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Differential Gene Expression Profile in Omental Adipose Tissue in Women with Polycystic Ovary Syndrome

Marta Cortón, José I. Botella-Carretero, Alberto Benguría, Gemma Villuendas, Angel Zaballos, José L. San Millán, Héctor F. Escobar-Morreale, and Belén Peral

Instituto de Investigaciones Biomédicas (M.C., B.P.), Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, E-28029 Madrid, Spain; Department of Endocrinology (J.I.B.-C., G.V., H.F.E.-M.), Hospital Ramón y Cajal and Universidad de Alcalá, and Department of Molecular Genetics (J.L.S.M.), Hospital Universitario Ramón y Cajal, E-28034 Madrid, Spain; and Centro Nacional de Biotecnología (A.B., A.Z.), Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, E-28049 Madrid, Spain

Context: The polycystic ovary syndrome (PCOS) is frequently associated with visceral obesity, suggesting that omental adipose tissue might play an important role in the pathogenesis of the syndrome.

Objective: The objective was to study the expression profiles of omental fat biopsy samples obtained from morbidly obese women with or without PCOS at the time of bariatric surgery.

Design: This was a case-control study.

Settings: We conducted the study in an academic hospital.

Patients: Eight PCOS patients and seven nonhyperandrogenic women submitted to bariatric surgery because of morbid obesity.

Interventions: Biopsy samples of omental fat were obtained during bariatric surgery.

Main Outcome Measure: The main outcome measure was high-density oligonucleotide arrays.

Results: After statistical analysis, we identified changes in the expression patterns of 63 genes between PCOS and control samples. Gene classification was assessed through data mining of Gene Ontology annotations and cluster analysis of dysregulated genes between both groups. These methods highlighted abnormal expression of genes encoding certain components of several biological pathways related to insulin signaling and Wnt signaling, oxidative stress, inflammation, immune function, and lipid metabolism, as well as other genes previously related to PCOS or to the metabolic syndrome.

Conclusion: The differences in the gene expression profiles in visceral adipose tissue of PCOS patients compared with nonhyperandrogenic women involve multiple genes related to several biological pathways, suggesting that the involvement of abdominal obesity in the pathogenesis of PCOS is more ample than previously thought and is not restricted to the induction of insulin resistance. ***(J Clin Endocrinol Metab* 92: 328–337, 2007)**

THE POLYCYSTIC OVARY syndrome (PCOS) appears to be a complex disorder in terms of inheritance, in which protective and predisposing genomic variants interfere with a very important environmental influence, including diet and lifestyle, leading to the syndrome's phenotype (1).

Obesity plays a very important role in the development of PCOS in many women. PCOS patients are frequently obese (2); as many as 42% of women with PCOS were overweight or obese in population-based studies conducted in the United States (3). Conversely, PCOS is probably the most common association of obesity in premenopausal women, because 28% of overweight and obese women have this prevalent disorder (4). This association may be explained by the secretion by adipose tissue of several mediators, including cytokines and adipokines, that favor the insulin resistant and low-grade chronic inflammatory state characteristic of PCOS (1, 5–7).

The hyperandrogenism characteristic of PCOS also may influence the pathophysiology of obesity. Androgen excess during fetal life or during the early postnatal period induced abdominal adiposity and an adverse metabolic profile in animal models (8), and a similar mechanism might occur in humans (9). Furthermore, girls with premature pubarche—a form of prepubertal hyperandrogenism that predisposes these girls to PCOS—have insulin resistance throughout puberty (10), and treatment with antiandrogens may contribute to ameliorate the insulin resistance of these girls (11). Therefore, the possibility exists that women with PCOS have a vicious circle that starts with hyperandrogenism favoring an abdominal visceral deposition of fat that induces insulin resistance, and the resulting hyperinsulinemia further facilitates androgen secretion in these patients (12).

