible effects on autophagy induction in humans (Castagnaro et al., 2016; Schwalm et al.,

2015). The study was conducted after obtaining informed consent from all subjects, and approved by the Ethics Committee of the School of Medicine, University of Belgrade.

2.2 Determination of BMI and hormonal status

BMI was calculated by dividing weight (kg) by the squared value of height in meters. Samples of venous blood were collected in the morning, after an overnight fast, and the serum was obtained by centrifugation. Serum samples were stored at -80 °C. Radioimmunoassay kits were used to determine the concentrations of insulin, estradiol, total testosterone (INEP Zemun, Belgrade, Serbia), and androstenedione (ZenTech, Angleur, Belgium). Insulin secretory response to glucose was measured by oral glucose tolerance test (OGTT) 2 h after 75-g load of glucose. The patients were considered hyperinsulinemic when their insulin levels were 2 standard deviations over the mean of the control group (Fornes et al., 2010). To estimate nonbound testosterone and estradiol activity, the free androgen index (FAI) and free estrogen index (FEI), respectively, were calculated as follows: FAI = 100 x testosterone (nmol/L)/SHBG (nmol/L); FEI = 100 × estradiol (nmol/L)/272.1 × SHBG (nmol/L).

2.3 Metformin treatment

PCOS patients received metformin (Glucophage®; Merck Serono, Darmstadt, Germany) orally for 3 months (2 g/day). The patients were followed for the common adverse events associated with metformin therapy, such as nausea, vomiting, diarrhea, abdominal pain, and loss of appetite. While all PCOS patients (n = 24) were initially enrolled for metformin treatment, post-treatment endometrial biopsy was performed only in 12 patients, as others did not adhere to treatment due

to adverse effects (n = 2), or were lost to follow-up (n = 10). The patients who completed the study did not report any significant side effects.

2.4 Endometrial sample collection

As previous results indicated that autophagic activity is significantly higher in secretory compared to proliferative phase of the menstrual cycle (Choi et al., 2012), the endometrial samples from control subjects were collected at mid-secretory phase (cycle days 20-22). In PCOS group, the endometrial tissue was collected at the same time-point (days 20-22) after spontaneous menstruation in patients with oligomenorrhea (n = 18) or progesterone-induced withdrawal bleeding in those with amenorrhea (n = 6). Endometrial samples were obtained using Pipelle endometrial aspirator, snap-frozen in liquid nitrogen, and stored at -80 ^{o}C for reverse transcriptase-polymerase chain reaction assay (RT-PCR). Based on histological analysis (Noyes et al., 1975), the mid-secretory phase was confirmed in all control endometrium samples, while proliferative or disordered proliferative endometrium was observed in PCOS patients. No endometrial hyperplasia was observed.

2.5 RT-PCR analysis of autophagy genes

Total RNA from endometrial tissue was extracted using TRIZOL reagent (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Approximately 1 μg of RNA was used in the reverse transcription reaction using M-MLV reverse transcriptase with random hexamers (both from ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Real-time RT-PCR was performed in a Realplex^2 Mastercycler

(Eppendorf, Hamburg, Germany), using 96-well reaction plates, TaqMan Universal PCR Master Mix, and TaqMan primers/probes (all from ThermoFisher Scientific, Waltham, MA) for ATG3, ATG4B, ATG5, ATG7, ATG10, ATG12, ATG13, ATG14, activating transcription factor 4 (ATF4), autophagy/beclin-1 regulator 1(AMBRA1), BECN1, SH3-domain GRB2-like endophilin B1 (SH3GLB1/Bif-1), BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), RB1-inducible coiled-coil 1 (RB1CC1/FIP200), forkhead box protein O1 (FOXO1), FOXO3, GABARAP, RUN domain and cysteine-rich domain containing beclin 1-interacting protein (RUBCN), sequestosome 1 (SQSTM1), unc-51 like autophagy activating kinase 1 (ULK1), UV radiation resistance-associated gene (UVRAG), phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3/Vps34), B-cell lymphoma 2 (BCL2), and house-keeping genes 18s ribosomal RNA (RN18S) and β2-microglobulin (B2M). The reaction conditions were as recommended by the manufacturer, and all assays were performed in triplicates. The geometric mean of threshold cycle (Ct) values of RN18S and B2M were subtracted from Ct values of target genes to obtain ΔCt, and the relative gene expression was calculated as $2^{-\Delta Ct}$. The data are presented in arbitrary units (a.u.).

