Lifestyle intervention up-regulates gene and protein levels of molecules involved in insulin signaling in the endometrium of overweight/obese women with polycystic ovary syndrome

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STUDY QUESTION: Does lifestyle intervention aiming at weight loss influence endometrial insulin signaling in overweight/obese women with polycystic ovary syndrome (PCOS)?

SUMMARY ANSWER: Lifestyle intervention up-regulates, both at the mRNA and protein levels, components of insulin signaling in the endometrium of overweight/obese PCOS women, in relation to an improved menstrual pattern.

WHAT IS KNOWN ALREADY: PCOS is a multifactorial endocrine disorder diagnosed by two of the following three criteria: chronic anovulation, hyperandrogenism and polycystic ovaries. Many women with PCOS also have insulin resistance and obesity. The syndrome is furthermore associated with endometrial cancer and possible alterations in endometrial function and receptivity.

STUDY DESIGN, SIZE, DURATION: This study assessed the effects of a combined diet and exercise lifestyle intervention for 3 months.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A group of 20 overweight/obese PCOS women with anovulation, hyperandrogenism and polycystic ovaries were subjected to a combined diet and exercise program for 3 months. Ten body mass index (BMI)-matched regularly menstruating overweight/obese controls, nine normal-weight PCOS women and ten normal-weight controls were also included in the study. In an academic clinical setting, women were examined in mid-follicular phase for endocrine assessment and determination of endometrial levels of mRNA and immunohistochemical staining of insulin signaling molecules (the insulin receptor, insulin receptor substrate-1 (IRS1) and glucose transporter (GLUT) 1 and 4).

MAIN RESULTS AND THE ROLE OF CHANCE: Women with PCOS exhibited lower levels of IRS1 \((P < 0.01)\) and GLUT4 \((P < 0.01)\) mRNA in their proliferative endometrium than BMI-matched controls. After lifestyle intervention, weight loss averaged 4.7% and the menstrual pattern improved in 65% of the overweight/obese women with PCOS. Levels of IRS1 \((P < 0.01)\) and GLUT1 \((P < 0.05)\) mRNA were significantly up-regulated in the endometrium of those women with improved menstrual function, as were the protein expression levels of pY612IRS1 (the activated IRS1 form, \(P < 0.05\)), pS312IRS1 (the inhibitory form of IRS1, \(P < 0.05\)) and GLUT1 \((P < 0.05)\). Improvement in the menstrual function of women in the obese/overweight group following the lifestyle intervention was positively correlated with the increase in the endometrial level of IRS1 mRNA \((r = 0.63, P < 0.01)\) and negatively correlated with the change in BMI \((r = -0.50, P < 0.05)\).

LIMITATIONS, REASONS FOR CAUTION: The number of women in each group was limited, although the power calculation indicated that the number of patients subjected to the lifestyle intervention was sufficient.

WIDER IMPLICATIONS OF THE FINDINGS: We propose that up-regulation of endometrial IRS1 and GLUT1 in overweight/obese women with PCOS following lifestyle intervention improves the glucose homeostasis and thereby restores the functioning of the endometrium in these women.
**Introduction**

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of fertile age and a major cause of anovulatory infertility (Goodarzi et al., 2011). The syndrome is multifactorial and characterized by hyperandrogenism, chronic anovulation and polycystic ovaries. In many cases it is also associated with insulin resistance and hyperinsulinemia, which contribute to the hyperandrogenism and ovarian dysfunction (Diamanti-Kandarakis and Dunaif, 2012). Furthermore, insulin resistance is linked to abdominal obesity and an elevated long-term risk of developing diabetes mellitus, cardiovascular disease and/or dyslipidemia (Diamanti-Kandarakis and Dunaif, 2012).