Given the central role of visceral adipose tissue in the development of PCOS in overweight and obese women, we have considered of interest the study of the expression profiles of omental fat biopsy samples obtained from morbidly obese women with or without PCOS at the time of bariatric surgery. Compared with classic molecular genetic ap-

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Abbreviations: BMI, Body mass index; GO, gene ontology; HOMA-IR, homeostasis model assessment for insulin resistance; FOXA2, forkhead box A2; IR, insulin receptor; PCOS, polycystic ovary syndrome; qPCR, quantitative PCR; RMA, robust multi-array analysis; SAM, significance analysis of microarrays; TFBS, transcription factor binding site; TransFAT, Transcription Factor Association Test tool.

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proaches, our present experimental design had the theoretical advantages of using a hypothesis-free approach to find genes dysregulated in the visceral fat of PCOS patients, as well as providing an integrative view of the interaction of genomic variants with environmental influences that result in gene expression in adipose tissue.

Subjects and Methods

Subjects, sampling, and surgical procedure

Fifteen morbidly obese premenopausal women submitted for bariatric surgery, including eight PCOS patients [age 31.6 ± 7.9 yr; body mass index (BMI) 51.0 ± 10.2 kg/m^2, range 41–75 kg/m^2] and seven nonhyperandrogenic women (age 40.4 ± 3.6 yr; BMI 52.8 ± 5 kg/m^2, range 44–58 kg/m^2) were included. The study was conducted according to the Declaration of Helsinki and was approved by the ethics committee of Hospital Ramón y Cajal. Signed informed consent was obtained from all subjects.

The diagnosis of PCOS was established by the presence of oligoovulation, clinical and/or biochemical hyperandrogenism, and exclusion of hyperprolactinemia, nonclassic congenital adrenal hyperplasia, and androgen-secreting tumors (13). Hirsutism was quantified by the modified Ferriman-Gallwey score (14). Evidence for oligoovulation was provided by chronic oligomenorrhea or amenorrhea in all the patients. The controls had no signs of hyperandrogenism and had regular menstrual cycles every 26–34 d. None of the patients and controls were being treated with insulin sensitizers, statins, or hormonal therapy.

After a 12-h overnight fast, basal blood samples were obtained for the measurement of serum glucose, insulin, prolactin, total testosterone, SHBG, 17-hydroxyprogesterone, androstenedione, and dehydroepiandrosterone-sulfate. The technical characteristics of the assays used for hormone measurements have been reported elsewhere (15). The free testosterone concentration was calculated from total testosterone and SHBG concentrations (16). Insulin resistance in the fasting state was determined using homeostasis model assessment (HOMA-IR) (17).

The indication for bariatric surgery was morbid obesity in all patients, as defined by BMI 40 kg/m^2 or greater (18). During surgery, biopsy samples of omental fat were obtained, washed in chilled NaCl 0.9% solution, partitioned into pieces, and immediately frozen in liquid nitrogen and stored at −80 C until total RNA preparation. The surgeon aimed to obtain the samples from similar anatomical locations in all the women, and patients and controls were submitted to the same anesthetic procedures to avoid a potential effect on the expression of susceptible genes such as those related to stress and inflammation.

RNA isolation, array hybridization, and data processing

Total RNA was extracted from omental adipose tissue (500 mg) using Trizol (Invitrogen Corp., Carlsbad, CA), followed by the QIAGEN RNeasy kit (QIAGEN N.V., Venlo, The Netherlands), and was tested for degradation using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). cDNA was synthesized from 5 μg total RNA using T7-(dT)$_{24}$ oligonucleotide (Superscript II Reverse Transcriptase; Invitrogen Corp.). *In vitro* transcription was performed using BioArray High Yield RNA transcript labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY) to produce biotin labeled cRNA; 5 μg fragmented cRNA was used for the TestChip (Test3; Affymetrix, Inc., Santa Clara, CA). After quality-control, 15 μg cRNA was hybridized for 16 h at 45 C to the Affymetrix HG-U133A chip. Each microarray was washed and stained in the Affymetrix Fluidics Station 400 following the standard protocols. Microarrays were scanned at 3-μm resolution in an Agilent HP G2500A GeneArray scanner. A very high experimental reproducibility ($r^2 > 0.97$), as assessed by hybridizing two arrays with RNA from the same sample, was found. Furthermore, the concordance of the results of pairs of arrays hybridized using RNA from different women was also high ($0.97 > r^2 > 0.84$), indicating low interindividual variability in the gene expression of omental fat.