2.6 Statistical analysis

Since Shapiro-Wilk test revealed that the distribution of values for most of the variables did not conform to normality, the statistical significance of the differences between control and PCOS group was analyzed by Mann-Whitney test. The differences in gene expression before and after metformin treatment in PCOS group were evaluated by Wilcoxon signed-rank test. The correlations between different parameters were analyzed using Spearman correlation test and the

line fitting was done using GraphPad Prism software 5 (GraphPad Software Inc., La Jolla, CA). The differences in frequencies was analyzed by Fisher's exact test. The significance level was set at $p < 0.05$.

3. Results

3.1 Clinical characteristics and hormonal profile of PCOS patients

The demographics and baseline clinical characteristics of PCOS and control group are presented in Table 1. No significant differences were observed in age and BMI, with 25 % and 46 % women in control and PCOS group, respectively, being overweight or obese ($p = 0.292$). The concentrations of estradiol and FEI values did not significantly differ between the two groups. Expectedly, PCOS patients had higher blood concentrations of testosterone, androstenedione, lower concentrations of SHBG, and increased FAI values, thus confirming the increase in androgen activity. Based on clinical appearance and/or androgen levels (testosterone and/or androstenedione above the upper reference value), all PCOS patients were hyperandrogenic. It should be noted that although androstenedione measurement is not usually a part of the mainstream diagnostic criteria for biochemical hyperandrogenemia in PCOS, its inclusion has been proposed to aid PCOS diagnosis (Georgopoulos et al., 2014). Both control and PCOS patients had normal fasting insulin and glucose levels, remaining euglicemic during OGTT (data not shown). However, in accordance with previous studies (Fornes et al., 2010; Saxena et al., 2011), PCOS, unlike conrtol group, displayed a significantly higher insulin levels during OGTT, with 29% of patients characterized as hyperinsulinemic ($p = 0.009$).

3.2 Endometrial expression of autophagy genes in PCOS

The quantitative RT-PCR analysis demonstrated that the levels of mRNA encoding several proteins involved in autophagy induction (ATG3, ATG5, ATG13, ATG14, BECN1, GABARAP, RB1CC1, SH3GLB1, SQSTM1), as well as transcription factor FOXO1, were significantly reduced in the endometrial tissue of PCOS patients compared to non-PCOS controls (Fig. 1). The mRNA levels of other autophagy regulators, including BECN1-binding apoptosis/autophagy inhibitor BCL2 (Pattingre et al., 2005), did not significantly differ between the two groups (Table 2). However, it should be noted that, with the exception of autophagy inhibitor RUBCN and transcription factor ATF4, there was a general trend towards a decrease of autophagy gene expression in PCOS patients (Table 2). No correlation was observed between the endometrial expression of autophagy genes that were downregulated in PCOS and patients' BMI or blood concentrations of testosterone, androstenedione, estradiol, or insulin (Table 3). On the other hand, a significant negative correlation was found between FAI and endometrial mRNA levels of ATG3, ATG14, and GABARAP in PCOS (Table 3 and Fig. 2). The expression of other autophagy-regulating genes that were reduced in PCOS did not significantly correlate with FAI (Table 3).

3.3 Effect of metformin on endometrial expression of autophagy genes in PCOS

The treatment of PCOS patients with metformin for 3 months significantly increased the endometrial levels of ATG3, FOXO1, and UVRAG mRNA (Fig. 3). The endometrial mRNA expression of other ATG genes/transcription factors did not significantly change, despite an apparent tendency towards the increase of ATG13, BECN1, GABARAP, PIK3C3, and RB1CC1

mRNA levels after metformin treatment (Table 4). While BMI, androstenedione, and SHBG concentrations were not significantly altered after metformin treatment (data not shown), those of testosterone were significantly reduced (median 2.7 vs. 2.2 nmol/L before and after metformin, respectively; $p = 0.046$), and FAI values displayed a tendency towards a decrease (4.6 vs. 2.9; $p = 0.116$). The insulin levels during OGTT did not significantly change in the whole group, although they returned to normal values in the two hyperinsulinemic patients (data not shown). Also, three patients (25 %) regained normal menstruation and ovulation following the treatment with metformin ($p = 0.231$).

