Gene expression in subcutaneous adipose tissue differs in women with polycystic ovary syndrome and controls matched pair-wise for age, body weight, and body mass index

Louise Mannerås-Holm1, Anna Benrick1, and Elisabet Stener-Victorin1,2,4

1Institute of Neuroscience and Physiology; Department of Physiology; Sahlgrenska Academy; University of Gothenburg; Gothenburg, Sweden;
2Department of Obstetrics and Gynecology; First Affiliated Hospital; Heilongjiang University of Chinese Medicine; Harbin, PR China

Keywords: adiponectin receptor 2 (ADIPOR2), adipose tissue, chemokine (C-C motif) ligand 2 (CCL2), gene expression, heme oxygenase (decycling 1) (HMOX1), insulin sensitivy, lipoprotein lipase (LPL), PCOS, twist-related protein 1 (TWIST1)

Abbreviations: ADIPOR, adiponectin receptor; ADRB2, β2-adrenergic receptors; BMI, body mass index; CCL2, chemokine (C-C motif) ligand 2; GIR, glucose infusion rate; HMOX1, heme oxygenase (decycling 1); LPL, lipoprotein lipase; PCOS, polycystic ovary syndrome; SHBG, sex hormone binding globulin; TWIST1, twist-related protein 1

Introduction

Polycystic ovary syndrome (PCOS) is associated not only with endocrine abnormalities but also with insulin resistance, obesity, dyslipidemia, chronic low-grade inflammation, and disturbances of coagulation and fibrinolysis.1-3 Consequently, PCOS increases the risk for type 2 diabetes and possibly cardiovascular disease.1 Insulin resistance is thought to be a key pathophysiological feature that contributes to both reproductive and other metabolic disturbances in PCOS. However, the insulin resistance is only partly due to the high prevalence of obesity in women with the syndrome and is greater than that determined by their adiposity.4

Adipose tissue dysfunction is emerging as an important mechanism by which adipose tissue contributes to systemic insulin resistance and metabolic disease. We recently stressed the role of aberrant adipose tissue morphology and function in the pathogenesis/maintenance of insulin resistance in PCOS.4 Enlarged fat cells and reduced serum adiponectin, together with a large waistline, were the variables most strongly associated with insulin sensitivity in women with PCOS.4

Other features of adipose tissue, such as lipid metabolism (lipolysis, lipoprotein lipase [LPL] activity) and insulin action, have been reported to be aberrant in women with PCOS.4-6 Further, disturbed secretion of several adipokines may contribute to insulin resistance and cardiovascular risk.7-12 Gene expression profiles in omental fat from morbidly obese and nonobese women with and without PCOS revealed differences in the expression of genes encoding components of several biological pathways related to insulin and Wnt signaling, inflammation, immune function, lipid metabolism, and oxidative stress.13,14 Thus, adipose tissue dysfunction may negatively affect the metabolic health of women.
with PCOS and thereby increase their risk for type 2 diabetes and cardiovascular disease. However, the key factors in adipose tissue that are involved and their regional contribution have not been established.

Since dysfunctional adipose tissue is increasingly considered to be important in the metabolic disturbances in PCOS, we assessed, in subcutaneous adipose tissue, the expression of genes potentially involved in the pathogenesis of PCOS and adipose tissue dysfunction. We also explored the relation between gene expression and the cardinal features of the syndrome, i.e., body mass index (BMI), glucose infusion rate (GIR), sex hormone binding globulin (SHBG), testosterone, and estradiol. Particular focus was given to pair-wise matching of women with PCOS and controls for age, body weight, and BMI.

### Results

#### Subject matching

The age range was 21–37 y in women with PCOS and 22–35 y in controls. The age difference (control vs. PCOS) ranged from −4 to 3 y (mean, −1 ± 2.36 y). Body weight range was 55.2–96.0 kg in women with PCOS and 54.1–99.2 kg in controls. The body weight difference (control vs. PCOS) ranged from −4.9 to 4.6 kg (mean, 0.0 ± 2.63 kg). The BMI was 18.2–33.4 kg/m² in women with PCOS and 19.2–31.7 kg/m² in controls. The BMI difference (control vs. PCOS) ranged from −1.79 to 1.48 kg/m² (mean, −0.25 ± 0.94 kg/m²). The proportion of overweight/obesity (BMI ≥ 25 kg/m²) was 38%. All 21 matched pairs met the criteria for age (± 5 y), weight (± 5 kg), and BMI (± 2 kg/m²). However, the paired statistics revealed a significant difference in age (29.0 ± 4.2 vs. 27.6 ± 3.5; PCOS vs. controls, *P* = 0.03, Table 1).