Although the insulin resistance associated with PCOS is independent of obesity, obese patients often exhibit more aggravated resistance, indicating that these two conditions interact (Dunaif et al., 1987; Legro et al., 1999; Moran et al., 2010). The mechanisms underlying insulin resistance remain unclear, but studies on PCOS, both in vitro and in vivo, have provided evidence of abnormalities in post-receptor signaling. Several investigations on cultured skin fibroblasts, as well as on fat and skeletal muscle, indicate impairment of metabolic signaling and polycystic ovaries. In many cases it is also associated with anovulatory infertility (Goodarzi et al., 2011). The syndrome is based on the presence of all three criteria formulated by the Rotterdam Consensus, i.e. oligo- or anovulation, hyperandrogenism and detection of polycystic ovaries by ultrasound (Goodarzi et al., 2011). Additional inclusion criteria were an age of 18–40 years, absence of pregnancy or lactation during the preceding 12 months, and no hormonal treatment for at least 3 months. The exclusion criteria were presence of current disease or endocrine disorder other than PCOS or an eating disorder, use of nicotine, or ongoing medication, including insulin-sensitizing drugs.

Twenty women with PCOS and a body mass index (BMI) >27 (the OB-PCOS group), 10 healthy overweight/obese women (OB-C), 9 women with PCOS and of normal weight (BMI 18.5–25) (NW-PCOS) and 10 healthy women matched in BMI to this last group (NW-C) were chosen as participants.

**Materials and Methods**

**Subjects**

From 2008 to 2012 women with PCOS were recruited by clinical referral to the Women’s Health Research Unit, Karolinska University Hospital (Stockholm, Sweden) or advertisement in local newspapers and initially screened by a nurse employing a standardized telephone questionnaire, after which those appearing suitable were scheduled for a visit to a gynecologist. The diagnosis of PCOS was based on the presence of all three criteria formulated by the Rotterdam Consensus, i.e. oligo- or anovulation, hyperandrogenism and detection of polycystic ovaries by ultrasound (Goodarzi et al., 2011). Additional inclusion criteria were an age of 18–40 years, absence of pregnancy or lactation during the preceding 12 months, and no hormonal treatment for at least 3 months. The exclusion criteria were presence of current disease or endocrine disorder other than PCOS or an eating disorder, use of nicotine, or ongoing medication, including insulin-sensitizing drugs.

Twenty women with PCOS and a body mass index (BMI) >27 (the OB-PCOS group), 10 healthy overweight/obese women (OB-C), 9 women with PCOS and of normal weight (BMI 18.5–25) (NW-PCOS) and 10 healthy women matched in BMI to this last group (NW-C) were chosen as participants.

**Ethical approval**

All women gave their written informed consent and the study was approved by the local ethics committee (IRB 2008/865-32).

**The intervention**

For 3 months, the OB-PCOS women followed individualized programs of dietary restriction and physical activity designed to reduce their weight. In connection with monthly visits, these women were closely supervised by a dietician (Å.N.), who recommended a diet high in protein and low in carbohydrates (40% carbohydrates, 30% fat and 30% proteins) (Moran et al., 2003; Larsen et al., 2010). A strict regimen of three main meals and two or three snacks was also introduced. Food intake was self-reported and corrected when necessary.

The recommendations concerning exercise were based on each individual’s situation, interest and experience. All were given membership in a local gym and prescribed aerobic activity for 45 min 2 or 3 times each week, gradually increasing to a moderate level. Participation was recorded by the gym staff.

**Experimental design**

All of the women in the OB-PCOS group were initially anovulatory, exhibiting either amenorrhea (no bleeding for the past 3 months) or oligomenorrhea (5–9 periods during the past year at intervals exceeding 6 weeks). Both before and following the intervention, they were examined in mid-follicular phase (days 6–8 of the menstrual cycle), determined by spontaneous menstruation or by induced bleeding after a prostaglandin test. After fasting overnight, they came in the morning to the Women’s Health Research Unit at the Karolinska University Hospital, to undergo a general health control, including determination of blood pressure, weight, height and waist/hip ratio (WHR). A resting blood sample was collected from a peripheral vein at 8:00 a.m. and
the serum was subsequently separated by centrifugation and stored at −70°C pending analysis for hormones, binding proteins and glucose.

Gynecological examinations, including transvaginal ultrasound, designed to determine endometrial thickness and ovarian parameters, were all performed by the same investigator (A.L.H.) using Sonoline SL-250 equipment (Siemens Healthcare Diagnostics). In addition, endometrial biopsies were collected under local anesthesia using an endometrial suction curette (Pipet Curet, CooperSurgical, USA).