4. Discussion

The present study, to the best of our knowledge, is the first to demonstrate that the endometrial expression of mRNA encoding a range of proteins involved in different phases of autophagy induction is reduced in anovulatory PCOS compared to normally cycling women. In addition, we show that the treatment with metformin, which has some beneficial effect in PCOS, can partly counteract the observed downregulation of autophagy gene expression in endometrial tissue of PCOS patients.

The molecules whose mRNA was downregulated in PCOS endometrium included those required for the initiation of phagophore formation (ATG13, RB1CC1), phagophore nucleation (ATG14, BECN1, SH3GLB1), phagophore elongation (ATG3, ATG5, GABARAP), and delivery of ubiquitinated protein aggregates to autophagosomes (SQSTM1) (Parzych and Klionsky 2014). The expression of these genes has previously been found essential for optimal autophagy induction in mammalian cells (Bjorkoy et al., 2005; Ganley et al., 2009; Itakura et al., 2008;

Kuma et al., 2004; Murrow et al., 2015; Qu et al., 2003; Takahashi et al., 2007; Weidberg et al., 2010), indicating that their coordinated downregulation might indeed affect endometrial autophagy in PCOS. Since some of these genes (*ATG3*, *ATG5*, *ATG14*) are under direct control of the transcription factor FOXO1 (Sengupta et al., 2009; Xiong et al., 2012; Xu et al., 2011), their reduced expression in PCOS could be at least partly explained by the observed FOXO1 downregulation. FOXO1 can also increase autophagy through direct binding to ATG7 (Zhao et al., 2010), so attenuation of this mechanism could further contribute to autophagy dysregulation in PCOS. Transcription factors FOXO3 and ATF4 have also been implicated in the transcriptional regulation of autophagy (Feng et al., 2015), but the endometrial levels of their transcripts, despite an apparent trend towards decrease, were not significantly reduced in PCOS. However, since it has been proposed that FOXO1 can physically interact with and promote the transcriptional activity of ATF4 (Kode et al., 2012), it is plausible that reduced FOXO1-ATF4 interaction in PCOS could impair transcriptional activation of ATF4 targets ATG3, ATG5, BECN1, and SQSTM1 (B'Chir et al., 2013; Milani et al., 2009; Rouschop et al., 2010).

It has been reported that testosterone, estradiol, and insulin can modulate autophagic response in different cells/tissues in various experimental conditions (Lin et al., 2016; Lumeng and Saltiel 2006; Ma et al., 2015; Riehle and Abel 2014; Serra et al., 2013; Yang et al., 2013). In PCOS patients in the present study, only FAI, but not FEI or insulin OGTT levels, negatively correlated with the endometrial expression of ATG3, ATG14, and GABARAP, which are all required for optimal autophagy induction in mammalian cells (Itakura et al., 2008; Murrow et al., 2015; Weidberg et al., 2010). Therefore, increased androgen availability, rather then estrogen or insulin dysregulation, might contribute to the observed decrease in autophagy gene expression in PCOS endometrium. While the role of testosterone in endometrial autophagy has not been explored so

far, our hypothesis is consistent with the ability of testosterone to suppress basal autophagy in rat Sertoli cells (Ma et al., 2015), as well as castration-induced FOXO1/3-dependent autophagy in skeletal muscle of male mice (Serra et al., 2013). Accordingly, testosterone was also found to inhibit dexamethasone-induced FOXO1 transcription in rat skeletal muscle in vivo (Qin et al., 2010). It would be interesting to evaluate in further studies the direct effects of androgens on autophagy induction in normal and PCOS endometrium. It has also been shown that autophagic activity in human adipose tissue positively correlates with BMI (Jansen et al., 2012). However, the BMI values in our study did not correlate with the expression of autophagy genes in PCOS endometrium, suggesting that the observed association between BMI and autophagy might be tissue-dependent.