#### Subject characteristics

In this study we included 42 controls and PCOS women pair-matched for age, weight and BMI (Table 1). As previously reported, PCOS women are insulin resistant (lower GIR) and have lower circulating levels of SHBG, and higher levels of testosterone and estradiol than controls (Table 1), all cardinal features of PCOS.

### Adipose tissue gene expression

We and others have shown that women with PCOS have dysfunctional adipose tissue. Since dysfunctional adipose tissue is increasingly considered to be important in the metabolic disturbances in PCOS, we assessed, in subcutaneous adipose tissue, the expression of genes potentially involved in the pathogenesis of PCOS and adipose tissue dysfunction. We also explored the relation between gene expression and the cardinal features of the syndrome, i.e., body mass index (BMI), glucose infusion rate (GIR), sex hormone binding globulin (SHBG), testosterone, and estradiol. Particular focus was given to pair-wise matching of women with PCOS and controls for age, body weight, and BMI.

### Table 1. Characteristics of women with PCOS and controls pair-wise matched for age, weight, and body mass index (BMI)

| Variable                  | PCOS (n = 21) | Controls (n = 21) | *P*  |
|---------------------------|---------------|------------------|------|
| Age (y)                   | 29.0 ± 4.2    | 27.6 ± 3.5       | 0.031|
| BMI (kg/m²)               | 24.6 ± 3.9    | 24.3 ± 3.5       | 0.433|
| Weight (kg)               | 71.2 ± 11.3   | 71.3 ± 11.5      | 0.886|
| Glucose disposal rate (mg kg⁻¹min⁻¹) | 10.9 ± 2.6 | 12.6 ± 3.7      | 0.046|
| SHBG (nmol/L)             | 48.0 ± 24.6   | 69.1 ± 30.5      | 0.030|
| Testosterone (nmol/L)     | 1.91 ± 0.57   | 0.75 ± 0.31      | <0.001|
| Estradiol (pmol/L)        | 236 ± 135     | 136 ± 52         | 0.002|

Plus-minus values are means ± SD; *Paired t test; SHBG, sex hormone binding globulin.

### Figure 1. Significantly expressed genes in women with PCOS relative to controls in subcutaneous adipose tissue. Data are normalized to the controls and each line represent geometrical mean. Log-transformed RQ values were compared by paired t test; CLN3 and LRP10 severed as reference genes. *P* < 0.05, **P** < 0.01.
protein-1) and HMOX1 were expressed at higher levels, than in controls (Fig. 1). All nonsignificant genes are shown in Table S2.

Correlations

Next, we investigated whether altered mRNA expression of ADIPOR2, CCL2, HMOX1, LPL, and TWIST1 correlated with BMI and clinical variables shown to differ between women with PCOS and controls (Fig. 2). These variables include insulin sensitivity (GIR), SHBG, and sex steroids including testosterone and estradiol.2 Correlations between mRNA expression and clinical variables were based on all women in the cohort.

Circulating estradiol correlated with ADIPOR2 and LPL inversely and independently of BMI (Fig. 2; Table 2).

CCL2 correlated positively with BMI and inversely with GIR (Fig. 2). The correlation with GIR was significant also after adjustment for BMI (Table 2).

HMOX1 correlated positively with BMI, and inversely with GIR (Fig. 2). The significance of these correlations was lost after adjustment for BMI (Table 2).

TWIST1 correlated inversely with BMI and positively with GIR and SHBG (Fig. 2). The significance of these correlations was lost after adjustment for BMI (Table 2).

Discussion

We investigated adipose tissue expression of genes potentially involved in the pathogenesis of PCOS and its dysfunctional adipose tissue. Of 44 selected genes related to insulin sensitivity, inflammation, lipid metabolism, adipogenesis, oxidative stress, steroidogenesis, and adipokines, we found that ADIPOR2, LPL, and TWIST1 were expressed at lower levels, and CCL2 and HMOX1 at higher levels, in subcutaneous adipose tissue from women with PCOS than in controls matched pair-wise for age, weight, and BMI. Expression of ADIPOR2 and LPL correlated inversely with serum estradiol and CCL2 with GIR, independent of BMI.