Data analyses were performed using Affymetrix Microarray Suite 5.0 software and images from each gene chip were normalized by the global scaling method to a target intensity value of 100, following the manufacturer's instructions. Also we used the robust multiarray analysis (RMA) algorithm for normalization, by applying the RMA Express software (19). To reduce the complexity of data, the expression analysis was restricted to a subset of reproducibly expressed probe sets that significantly varied among the data. Probe sets with "absent calls" across all samples and/or absolute fold change less than 1.2 were filtered out. Complete data sets are available from the National Center for Biotechnology Information Gene Expression Omnibus web site, accession no. GSE5090.

Quantitative RT-PCR

Reverse transcription (RT) was performed using 1 μg total RNA, with random hexamers and the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed in the ABI Prism 7900HT using specific primers and probes for target genes (TaqMan Low-Density Array System; Applied Biosystems). cDNAs were diluted to a final concentration of 0.5 ng/μl and incubated at 95 C for 10 min followed by 40 cycles (15 sec at 95 C and 1 min at 60 C). Glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A, and glucuronidase β were included in the qPCR as endogenous controls. Fluorescence intensity was recorded by using ABI 7700 Prism SDS 2.1 software (Applied Biosystems), and the results were plotted *vs.* cycle number. The $\Delta\Delta$Ct method was used to calculate relative changes in mRNA abundance as fold change values (20).

Statistical analysis

The differences in clinical and hormonal variables between PCOS patients and nonhyperandrogenic controls were evaluated by the Mann-Whitney *U* test. The differential gene expression was evaluated by using a two-tail unequal-variances *t* test implemented in the Gene Expression Pattern Analysis Suite package (21). Also, a permutation-based statistical method termed significance analysis of microarrays (SAM) was achieved in a two-unpaired classes format, which performs a correction for multiple testing using the false discovery rate method (22). Annotations of PCOS dysregulated probe sets were collected from different available databases by using the NetAffx online tool (http://www.affymetrix.com/analysis/index.affx), and their identity was verified using the BLAST tool (http://www.ncbi.nlm.nih.gov).

The sequence of the promoter region (from −1000 to + 100 bp with respect to the start of transcription site) for each dysregulated gene was obtained from the Transcriptional Regulatory Element Database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). A search for androgen response elements in these regions was conducted using the MatInspector software (23), using the V$ARE.01 and V$ARE.02 matrixes contained in the MatBase version 6.1 library (Genomatix Software GmbH, München, Germany). Spearman's nonparametric correlation analysis and partial correlation analysis were used to study the influence of age, hyperandrogenism, and insulin resistance on gene expression as described.

Functional analysis was performed on the basis of gene ontology (GO) terms by using Onto-Express Tool (24). Significantly overrepresented or underrepresented GO terms among the differentially expressed dataset were obtained in relation to the number of probe sets expected for each GO category, based on their representation on the Affymetrix U133A array. A χ^2 test was performed to determine confidence levels for differential expression within ontology groups, and the *P* values were corrected for multiple testing based in the false discovery rate method.

Genesis software (http://genome.tugraz.at) was used for hierarchical clustering analysis of PCOS-regulated genes applying average linkage and Pearson's correlation as a measure of similarity.

The Transcription Factor Association Test tool (TransFAT) (25) was used to detect under- or overrepresentation of putative transcription factor binding sites (TFBSs) in coexpressed genes. Each gene cluster obtained by clustering analysis was compared against the rest of the altered PCOS genes. TFBSs were searched in the 10-kb upstream region of the selected genes by using Match program. Only TransFAT high-quality matrices were used with a cutoff to minimize false positives. Finally, a Fisher's exact test was performed to obtain significant TFBSs overrepresentation in the cluster we were analyzing. For RT-qPCR analyses, the Mann-Whitney *U* test was used. Statistical significance was set

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at the $P < 0.05$ level for all analyses except when correcting for multiple testing.