Recent studies indicate that PCOS patients display a lower level of activation of the intracellular sensor AMPK in the endometrium (Carvajal et al., 2013), which was particularly pronounced in endometrial hyperplasia (Li et al., 2015b). Having in mind the important role of AMPK signaling in autophagy induction (Mihaylova and Shaw 2011), these data are in accordance with the downregulation of autophagy gene expression in PCOS endometrium, observed in the present study. Moreover, the AMPK-activating drug metformin has been found to restore the activation of AMPK (Carvajal et al., 2013) and reduce the activity of its downstream target and a major autophagy repressor mTOR in endometrial cells of PCOS patients (Li et al., 2015a). Accordingly, treatment with metformin for three months in the present study increased the endometrial levels of FOXO1, ATG3, and UVRAG, which are involved in the transcriptional initiation of autophagy, autophagosome nucleation, and autophagosome-lysosome fusion, respectively (Parzych and Klionsky 2014). This ability of metformin to partly reverse the inhibition of autophagy gene expression in PCOS is consistent with the findings that AMPK can increase the

expression and/or transcriptional activity of FOXO1 and FOXO3 in different in vitro and in vivo experimental settings (Awad et al., 2014; Chiacchiera and Simone 2010; Greer et al., 2007; Nakashima and Yakabe 2007; Nystrom and Lang 2008; Sanchez et al., 2012). On the other hand, AMPK directly activates autophagy through mTOR-dependent and -independent changes in ULK1 phosphorylation status (Alers et al., 2012), so metformin could affect endometrial autophagy in PCOS independently of autophagy gene transcription. Since we have observed a reduction of testosterone levels and a tendency towards a decrease in FAI, it seems conceivable that the drug, particularly after longer treatment, might also influence endometrial autophagy in PCOS indirectly, by improving metabolic disturbances, menstrual cycle, and ovulation. Additional studies are required to assess how autophagy induction and autophagic flux (a measure of autophagy degradation capacity) are regulated by metformin in endometrium of healthy and PCOS subjects.

The reduced expression of autophagy genes in anovulatory PCOS endometrium in our study is consistent with the finding that autophagic activity is upregulated during the secretory phase of the normal cycle (Choi et al., 2012), indicating that this upregulation is at least in part transcriptional. While we did not observe any endometrial hyperplasia in our study group, it would be interesting to examine if dysregulation of autophagy, which is known for its antiproliferative/proapoptotic capacity, might contribute to the proliferative state of anovulatory endometrium and consequent risk for hyperplasia. Moreover, a recent study demonstrated that suppression of autophagy is involved in compromised endometrial receptivity in obese mice (Rhee et al., 2016). Therefore, it seems plausible to investigate if autophagic deficiency is also present in ovulatory PCOS endometrium during the window of implantation, and if it is possibly linked to the reduced endometrial receptivity in this disorder.

In addition to relatively low sample size, the main limitation of the present study is that, due to a limited amount of endometrial tissue, it investigated the expression of autophagy regulators only at the mRNA level, without assessing their protein level, the presence of autophagosomes, and autophagic flux. For example, one study demonstrated that although the endometrial mRNA expression of apoptosis/autophagy inhibitor BCL2 did not differ in control and PCOS subjects, its protein levels were significantly increased in the latter group (Maliqueo et al., 2003). In addition, the observed decrease in autophagy gene expression was relatively moderate (mean fold change 1.3-1.7), which could explain why it was not detected in previous gene array analyses of PCOS endometrial tissue, which used the usual 2/2.5 fold change threshold for a significant effect (Bellver et al., 2011; Kim et al., 2009; Qiao et al., 2008; Savaris et al., 2011). It should also be noted that, in order to reduce the risk of missing the true effects (type II error), we did not make any corrections for multiple comparisons. While preferred by some researchers, particularly for testing the previously unexplored hypotheses (Feise 2002; Rothman 1990), this approach increases the risk of detecting an effect that is not present (type I error). Therefore, it is on future studies with larger sample size to confirm endometrial autophagy downregulation in PCOS at the protein and functional level.

In conclusion, the present study suggests that the increased androgen availability in PCOS is associated with the transcriptional downregulation of endometrial autophagy, an effect that could be partly reversed by metformin treatment. However, it remains to be established if and what role autophagy might play in normal endometrial cycle, as well as in endometrial dysfunction in PCOS.