Insulin resistance is a key factor in the pathogenesis of PCOS. In addition to clear evidence of intrinsic defects in insulin signaling in skeletal muscle, similar defects in insulin signaling have also been described in adipose tissue in PCOS, but data are limited.5 TWIST1 expression, which is predominantly found in adipocytes, is highest in samples from nonobese subjects with hyperplastic adipose tissue and lowest in obese subjects with hypertrophic adipose tissue.17 This is in line with the inverse correlation between TWIST1 expression and BMI in both controls and PCOS women. We showed for the first time that adipose
tissue expression of TWIST1 is lower in insulin-resistant women with PCOS than in controls. And, there was a positive correlation of TWIST1 expression with GIR and SHBG, but this did not remain significant after adjustment for BMI. This suggests an association with fatty acid oxidation in PCOS since Twist1 may, in contrast to findings in mice, be a positive regulator of fatty acid oxidation in human adipocytes. Moreover, Twist1 knockdown accentuates the pro-inflammatory effects of TNF-α, suggesting that Twist1 protects against adipose inflammation. 

Increased oxidative stress in fat is a key mechanism of obesity-related insulin resistance. Oxidative stress impairs glucose uptake in both muscle and fat. Proteomic and genomic studies have highlighted the role of oxidative stress in omental fat from obese PCOS women. It is not known whether subcutaneous fat is altered in normal-weight PCOS women as well. HMOX1 has been implicated as a new adipokine known to reduce oxidative stress. In contrast to a recent study, we found that HMOX1 mRNA expression was higher, instead of lower, in women with PCOS than controls, consistent with our previous findings. Both androgens and estrogens have been reported to inhibit adipose tissue LPL activity. In support of this, adipose tissue gene expression of LPL correlated negatively with plasma estradiol, and the correlation remained significant after adjustment for BMI.

Systemic low-grade inflammation, characterized by increased accumulation of macrophages and release of proinflammatory cytokines and chemokines in adipose tissue, has been suggested to contribute to insulin resistance and be a hallmark of obesity. Circulating levels of several markers of inflammation are elevated in women with PCOS, but whether the elevations are independent of obesity is debated. We recently showed that macrophage density in subcutaneous adipose tissue and circulating inflammatory markers do not differ in PCOS women and controls after adjustment for age, weight, and BMI. Further, a recent study concluded that obesity rather than PCOS per se seems to be the main determinant of increased inflammatory markers in adipose tissue. Indeed, we found that the genes related to immune and inflammatory response (e.g., CD68, IL6, IL8, SAA2, and TNF) were expressed at similar levels in adipose tissue of women with and without PCOS. However, expression of CCL2, which encodes a chemokine that attracts macrophages and other immune cells, was significantly upregulated in adipose tissue from PCOS women. And, a recent study showed that chemokines and cytokines, including CCL2, were paradoxically expressed at higher levels in adipose tissue from nonobese PCOS women than controls. We found that high CCL2 expression correlated inversely, and independently of BMI, with GIR. This is in line with the association between low-grade inflammation and insulin resistance. It is still unclear whether PCOS is associated with a systemic proinflammatory state independently of obesity and whether adipose tissue plays a role in it.

Table 2. Correlations and partial correlations, adjusted for body mass index (BMI), between mRNA expression in adipose tissue and clinical variables in all women

| Clinical variable | ADIPOR2 | CCL2 | HMOX1 | LPL | TWIST1 |
|------------------|---------|------|-------|-----|--------|
| BMI (kg/m²)      | 0.225, P = 0.153 | 0.523, P = 0.001 | 0.395, P = 0.010 | 0.180, P = 0.254 | −0.477, P = 0.001 |
| GIR (mg kg⁻¹ min⁻¹) | −0.264, P = 0.091 | −0.464, P = 0.002 | −0.401, P = 0.009 | −0.174, P = 0.270 | 0.368, P = 0.017 |
| SHBG (nmol/L)    | −0.148, P = 0.349 | −0.250, P = 0.111 | −0.278, P = 0.075 | −0.166, P = 0.293 | 0.344, P = 0.026 |
| Testosterone (ng/mL) | −0.145, P = 0.358 | −0.205, P = 0.192 | −0.230, P = 0.143 | −0.080, P = 0.617 | −0.267, P = 0.087 |
| Estradiol (pmol/L) | −0.551, P = 0.001 | −0.041, P = 0.796 | 0.017, P = 0.917 | −0.494, P = 0.001 | 0.018, P = 0.912 |
| BMI adjusted     | −0.190, P = 0.235 | −0.308, P = 0.050 | −0.276, P = 0.080 | −0.108, P = 0.501 | 0.202, P = 0.205 |
| SHBG (nmol/L)    | −0.056, P = 0.727 | −0.024, P = 0.880 | −0.125, P = 0.435 | −0.098, P = 0.541 | 0.169, P = 0.291 |
| Testosterone (ng/mL) | −0.205, P = 0.198 | 0.108, P = 0.502 | 0.159, P = 0.321 | −0.124, P = 0.438 | −0.189, P = 0.238 |
| Estradiol (pmol/L) | −0.517, P = 0.001 | 0.189, P = 0.237 | 0.188, P = 0.239 | −0.467, P = 0.002 | −0.191, P = 0.232 |