During the intervention period, menstrual bleeding was recorded in a diary and ovulation was confirmed on the basis of an elevated serum level of progesterone (>17 nmol/l) during the luteal phase of the menstrual cycle. Following the intervention, menstrual function was evaluated, with a shift from amenorrhea to oligomenorrhea/regular menstruation or from oligomenorrhea to regular menstruation being defined as improvement.

The women in the OB-C and NW-C groups (all with regular menstrual cycles) and the NW-PCOS group (all anovulatory) were subjected once in mid-follicular phase to the same investigations including endometrial biopsy as described above.

Real-time PCR
Levels of mRNA quantified by real-time PCR, using Taqman Gene Expression Assays (with FAM as the reporter dye) and Taqman Gene Expression Master Mix (Applied Biosystems), were normalized to the level of endogenous control ribosomal protein L13a (RPL13A) mRNA. The following assays were employed: Hs00961554_m1 for the insulin receptor (INSR), Hs00178563_m1 for IRS1, Hs01573827_g1 for GLUT4, Hs00892681_m1 for GLUT1 and Hs01926559_g1 for RPL13A. The reactions were run in a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). The PCR efficiency with all amplicons was 90–100% and all determinations were performed in triplicate.

Immunohistochemical analysis
Monoclonal antibodies against the N-terminus of the α subunit of INSR (diluted 1:50), the β subunit of INSR (1:100), GLUT4 (1:500) and GLUT1 (1:400) (LifeSpan Biosciences, Seattle, WA, USA) and polyclonal antibodies against IRS1 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the activated tyrosine612-phosphorylated form of IRS1 (pY612IRS1) (1:2500) (Abcam, Cambridge, UK) and the inhibitory serine312-phosphorylated form of IRS1 (pS312IRS1) (1:500) (MyBio Source, San Diego, CA, USA) were purchased from the sources indicated.

Using the dilutions above, immunostaining was performed on 5-μm sections of formalin-fixed, paraffin-embedded endometrial tissue as described previously (Dubicke et al., 2010). Immunohistochemical staining was evaluated by conventional light microscopy at a magnification × 200. The intensity and distribution were manually and independently evaluated in luminal epithelium, glandular epithelium and stromal cells by two observers blinded for group and order of sampling using the semi-quantitative manual scoring on a four-point scale: (−), negative; (+), faint; (++), moderate; and ++, strong.

### Table I
Comparison of the clinical characteristics, endocrinological variables and relative levels of mRNA encoding insulin signaling molecules in overweight/obese women with PCOS (OB-PCOS), overweight/obese controls (OB-C), normal-weight PCOS (NW-PCOS) and in healthy normal-weight controls (NW-C).

<table>
<thead>
<tr>
<th></th>
<th>OB-PCOSa</th>
<th>OB-C</th>
<th>NW-PCOS</th>
<th>NW-C</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.6 ± 4.9</td>
<td>33.5 ± 3.4</td>
<td>28.3 ± 5.7</td>
<td>28.1 ± 5.4</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>37.0 ± 5.3</td>
<td>34.0 ± 5.1</td>
<td>21.6 ± 2.3</td>
<td>21.5 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>4.6 ± 1.6</td>
<td>4.9 ± 2.0</td>
<td>8.6 ± 3.1</td>
<td>4.7 ± 1.1</td>
<td>b, d**</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>5.1 ± 1.1</td>
<td>7.3 ± 2.9</td>
<td>6.9 ± 2.1</td>
<td>6.7 ± 2.4</td>
<td>a**, b, c*</td>
</tr>
<tr>
<td>Total testosterone (nmol/l)</td>
<td>1.3 ± 0.6</td>
<td>0.7 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>a**</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>27.4 ± 11.3</td>
<td>41.9 ± 18.6</td>
<td>63.5 ± 25.7</td>
<td>68.4 ± 22.6</td>
<td>a*, b, c***</td>
</tr>
<tr>
<td>Free testosterone (pmol/l)</td>
<td>31.4 ± 18.3</td>
<td>12.4 ± 5.1</td>
<td>18.1 ± 5.5</td>
<td>12.8 ± 3.7</td>
<td>a, c**, d*</td>
</tr>
<tr>
<td>Glucose (nmol/l)</td>
<td>5.5 ± 0.9</td>
<td>5.0 ± 0.7</td>
<td>4.8 ± 0.9</td>
<td>4.7 ± 0.5</td>
<td>b, c*</td>
</tr>
<tr>
<td>Insulin (mIU/l)</td>
<td>42.6 (27.4–96.4)</td>
<td>29.2 (24.1–36.6)</td>
<td>15.1 (13.8–19.4)</td>
<td>17.5 (13.8–31.0)</td>
<td>b***, c**</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.27 ± 0.030</td>
<td>0.30 ± 0.022</td>
<td>0.32 ± 0.020</td>
<td>0.31 ± 0.03</td>
<td>b**, c*</td>
</tr>
</tbody>
</table>