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Conflict of interest statement

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Downregulation of autophagy gene expression in endometrial tissue of PCOS patients. The mRNA levels of autophagy regulators were compared in the endometrial tissues of PCOS patients (n = 24) and control subjects (n = 12) (the lines represent median and interquartile range).

Fig. 2. Negative correlation between FAI and endometrial expression of ATG3, ATG14, and GABARAP in PCOS. The FAI values were correlated with the endometrial levels of ATG3, ATG14, and GABARAP mRNA in PCOS patients (n = 24).

Fig. 3. Metformin-mediated increase in endometrial expression of ATG3, UVRAG, and FOXO1 in PCOS. The endometrial levels of ATG3, UVRAG, and FOXO1 mRNA in PCOS patients (n = 12) were compared before and after 3 months of treatment with metformin (2 g/day).

Table 1. Clinical characteristics and hormonal profile of PCOS patients (n = 24) and control subjects (n = 12). The data are presented as median with interquartile range (* denotes a statistically significant difference). Normal reference ranges: [a]18.5-25.0, [b]0.3-3.0, [c]0.2-2.26, [d]105-217, [e]18-87, [f]5-25; [b-f]test manufacturer's data.

Variable	**Control**	**PCOS**	**p-value**
Age (years)	34 (10)	35 (9)	*0.557*
BMI (kg/m^2)[a]	22.5 (7.4)	25.3 (4.7)	*0.307*
Testosterone (nmol/L)[b]	1.7 (0.5)	2.2 (1.2)	*0.026**
Androstenedione (ng/ml)[c]	1.9 (0.6)	3.0 (2.1)	*0.001**
Estradiol (pmol/L)[d]	169.6 (224.6)	207.2 (334.9)	*0.375*
SHBG (nmol/L)[e]	51.9 (69.8)	39.9 (26.2)	*0.045**
FAI	3.4 (2.9)	4.4 (4.9)	*0.011**
FEI	1.2 (0.7)	1.9 (3.7)	*0.154*
OGTT Insulin (mIU/ml)[f]	22.8 (38.5)	46.1 (56.9)	*0.036**

Table 2. Autophagy genes not differentially expressed in PCOS vs. control subjects. The data are presented as median with interquartile range.

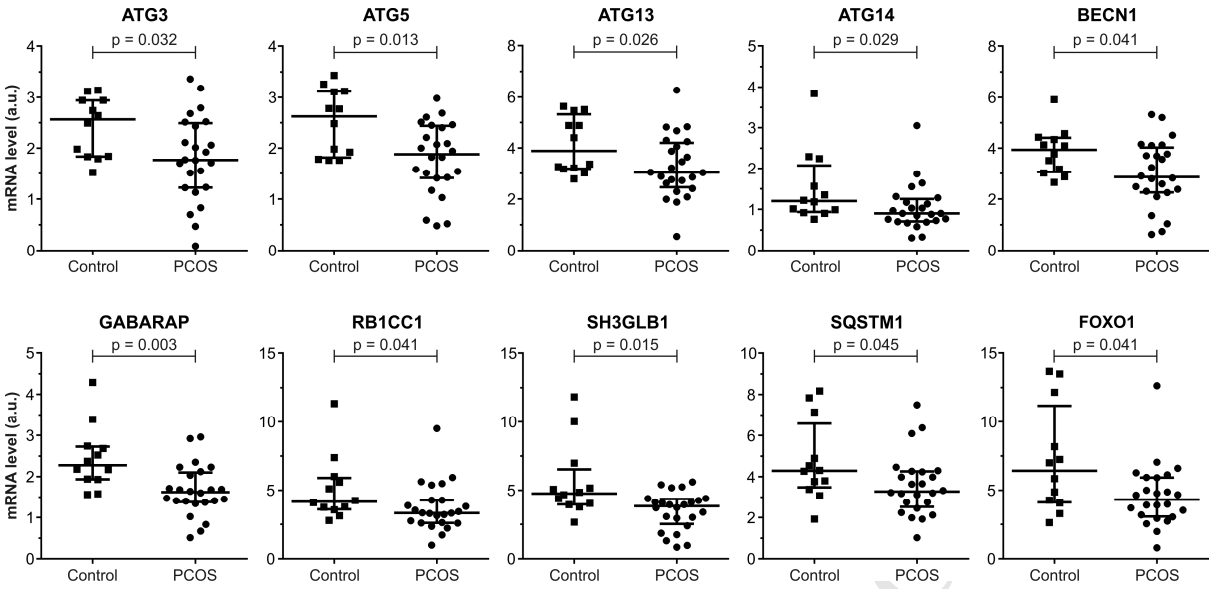
mRNA	Control (n = 12)	PCOS (n = 24)	p-value
ATG4B	8.90 (5.98)	7.57 (3.88)	*0.062*
ATG7	4.02 (2.98)	3.41 (1.41)	*0.067*
ATG10	1.02 (1.00)	0.89 (0.43)	*0.334*
ATG12	1.90 (2.60)	0.63 (1.59)	*0.078*
AMBRA1	1.53 (0.82)	1.25 (0.82)	*0.059*
BCL2	7.95 (3.12)	6.16 (5.27)	*0.233*
BNIP3	4.09 (5.69)	3.53 (2.31)	*0.146*
PIK3C3	1.85 (0.87)	1.55 (1.05)	*0.062*
RUBCN	4.24 (4.30)	4.57 (2.10)	*0.416*
ULK1	3.29 (2.27)	3.10 (1.88)	*0.379*
UVRAG	1.51 (1.13)	1.24 (0.86)	*0.177*
ATF4	2.24 (2.12)	2.39 (1.48)	*0.960*
FOXO3	1.30 (1.48)	0.67 (1.35)	*0.090*