Pearson correlation. GIR, glucose infusion rate; SHBG, sex hormone binding globulin.
Dysregulation of adipokine production and secretion from adipose tissue may contribute to several cardinal and metabolic features of PCOS. Women with PCOS have lower circulating levels of adiponectin, and subcutaneous adipose tissue production of adiponectin is considered to be the main contributor to the circulating levels. However, studies on ADIPOQ expression in adipose tissue of women with PCOS have been inconsistent, finding either lower expression than in controls or no difference from controls. Although expression of ADIPOQ and other adipokines (e.g., LCN2, LEP, LIPIN1, RBP4, and SERPINE1) did not differ in PCOS women and controls, expression of ADIPOR2, one of two cell membrane adiponectin receptors, was decreased in women with PCOS. This finding is in agreement with a previous small study in which mRNA expression of ADIPOR1 and ADIPOR2 in omental adipose tissue was lower in nonobese women with PCOS than in age- and BMI-matched controls. However, in another small study, expression of ADIPOR1 and ADIPOR2 mRNA in subcutaneous and omental fat was higher in PCOS women than in controls. In the present study, ADIPOR2 expression correlated positively, and independently of BMI, with plasma estradiol.

In contrast to earlier findings, we did not observe any significant difference in adipogenic transcription factors and markers of undifferentiated adipocytes (e.g., CEBPA, CEBPB, DLK1, PPARG, SREBF1, and WNT10B) and no differences in the expression of enzymes and receptors involved in steroidogenesis (e.g., AR, AKR1C2, AKR1C3, CYP19A1, ESR1, and SRD5A2) suggesting a normal adipogenesis and steroidogenesis in the subcutaneous adipose tissue in our cohort. However, we investigated only mRNA expression, not protein expression or enzyme activity. Thus, further studies are needed to elucidate the contribution of imbalanced adipogenesis and steroidogenesis to the pathophysiology of PCOS. In selecting the genes to investigate, we used a hypothesis-driven approach, whereas previous genomic and proteomic studies have used a discovery-driven approach. This difference can partly explain the discrepancies between our studies. Furthermore, despite their insulin resistance, women with PCOS in the present study had a relatively healthy metabolic profile, with no hypertension, lipid disturbances, or evident visceral adiposity.

Surprisingly few of the genes we investigated were differentially expressed in subcutaneous adipose tissue from women with and without PCOS. However, previous microarray analyses of >14,500 genes in subcutaneous and omental adipose tissue from nonobese and obese PCOS women and controls reported differential expression of fewer than 100 genes. Commonly altered genes in these studies included those involved in inflammation, lipid metabolism, and Wnt signaling.

The main strengths of this study are the inclusion of untreated, well-characterized normal weight and overweight women with PCOS and the pair-wise matching by age, weight, and BMI to healthy controls. Further, we only included women with PCOS who fulfilled all three diagnostic criteria for the syndrome, resulting in a more homogenous study group. The majority of the genes in the present study have not previously been investigated in adipose tissue of PCOS women. Moreover, we use gold-standard techniques for measurements of circulating sex steroids and whole-body insulin sensitivity. However, a limitation of this study is relatively small numbers of patients and controls. Furthermore, we only examined mRNA expression, so our findings must be interpreted cautiously, as they might not be transferable to protein level.

In conclusion, PCOS women had significant differences from controls in mRNA expression in adipose tissue. The role of two novel adipokines TWIST1 and HMOX1, together with adiponectin, LPL, and CCL2, and their downstream pathways merit further investigation.

Subjects, Materials, and Methods

Subjects
All participants in the study were selected from premenopausal women with (n = 74) and without (n = 31) PCOS who were recruited by local advertising as described. To be included, women with PCOS had to (1) meet all three Rotterdam criteria for the syndrome (PCO morphology, hyperandrogenism, and menstrual irregularities), so as to reduce the metabolic heterogeneity associated with multiple phenotypic subgroups, and (2) be matched with a control woman by age (± 5 y), body weight (± 5 kg), and BMI (± 2 kg/m²). Twenty-one matched pairs were included and their clinical characteristics, as previously described, are shown in Table 1.