**Relative mRNA levels**

<table>
<thead>
<tr>
<th></th>
<th>OB-PCOS</th>
<th>OB-C</th>
<th>NW-PCOS</th>
<th>NW-C</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSR</td>
<td>0.47 (0.36–0.80)</td>
<td>0.54 (0.45–0.68)</td>
<td>0.51 (0.34–0.55)</td>
<td>0.63 (0.51–0.69)</td>
<td>NS</td>
</tr>
<tr>
<td>IRS1</td>
<td>0.75 (0.60–1.00)</td>
<td>1.15 (0.65–1.61)</td>
<td>1.18 (1.08–1.26)</td>
<td>1.66 (0.92–1.80)</td>
<td>b*, c***</td>
</tr>
<tr>
<td>GLUT4</td>
<td>0.27 (0.24–0.34)</td>
<td>0.69 (0.50–0.95)</td>
<td>0.40 (0.26–0.44)</td>
<td>0.42 (0.34–0.54)</td>
<td>a***</td>
</tr>
<tr>
<td>GLUT1</td>
<td>0.17 (0.14–0.30)</td>
<td>0.23 (0.10–0.34)</td>
<td>0.21 (0.16–0.37)</td>
<td>0.22 (0.14–0.26)</td>
<td>NS</td>
</tr>
</tbody>
</table>

The values presented are means ± SD or medians and quartile ranges (25th–75th quartiles).

*Prior to the intervention.

P < 0.05, **P < 0.01, ***P < 0.001 as follows: (a) OB-PCOS versus OB-C; (b) OB-PCOS versus NW-PCOS; (c) OB-PCOS versus NW-C; (d) NW-PCOS versus NW-C.

BMI, body mass index; LH, luteinising hormone; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin; QUICKI, quantitative insulin sensitivity check index; INSR, insulin receptor; IRS1, insulin receptor substrate-1; GLUT, glucose transporter.
Insulin resistance is defined as QUICKI assayed utilizing the YSI 2300 STAT Plus™ Glucose & Lactate Analyzer.

 Serum hormone levels

Serum concentrations of testosterone were analyzed by liquid chromatography (UPLC)/tandem mass spectrometry (LC-MS/MS). The detection limit is 0.1 nmol/l and the CV is 9.2%. Serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and sex hormone-binding globulin (SHBG) were determined by chemiluminescent enzyme immunoassays, and estradiol-17β (E2), and insulin were measured by radioimmunoassay, as described previously (Nybacka et al., 2011). The apparent concentration of free testosterone was calculated from the levels of total testosterone and SHBG, utilizing a fixed albumin concentration of 40 g/l and successive computerized approximation involving a system of equations derived from the law of mass action (Sodergard et al., 1982). Glucose was assayed utilizing the YSI 2300 STAT Plus™ Glucose & Lactate Analyzer (YSI, Inc., Life Sciences, Yellow Springs, OH, USA). Insulin resistance was quantified by calculating the quantitative insulin sensitivity check index (QUICKI) = 1/(log(fasting insulin mIU/l) + log(fasting glucose mmol/l)). Insulin resistance is defined as QUICKI ≤ 0.33.