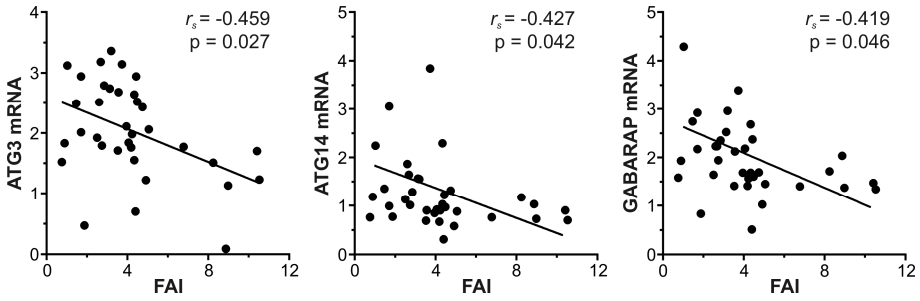
Table 3. The analysis of correlation between the mRNA levels of downregulated autophagy genes and BMI/hormonal characteristics of PCOS patients. The data are r_s values with corresponding p values in parentheses (* denotes a statistically significant difference). T - testosterone; AE - androstenedione.

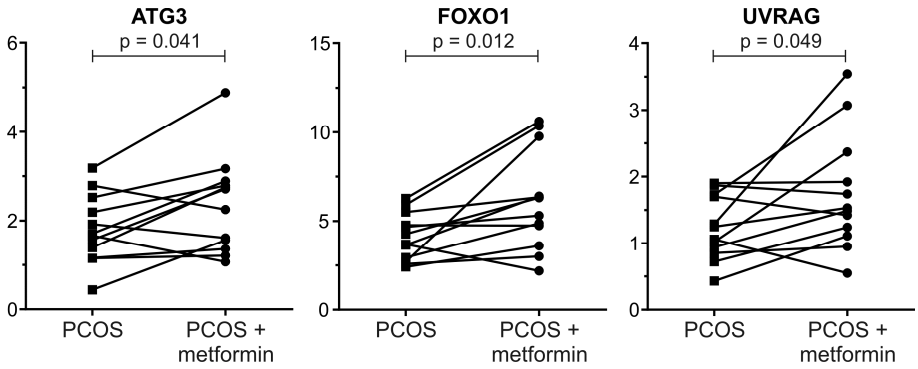
mRNA	BMI	T	AE	estradiol	FEI	FAI	insulin
ATG3	-0.334 (*0.139*)	-0.136 (*0.535*)	0.133 (*0.536*)	-0.092 (*0.684*)	-0.317 (*0.151*)	-0.459 (*0.027**)	-0.110 (*0.645*)
ATG5	0.075 (*0.746*)	-0.051 (*0.815*)	0.098 (*0.655*)	-0.068 (*0.763*)	-0.150 (*0.506*)	-0.168 (*0.444*)	0.146 (*0.539*)
ATG13	0.103 (*0.658*)	0.282 (*0.182*)	-0.009 (*0.968*)	-0.117 (*0.604*)	-0.193 (*0.391*)	-0.270 (*0.213*)	-0.211 (*0.373*)
ATG14	0.075 (*0.748*)	0.309 (*0.142*)	0.035 (*0.874*)	-0.91 (*0.687*)	-0.317 (*0.150*)	-0.427 (*0.042**)	-0.413 (*0.070)*
BECN1	-0.032 (*0.889*)	-0.017 (*0.939*)	0.088 (*0.690*)	-0.069 (*0.759*)	-0.168 (*0.456*)	-0.140 (*0.523*)	-0.054 (*0.821)*
GABARAP	-0.090 (*0.699*)	0.254 (*0.230)*	-0.031 (*0.889*)	-0.154 (*0.493*)	-0.287 (*0.196*)	-0.419 (*0.046**)	-0.383 (*0.096*)
RB1CC1	0.001 (*0.998*)	0.396 (*0.055*)	0.048 (*0.826*)	-0.034 (*0.879*)	-0.156 (*0.487*)	-0.291 (*0.178*)	-0.263 (*0.264*)
SH3GLB1	0.319 (*0.159*)	0.006 (*0.979*)	0.227 (*0.287*)	-0.062 (*0.785*)	-0.103 (*0.649*)	-0.121 (*0.582*)	-0.123 (*0.604*)