Results

Description of PCOS patients and nonhyperandrogenic controls

The comparisons of clinical, biochemical, and hormonal variables between PCOS patients and controls are shown in Table 1. PCOS patients were younger and presented with statistically significant increases in hirsutism scores and free testosterone levels, and decreased SHBG concentrations, compared with nonhyperandrogenic controls. No other differences were found, although indexes of insulin resistance such as fasting insulin and HOMA-IR showed median values that were close to twice as high in PCOS patients compared with nonhyperandrogenic controls. PCOS patients and controls presented metabolic comorbidities with similar frequencies.

Identification of differentially expressed genes between PCOS patients and nonhyperandrogenic women

After removing the noninformative probe sets from the analysis, 3,055 of the 22,284 transcripts evaluated on the Affymetrix HG-U133A chip were subsequently analyzed. Microarray data were analyzed by an uncorrected two-tail standard *t* test after normalization by the MAS5.0 and RMA algorithms and showed that 391 probe sets were differentially expressed between PCOS patients and nonhyperandrogenic women.

To control for multiple testing, we applied the more strict SAM procedure, using an estimated false discovery rate of 0.5%, and 78 of the 391 dysregulated probe sets retained statistical significance. These probe sets exhibited differential gene expression 1.5-fold or greater. Often more than one probe set detected a similar change in the same gene, providing additional validation of the expression data (Tables 2 and 3).

Of the 78 probe sets, 42 of them, representing 39 genes, were overexpressed in PCOS patients compared with the nonhyperandrogenic women (Table 2), whereas 36 probe sets, representing 24 genes, were underexpressed (Table 3).

To confirm the gene expression pattern by an independent method, we performed RT-qPCR for 50 genes. We found a close correlation between microarray and real-time PCR data, and the differential expression was confirmed for most genes. Figure 1 shows the comparison of gene expression by both methods for 12 genes.

Influence of age, hyperandrogenism, and insulin resistance on gene expression

Because patients were younger than controls, we studied the correlation of age and the levels of expression of the 63 genes described, including PCOS patients and nonhyperandrogenic women as a whole. Age showed significant correlations with the expression of CHI3L1 ($r = 0.59$, $P = 0.021$), CLIC5 ($r = -0.63$, $P = 0.012$), JARID1A ($r = -0.66$, $P = 0.007$), NRID2 ($r = -0.66$, $P = 0.008$), SCD ($r = -0.62$, $P = 0.014$), TCF4 ($r = -0.64$, $P = 0.011$), and SERINC3 ($r = -0.62$, $P = 0.015$), suggesting that the up-regulation or down-regulation of these genes seen in PCOS samples might result from the younger age of the patients.

Furthermore, the dysregulation of gene expression in the omental tissue of PCOS patients may be a consequence of hyperandrogenism or may also play a causative role in the insulin resistance characteristic of the syndrome. Of note, several of the genes dysregulated in the omental fat of our