 Statistical analyses

All values are presented as means and standard deviations or as medians and inter-quartile ranges (P25–P75). Within-group comparisons were performed with the paired t-test or Wilcoxon matched pairs test, depending on the distribution of values. Comparisons between groups were performed by the Kruskal–Wallis test followed by multiple comparison of mean ranks. Comparisons between subgroups were performed using the unpaired t-test or Mann–Whitney U-test, again depending on the distribution. Possible correlations between variables were evaluated using the Spearman’s rank order correlation test. To examine if menstrual pattern, ovulation and insulin sensitivity were significantly changed by lifestyle intervention, we used the sign test. A P-value of < 0.05 was considered statistically significant. A power calculation revealed that 20 subjects subjected to the lifestyle intervention would provide 80% power to detect a difference in mean BMI with a 0.05 two-sided level of significance.

 Results

Comparison of the groups of overweight/obese and normal-weight women with and without PCOS

 Clinical characteristics

The endocrinological parameters for age- and BMI-matched groups of overweight/obese and normal-weight women with and without PCOS are documented in Table I. As expected, prior to intervention, the OB-PCOS women had significantly higher serum levels of total and free testosterone and lower levels of FSH and SHBG than the OB-C group. Furthermore, the OB-PCOS group exhibited significantly lower serum levels of LH, FSH and SHBG and a lower QUICKI, and higher serum levels of testosterone and lower levels of FSH and SHBG than the OB-C group. Obese women had significantly higher serum levels of total and free testosterone and SHBG, utilizing a fixed albumin concentration of 40 g/l and successive computerized approximation involving a system of equations derived from the law of mass action (Sodergard et al., 1982). Glucose was assayed utilizing the YSI 2300 STAT Plus™ Glucose & Lactate Analyzer (YSI, Inc., Life Sciences, Yellow Springs, OH, USA). Insulin resistance was quantified by calculating the quantitative insulin sensitivity check index (QUICKI) = 1/(log(fasting insulin mIU/l) + log(fasting glucose mmol/l)). Insulin resistance is defined as QUICKI ≤ 0.33.

 Gene and protein expression

The levels of IRS1 mRNA in the proliferative endometrium of the women in the OB-PCOS group (prior to the intervention) were lower than the corresponding value for the NW-PCOS group (P < 0.05) and the NW-C group (P < 0.001) (Table I, Fig. 1A). Furthermore, this value for all women with PCOS combined was lower than for all healthy individuals combined (P < 0.01) (Fig. 1B). The level of GLUT4 mRNA was also lower in the endometrium of the OB-PCOS than of the OB-C group (P < 0.001), but there was no difference between normal-weight women with or without PCOS (Table I). There were no significant differences between any of the groups with respect to endometrial mRNA levels of INSR or GLUT1 mRNA (Table I). Furthermore, there were no significant differences in immunostaining between the groups for any of the proteins examined (not shown).

 The lifestyle intervention

Clinical outcome

Of the 20 initially overweight/obese women with PCOS, two dropped out for personal reasons and of the 18 who completed the intervention, one patient was excluded because her endometrial biopsy was too
small. After 3 months, body weight was reduced in 88% (on average by 5%), menstrual pattern improved in 65% and ovulation was confirmed in 35% of the remaining 17 patients (Table II). Moreover, mean body weight, BMI and fasting insulin levels were significantly reduced, while FSH and LH levels and QUICKI were significantly elevated (Table II).

Gene expression

The level of IRS1 mRNA in proliferative endometrium was increased after the intervention ($P < 0.05$) (Table II), particularly in the subgroup of women whose menstrual pattern improved ($n = 11$, $P < 0.01$) (Fig. 2). The level of GLUT1 mRNA was also elevated in this subgroup ($P < 0.05$) (Fig. 2), but did not reach significance for the entire OB-PCOS group ($P = 0.06$) (Table II). In addition, the levels of GLUT4 mRNA were not significantly up-regulated in this subgroup ($P = 0.09$) (Fig. 2), nor in the entire group (Table II). However the significant differences in IRS1 mRNA and GLUT4 mRNA between the OB-PCOS group and the other groups before intervention were no longer evident after the intervention.

Protein expression

In the proliferative endometrium, positive immunostaining for INSR was found in the epithelial cells and strong staining for both INSR $\alpha$ and $\beta$ in blood vessels and capillaries was detected, whereas weak staining for these proteins was observed in the stroma (Fig. 3). There were no significant differences in this immunostaining between the groups or after the intervention.