PCO morphology was confirmed by transvaginal sonography (12 or more follicles 2–9 mm in diameter or >10 mL in volume, in at least one ovary). Clinical signs of hyperandrogenism were defined as hirsutism (Ferriman–Gallwey score ≥ 8) and/or acne. Menstrual irregularities were defined as oligomenorrhea (intermenstrual interval >35 d and <8 cycles in the past year) or amenorrhea (absent menstrual bleeding or none in the past 90 d). All control women were eumenorrheic, had ultrasound-confirmed normal ovarian morphology, and had no signs of hyperandrogenism.

Exclusion criteria for all women were age <18 or >37 y, pharmacological treatment within 12 wk, breast feeding within 24 wk, cardiovascular disease, and diabetes mellitus or other endocrine disorders. All study subjects gave oral and written consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethical Review Board, University of Gothenburg. The Clinical Trials Government Identifier number is NCT00484705.

Study design
All examinations, including adipose tissue sampling, were performed in the morning after an overnight fast, as described. Since women with PCOS had oligo/amenorrhea, the examination day was chosen independently of cycle day. Controls were examined during the early follicular phase (days 1–7 of the cycle). A needle biopsy of subcutaneous abdominal adipose tissue was obtained under local anesthesia two-thirds of the distance from the iliac crest to the umbilicus. Part of the biopsy was immediately snap frozen for RNA extraction. Anthropometry, adipose

Subjects
tissue volumes, insulin sensitivity, adipocyte volume, LPL activity, and blood chemistry were analyzed as described.2–4

RNA extraction and cDNA synthesis

Approximately 500 ng of snap-frozen adipose tissue was homogenized in QIAzol Lysis Reagent, supplied with the RNeasy Lipid Tissue Midi kit (Qiagen, 75842), using the TissueLyser (Qiagen, 85300). Total RNA was extracted using RNeasy Lipid Tissue Midi kit and Maxiprep High-density tubes according to manufacturer’s protocol (Qiagen, 75842 and 129065). RNA concentration and purity were evaluated with a Nanodrop ND-1000 spectrophotometer (Nano-Drop Technologies) and RNA integrity was verified with an Agilent Bioanalyzer 2100 and the RNA 6000 Nano LabChip kit (Agilent Technologies, G2940CA and 5067-1511). First-strand cDNA was synthesized from 1 μg of total RNA with Superscript Vilo according to the manufacturer’s protocol (Invitrogen, 11754-250).

Gene expression

Quantitative real-time PCR was performed with custom TaqMan gene expression array micro fluid cards (Life Technologies, Table S1). Samples were run in singletons, and the amount of cDNA in each loading port was equivalent to 100 ng of mRNA. The arrays were run according to the manufacturer’s protocol with an ABI Prism 7900HT Sequence Detection System and ABI Prism 7900HT SDS software version 2.4 (Applied Biosystems). Candidate reference genes (RNU8SI, PP1A, LRPI0, and CLN3) were validated with NormFinder and GeNorm algorithms and GenEx software (MultiD Analyses). Based on the results from these algorithms the combination of CLN3 and LRPI0 was used as the reference control. Gene expression values were calculated with the ΔΔCq method (i.e., RQ = 2−ΔΔCq).9

Statistics

Paired t tests were used to compare mRNA levels and clinical variables between matched cases and controls. mRNA expression in controls was set to 1 to calculate fold changes in women with PCOS. Correlation analyses were performed with Pearson correlation, and partial correlation adjusted for BMI on the whole cohort. RQ values were log-transformed and clinical variables were transformed as described.4 P < 0.05 was considered significant. All statistical analyses were performed with IBM SPSS Statistics version 19 for Windows (SPSS).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

For the use of technical equipment and support we thank the Genomics Core Facility at the Sahlgrenska Academy, University of Gothenburg, which was funded by a grant from the Knut and Alice Wallenberg Foundation.

Financial Support

This study was financed by grants from the Swedish Research Council (K2012-55X-15276-08-3), Jane and Dan Olsson Foundations, Novo Nordisk Foundation, and the Swedish federal government under the LUA/ALF agreement (ALFGBG-136481) and the Regional Research and Development agreement (VFOUREG-5171, -11296, and -7861).

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/adipocyte/article/28731/
M111.010504 adipocytes. Mol Cell Proteomics 2012; 11:010504; of novel adipokines released from primary human type 2 diabetes mellitus. PLoS One 2010; 5:e12371; PMID:20811623; http://dx.doi.org/10.1371/journal.pone.0012571