TABLE 1. Clinical and biochemical characteristics of PCOS patients and controls

	Controls (n = 7)	PCOS patients (n = 8)	*P* value
Waist to hip ratio	0.84 (0.10)	0.77 (0.09)	0.281
Hirsutism score	2 (3)	9 (12)	0.004
Systolic blood pressure (mm Hg)	130 (20)	130 (0)	0.902
Diastolic blood pressure (mm Hg)	70 (20)	80 (15)	0.456
Total cholesterol (mg/dl)	180 (43)	178 (74)	0.779
High-density lipoprotein cholesterol (mg/dl)	49 (16)	37 (10)	0.072
Low-density lipoprotein cholesterol (mg/dl)	110 (32)	113 (41)	0.536
Triglycerides (mg/dl)	104 (52)	123 (66)	0.463
Total testosterone (ng/dl)	46 (31)	76 (54)	0.336
Free testosterone (ng/dl)	1.0 (0.5)	1.6 (1.1)	0.040
SHBG (μg/dL)	325 (124)	161 (146)	0.009
Dehydroepiandrosterone-sulfate (ng/ml)	982 (1159)	1330 (1630)	0.128
17-Hydroxyprogesterone (ng/ml)	0.8 (1.0)	0.8 (0.5)	0.535
Androstenedione (ng/ml)	2.0 (0.7)	4.0 (3.7)	0.053
Fasting glucose (mg/dl)	90 (17)	95 (4)	0.281
Fasting insulin (μUI/ml)	11.8 (17.8)	23.8 (25.6)	0.121
Glucose/insulin ratio	0.06 (0.03)	0.03 (0.03)	0.094
Insulin resistance (HOMA-IR)	2.8 (8.0)	5.4 (6.6)	0.232
Dyslipidemia	1 (14%)	0 (0%)	0.467
Hypertension	3 (43%)	2 (25%)	0.326
Impaired fasting glucose	1 (14%)	0 (0%)	0.467
Type 2 diabetes	0 (0%)	0 (0%)	1.000

Data are medians (interquartile range) or raw numbers (percentage) for continuous and discontinuous variables, respectively. To convert to SI units, multiply cholesterol by 0.0259 (resulting in mmol/liter), triglycerides by 0.0113 (resulting in mmol/liter), total testosterone by 0.03467 (resulting in nmol/liter), free testosterone by 34.67 (resulting in pmol/liter), SHBG by 0.111 (resulting in nmol/liter), dehydroepiandrosterone-sulfate by 0.002714 (resulting in μmol/liter), 17-hydroxyprogesterone by 3.026 (resulting in nmol/liter), androstenedione by 3.49 (resulting in nmol/liter), glucose by 0.0555 (resulting by mmol/liter), and insulin by 6.945 (resulting in pmol/liter).

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TABLE 2. Up-regulated gene transcripts in PCOS patients compared with nonhyperandrogenic subjects

Affymetrix probe set	Gene name	Gene symbol	Fold change	Function
205969_at	Arylacetamide deacetylase (esterase)	AADAC	1.80	Lipid metabolism
207328_at	Arachidonate 15-lipoxygenase	ALOX15	1.66	Lipid metabolism
209788_s_at	Type 1 tumor necrosis factor receptor shedding aminopeptidase	ARTS-1	2.23	Fat cell differentiation
219866_at	Chloride intracellular channel 5	CLIC5	1.51	Chloride transport
213998_s_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	DDX17	1.61	RNA helicase
219237_s_at	DnaJ (HSP40) homolog, subfamily B member 14	DNAJB14	1.68	Response to stress
220392_at	Early B-cell factor 2	EBF2	1.72	Transcription
201693_s_at	Early growth response 1	EGR1	1.51	Transcription
205066_s_at	Ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1	1.70	Insulin signaling
206404_at	Fibroblast growth factor 9 (glia-activating factor)	FGF9	1.54	Growth factor activity
219054_at	Hypothetical protein FLJ14054	FLJ14054	1.73	
204358_s_at	Fibronectin leucine rich transmembrane protein 2	FLRT2	1.67	Receptor signaling
219250_s_at	Fibronectin leucine rich transmembrane protein 3	FLRT3	1.57	Receptor signaling
202554_s_at	Glutathione S-transferase M3	GSTM3	1.71	Detoxification
213831_at	Major histocompatibility complex, class II, DQ α 1	HLA-DQA1	2.09	Immune response
205404_at	Hydroxysteroid (11-β) dehydrogenase 1	HSD11B1	1.50	Steroid metabolism
215698_at	Jumonji, AT rich interactive domain 1A (RBBP2-like)	JARID1A	2.05	Transcription
202040_s_at	Jumonji, AT rich interactive domain 1A (RBBP2-like)	JARID1A	1.58	Transcription
207092_at	Leptin (obesity homolog, mouse)	LEP	1.50	Energy metabolism
219759_at	Leukocyte-derived arginine aminopeptidase	LRAP	1.55	Antigen processing
213764_s_at	Microfibrillar associated protein 5	MFAP5	1.72	Extracellular matrix structural constituent
213765_at	Microfibrillar associated protein 5	MFAP5	1.66	Extracellular matrix
219789_at	Natriuretic peptide receptor C/guanylate cyclase C	NPR3	2.25	Peptide receptor activity
201467_s_at	NAD(P)H dehydrogenase, quinone 1	NQO1	1.78	Detoxification
201468_s_at	NAD(P)H dehydrogenase, quinone 1	NQO1	1.78	Detoxification
209750_at	Nuclear receptor subfamily 1, group D, member 2	NR1D2	1.51	Steroid hormone receptor
214680_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	1.60	Neurotrophin receptor
203803_at	Prenylcysteine oxidase 1	PCYOX1	1.51	Prenylcysteine metabolism
222317_at	Phosphodiesterase 3B, cGMP-inhibited	PDE3B	1.52	Insulin signaling
212249_at	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85α)	PIK3R1	1.61	Insulin signaling
212629_s_at	Protein kinase C-like 2	PKN2	1.70	Signal transduction
214449_s_at	Ras homolog gene family, member Q	RHOQ	1.63	Insulin signaling
200832_s_at	Stearoyl-CoA desaturase (δ-9-desaturase)	SCD	1.61	Fatty acid biosynthesis
221473_x_at	Serine incorporator 3	SERINC3	1.62	
207057_at	Solute carrier family 16 (monocarboxylic acid transporters), member 7	SLC16A7	1.61	Pyruvate transport
221276_s_at	Intermediate filament protein syncoilin	SYNC1	1.52	Structural protein
218930_s_at	Transmembrane protein 106B	TMEM106B	1.51	
212382_at	Transcription factor 4	TCF4	1.67	Transcription
204731_at	Transforming growth factor, β receptor III (betaglycan)	TGFBR3	1.61	TGFβ signaling
221496_s_at	Transducer of ERBB2, 2	TOB2	1.96	Cell proliferation
201387_s_at	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	1.51	Protein deubiquitination
206658_at	Uroplakin 3B	UPK3B	1.74	