Positive immunostaining for IRS1 was observed in all endometrial compartments. Staining for total IRS1 was not significantly changed after lifestyle intervention, whereas pY612IRS1, the activated form, was up-regulated in the stroma cells after lifestyle intervention in those with weight loss ($n = 15$) ($P < 0.05$) (Figs 3 and 4A). Immunostaining of pS312IRS1, the major inhibitory form of IRS1, was present both in the epithelial and stroma cells (Fig. 3). This immunostaining was significantly elevated in stromal cells following the lifestyle intervention ($P < 0.05$) (Figs 3 and 4B).

Positive cytoplasmic immunostaining of GLUT4 was observed in the epithelial cells, as well as, to a much weaker extent, in stromal cells, with no alteration after the lifestyle intervention (Fig. 3). Nearly all epithelial and stromal cells stained positively for GLUT1 and this immunoreactivity was found to be significantly higher in the stromal cells after the lifestyle intervention ($P < 0.05$) (Figs 3 and 4C).

Correlations

Improvement in the menstrual function of women in the OB-PCOS group following the lifestyle intervention was positively correlated with

### Table II

| Clinical characteristics, endocrinological variables and relative endometrial levels of mRNA encoding insulin signaling molecules in overweight/obese women with PCOS prior to and following the lifestyle intervention. |
|---|---|---|
| **Before** $n = 17$ | **After** $n = 17$ | **Significance** |
| Body weight (kg) | 103.8 ± 15.0 | 98.7 ± 15.9 | *** |
| BMI (kg/m²) | 37.0 ± 5.3 | 35.2 ± 5.6 | *** |
| Waist/hip ratio | 0.90 (0.80–0.90) | 0.90 (0.80–0.90) | NS |
| Endometrial thickness (mm) | 5.3 (4.4–7.5) | 4.2 (3.5–5.0) | NS |
| AM/OM/RM (n) | 4/13/0 | 3/5/9 | ** |
| Ovulation (n/total) | 0/17 | 6/17 | NS |
| **Endocrinological variables** | | |
| LH (IU/l) | 4.6 ± 1.6 | 6.5 ± 2.8 | ** |
| FSH (IU/l) | 5.1 ± 1.1 | 6.0 ± 1.5 | * |
| Total testosterone (nmol/l) | 1.3 ± 0.6 | 1.2 ± 0.6 | NS |
| SHBG (nmol/l) | 27.4 ± 11.3 | 27.5 ± 17.8 | NS |
| Free testosterone (pmol/l) | 31.4 ± 18.3 | 27.3 ± 16.3 | NS |
| E2 (pmol/l) | 144 (103–169) | 111 (81–142) | NS |
| Glucose (mmol/l) | 5.5 ± 0.9 | 5.4 ± 1.6 | NS |
| Insulin (mIU/l) | 42.6 (27.4–96.4) | 30.9 (21.3–59.1) | ** |
| QUICKI | 0.27 ± 0.03 | 0.29 ± 0.03 | * |
| Proportion of insulin resistance | 94.1% | 82.4% | NS |
| **Relative mRNA levels** | | |
| INSR | 0.07 (0.36–0.80) | 0.61 (0.51–1.04) | NS |
| IRS1 | 0.75 (0.60–1.00) | 1.03 (0.86–1.50) | * |
| GLUT4 | 0.27 (0.24–0.34) | 0.41 (0.23–0.55) | NS |
| GLUT1 | 0.17 (0.14–0.30) | 0.23 (0.15–0.92) | (*) |

The values presented are means ± SD or medians and quartile ranges (25th–75th quartiles).

AM, amenorrhea; OM, oligomenorrhea; RM, regular menstruation.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; (*) $P = 0.06$ in comparison to before the intervention.
the increase in the endometrial level of IRS1 mRNA \( (r = 0.63, P < 0.01) \) and negatively with the change in BMI \( (r = -0.50, P < 0.05) \) (Fig. 5). Finally, the increase in the endometrial level of IRS1 mRNA was positively correlated with the elevation in the endometrial level of GLUT1 mRNA \( (r = 0.55, P < 0.05) \).