SQSTM1	-0.115 (*0.620*)	0.301 (*0.153*)	0.207 (*0.344*)	0.050 (*0.824*)	-0.141 (*0.532*)	-0.304 (*0.158*)	-0.421 (*0.064)*
FOXO1	0.090 (*0.699*)	-0.050 (*0.820*)	0.161 (*0.453*)	-0.186 (*0.408*)	-0.306 (*0.166*)	-0.302 (*0.161*)	-0.233 (*0.323*)

Table 4. Autophagy genes not differentially expressed in PCOS patients (n = 12) before and after metformin treatment. The data are presented as median with interquartile range. MTF, metformin.

mRNA	PCOS	PCOS + MTF	p-value
ATG4B	7.60 (3.48)	8.18 (9.4)	*0.308*
ATG5	1.91 (1.02)	2.32 (1.23)	*0.308*
ATG7	3.22 (1.36)	3.86 (3.41)	*0.239*
ATG10	0.77 (0.47)	1.03 (0.50)	*0.239*
ATG12	1.10 (2.49)	0.88 (1.60)	*0.754*
ATG13	2.77 (1.26)	3.87 (2.25)	*0.136*
ATG14	0.84 (0.50)	1.03 (0.83)	*0.480*
AMBRA1	1.09 (0.71)	1.45 (0.67)	*0.182*
BCL2	4.70 (4.78)	5.34 (3.71)	*0.814*
BECN1	2.72 (1.50)	3.60 (1.78)	*0.136*
BNIP3	3.29 (1.63)	3.35 (2.21)	*0.722*
GABARAP	1.47 (0.39)	1.87 (1.34)	*0.099*
PIK3C3	1.35 (1.10)	1.86 (1.32)	*0.136*
RB1CC1	2.87 (1.32)	3.90 (2.93)	*0.099*
RUBCN	4.04 (2.21)	4.21 (3.70)	*0.239*
SH3GLB1	3.01 (2.21)	3.69 (1.94)	*0.308*
SQSTM1	3.17 (1.59)	3.75 (2.33)	*0.388*
ULK1	2.62 (2.01)	2.97 (2.30)	*0.308*
ATF4	2.56 (1.68)	2.74 (1.59)	*0.754*
FOXO3	0.59 (1.14)	0.47 (1.15)	*0.937*







- Endometrial expression of several autophagy-related genes is reduced in PCOS
- Free androgen index negatively correlates with autophagy gene expression in PCOS
- Metformin treatment partly restores autophagy gene expression in PCOS endometrium