Fold change values were obtained by the SAM test.

PCOS patients contained putative androgen response elements in their promoters (Table 4). Therefore, we studied the correlations between the levels of expression of the 63 genes with free testosterone as an index of hyperandrogenism and with HOMA-IR as an index of insulin resistance. Free testosterone levels correlated with the expression of ACTG2 (r = −0.60, P = 0.017), EBF2 (r = −0.57, P = 0.027), HLA_DQ1A (r = −0.61, P = 0.016), JARID1A (r = 0.57, P = 0.027), RHOQ (r = 0.65, P = 0.009), and SERINC3 (r = 0.64, P = 0.011), whereas insulin resistance correlated with SCD expression (r = 0.53, P = 0.043). However, the expression of JARID1A, SERINC3, and SCD correlated also with age as described. After correcting the influence of age by partial correlation analysis, the correlations of JARID1A and SERINC3 with free testosterone, and that of SCD with insulin resistance, did not retain statistical significance (r = 0.38, P = 0.183; r = 0.51, P = 0.064; and r = 0.093, P = 0.750, respectively). Therefore, only hyperandrogenism, and not insulin resistance, correlated with the expression of a few genes (ACTG2, EBF2, HLA_DQ1A, and RHOQ) in our model.

Nevertheless, because 63 separate correlation analyses were performed for each independent variable, it must be highlighted that none of the correlations described retained statistical significance after applying a Bonferroni correction for multiple testing to the level of significance (decreasing it to $P < 0.00079$), casting doubt on the actual relevance of all these correlations.