**Discussion**

To our knowledge, this is the first study investigating the influence of lifestyle intervention on endometrial insulin signaling and its role for menstrual function in women with PCOS. Before the intervention, endometrial levels of IRS1 and GLUT4 mRNA were significantly lower in the overweight/obese PCOS women than in healthy controls of the same BMI, suggesting a PCOS-specific endometrial insulin-resistant state. After the intervention, we demonstrated an up-regulation of gene and protein expression of insulin signaling molecules in the endometrium of overweight/obese PCOS women, particularly in those with improved menstrual function.

Insulin exerts a wide range of effects on its target tissues via different signaling pathways, influencing metabolism, growth and differentiation (Diamanti-Kandarakis and Dunaif, 2012). Binding of insulin to extracellular α subunits of the INSR activates the intrinsic tyrosine kinase activity of this receptor, resulting in autophosphorylation of its β subunits. Activated INSR phosphorylates a number of substrates, including IRS1, which plays a critical role in insulin signaling. Once IRS1 becomes tyrosine phosphorylated it recruits a number of signal transducers, propagating the insulin signal via the metabolic pathway.

Little information concerning the expression and distribution of the insulin receptor in the endometrium is presently available (Fornes et al., 2010). Here, we found that in the proliferative endometrium of both women with and without PCOS, this receptor is expressed by epithelial cells, but only at a very low level in the stroma. This was demonstrated by immunostaining with two separate antibodies directed against the α and N-terminus of the β subunits. Our study groups did not differ with respect to endometrial levels of INSR mRNA or protein, which is consistent with findings that the level of INSR expression and the affinity of this receptor in other insulin-dependent tissues, such as fat and skeletal muscle, are the same in patients with PCOS and in healthy individuals (Dunaif et al., 2001; Diamanti-Kandarakis and Dunaif, 2012).

Furthermore, the lifestyle intervention employed here did not alter endometrial levels of INSR.

Endometrial levels of IRS1 protein and its activated pY612IRS1 form are lower in hyperinsulinemic and obese women with PCOS than in normoinsulinemic women with PCOS or non-obese healthy controls (Fornes et al., 2010). This agrees with our present observation that IRS1 mRNA levels in the proliferative endometrium of all our patients with PCOS combined were significantly lower than in healthy individuals of the same BMI. In addition, we found significant up-regulation of endometrial IRS1 mRNA in overweight/obese women with PCOS following the lifestyle intervention to a comparable level as the other groups. Furthermore, this up-regulation and the improved menstrual function were positively correlated. To our knowledge, this is the first time that in vivo regulation of insulin signaling in the endometrium of women with PCOS by lifestyle intervention has been demonstrated.

The function of IRS1 is regulated by a balance between activating tyrosine phosphorylation and inhibitory phosphorylation of serine. The levels of both the activated (pY612IRS1) and inhibitory forms (pS312IRS1) of this protein in overweight/obese women with PCOS were significantly up-regulated following the lifestyle intervention, which may indicate that pS312IRS1 feedback regulates insulin signaling in the endometrium, as previously proposed (Gual et al., 2005; Corbould et al., 2006). Altogether, our results here suggest that lifestyle intervention enhances IRS1 function and insulin sensitivity in the endometrium of overweight/
Figure 3  Immunostaining of cells in the proliferative endometrium of one representative overweight/obese women with PCOS before (left) and after (right) the lifestyle intervention. Immunostaining for pY612IRS1, pS312IRS1 and GLUT1 was significantly up-regulated after lifestyle intervention ($P < 0.05$, respectively).
obese women with PCOS and that this might be of importance for their improved menstrual function.

GLUT4 is responsible for insulin-mediated glucose transport. Under basal conditions, it is localized to intracellular vesicles in insulin-responsive cells. In response to stimulation by insulin, these vesicles are translocated to the cell surface to promote glucose uptake (Diamanti-Kandarakis and Dunaif, 2012). It has previously been demonstrated that the levels of GLUT4 mRNA and protein in the proliferative endometrium of hyperinsulinemic and obese women with PCOS are reduced, whereas lean PCOS women have comparable levels to controls (Mioni et al., 2004; Mozzanega et al., 2004; Fornes et al., 2010).

These observations were confirmed here by the reduced endometrial levels of GLUT4 mRNA in overweight/obese, but not normal-weight women with PCOS in comparison to healthy women with the same BMI. The results could be explained by relatively higher testosterone levels as well as insulin levels in the OB-PCOS group compared with obese controls, since it has been shown that testosterone reduces, and metformin increases, endometrial GLUT4 mRNA (Zhang and Liao, 2010). At the same time, GLUT4 mRNA levels were numerically higher, but not significantly up-regulated after the lifestyle intervention in the subgroup of overweight/obese women with PCOS whose menstrual function improved. However, the level of GLUT4 protein, expressed primarily in endometrial epithelial cells, was not significantly changed. Interestingly, treatment with metformin elevates the gene and protein expression of GLUT4 in the endometrium of obese women with PCOS (Zhai et al., 2012). It remains to be determined whether lifestyle intervention may have similar effects to metformin on the endometrium.

**Figure 4** Influence of the lifestyle intervention on the protein levels of pY612IRS1 (A), pS312IRS1 (B) and GLUT1 (C) in the proliferative endometrium of overweight/obese women with PCOS. The values presented are medians and quartile ranges (25th–75th quartiles). *P < 0.05 in comparison to before the intervention.

**Figure 5** Correlation between the change in gene expression level of IRS1 and improved menstrual function (A), and correlation between the change in BMI and improved menstrual function (B) by lifestyle intervention in overweight/obese women with PCOS.
GLUT1, which is thought to be responsible for basal uptake and storage of glucose in most types of cells (Mueckler, 1990), is expressed both on the surface and inside the cell. Little is known about the expression and function of this protein in the proliferative endometrium. Recently, the level of GLUT1 mRNA in stromal cells of the human endometrium was reported to be higher than those of other glucose transporters (Frolova and Moley, 2011). Furthermore, it was shown that GLUT1 is essential for decidualization of endometrial stromal cells in vitro (Frolova and Moley, 2011) and therefore it was suggested that this protein plays a role in embryo implantation. In the current investigation, lifestyle intervention resulted in up-regulation of GLUT1 mRNA in the proliferative endometrium of overweight/obese women with PCOS, particularly in those who exhibited improved menstrual function. Furthermore, protein expression in the stromal cells was significantly up-regulated by lifestyle intervention. These results suggest improved basal glucose uptake in the endometrium. The positive correlation between the alterations in GLUT1 and IRS1 mRNA levels suggests that improved glucose uptake by both epithelial and stromal cells may help to restore endometrial function.

Certain limitations of this study must be recognized. Even though the power calculation indicated that the number of patients subjected to the lifestyle intervention should be sufficient, the number of women in each group was limited. Among the strengths of this investigation are the participation of homogenous groups of overweight/obese and normal-weight women with PCOS, fulfilling all three criteria of the Rotterdam consensus, and of BMI-matched healthy controls.

In conclusion, we have for the first time demonstrated that lifestyle intervention up-regulates, both at the mRNA and protein levels, endometrial expression of molecules involved in insulin signaling in overweight/obese women with PCOS and that the mRNA up-regulation correlates with improvement in menstrual function. These findings provide evidence of in vivo regulation of insulin signaling in the proliferative endometrium of women with PCOS. Furthermore, the presence of lower endometrial levels of IRS1 and GLUT4 mRNA in these patients than in healthy women with the same BMI may reflect defective insulin signaling in the former. We propose that up-regulation of endometrial IRS1 and GLUT1 in overweight/obese women with PCOS following this lifestyle intervention improves the glucose homeostasis and thereby restores the functioning of their endometrium.

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Authors’ roles

D.U. analyzed and interpreted the data and prepared the manuscript. M.H. was involved in the analysis and interpretation of the data, and contributed in writing the manuscript. A.C. contributed to design of the study, recruitment of subjects and collection of tissue samples. Å.N. was responsible for the lifestyle intervention. B.B. was involved in the analysis and interpretation of the data. A.L.H. had overall responsibility for the study, and was involved in interpretation of the data and writing the manuscript.

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Conflict of interest

None declared.

References


Insulin signaling in the endometrium in PCOS


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