Classification of dysregulated genes according to gene ontology categories

Genes with altered expression in PCOS phenotype were subsequently classified into GO categories. The χ^2 test revealed significant ontology groups that were differentially

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TABLE 3. Down-regulated gene transcripts in PCOS patients compared with nonhyperandrogenic subjects

Affymetrix probe set	Gene name	Gene symbol	Fold change	Function
200974_at	Actin, α 2, smooth muscle, aorta	ACTA2	0.60	Cytoeskeleton
202274_at	Actin, γ 2, smooth muscle, enteric	ACTG2	0.48	Cytoeskeleton
203180_at	Aldehyde dehydrogenase 1 family, member A3	ALDH1A3	0.48	Aldehyde detoxification
222168_at	Aldehyde dehydrogenase 1 family, member A3	ALDH1A3	0.22	Aldehyde detoxification
202357_s_at	B-factor, properdin	CFB	0.60	Complement activation
210916_s_at	CD44 antigen (homing function and Indian blood group system)	CD44	0.60	Hyaluronic acid binding
218182_s_at	Claudin 1	CLDN1	0.51	Cell adhesion
204619_s_at	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	0.60	Hyaluronic acid binding
204620_s_at	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	0.51	Hyaluronic acid binding
211571_s_at	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	0.43	Hyaluronic acid binding
215646_s_at	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	0.48	Hyaluronic acid binding
221731_x_at	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	0.47	Hyaluronic acid binding
209395_at	Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	0.51	Sugar binding
209396_s_at	Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	0.42	Sugar binding
204602_at	Dickkopf homolog 1 (Xenopus laevis)	DKK1	0.42	Wnt signaling pathway
214511_x_at	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	FCGR1A	0.59	Immune response
216950_s_at	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	FCGR1A	0.62	Immune response
204419_x_at	Hemoglobin, γ A	HBG1	0.51	Heme binding
203290_at	Major histocompatibility complex, class II, DQ α 1	HLA-DQA1	0.60	Immune response
209823_x_at	Major histocompatibility complex, class II, DQ β 1	HLA-DQB1	0.62	Immune response
221491_x_at	Major histocompatibility complex, class II, DR β 3	HLA-DRB3	0.43	Immune response
216207_x_at	Immunoglobulin κ constant	IGKC	0.61	Immune response
214669_x_at	Immunoglobulin κ light chain mRNA, partial cds	IGKV	0.55	Immune response
214677_x_at	Immunoglobulin lambda joining 3	IGLJ3	0.64	Immune response
217148_x_at	Immunoglobulin lambda joining 3	IGLJ3	0.56	Immune response
214777_at	Immunoglobulin κ light chain VKJ region mRNA	IGLV	0.40	Immune response
216576_x_at	Clone H10 anti-HLA-A2/A28 immunoglobulin light chain	IGLV	0.44	Immune response
215176_x_at	Partial mRNA for immunoglobulin light chain variable region	IGLV	0.41	Immune response
222379_at	Potassium voltage-gated channel, Isk-related family, member 4	KCNE4	0.58	Potassium ion transport
201496_x_at	Myosin, heavy polypeptide 11, smooth muscle	MYH11	0.38	Cytoeskeleton
201497_x_at	Myosin, heavy polypeptide 11, smooth muscle	MYH11	0.44	Cytoeskeleton
207961_x_at	Myosin, heavy polypeptide 11, smooth muscle	MYH11	0.50	Cytoeskeleton
205729_at	Oncostatin M receptor	OSMR	0.58	Cell proliferation
204939_s_at	Phospholamban	PLN	0.55	Calcium ion transport
204338_s_at	Regulator of G-protein signaling 4	RGS4	0.43	Signal transduction
201645_at	Tenascin C (hexabrachion)	TNC	0.47	Cell adhesion

Fold change values were obtained by the SAM test.

represented in PCOS-dysregulated genes. As shown in Table 5, several of the significantly overrepresented GO terms in PCOS patients compared with controls were related to oxidoreductase activity and toxicity including response to toxin, nicotinamide adenine dinucleotide (phosphate) dehydrogenase (quinone) activity, and oxidoreductase activity. GO terms related to cell adhesion, cell recognition, and defense response were significantly represented, yet to a lesser extent, in PCOS samples.

Searching for gene expression patterns

To study the regulatory relationships among dysregulated genes in PCOS, we conducted hierarchical clustering analysis based on their expression profiles. As shown in Fig. 2, coexpressed genes were clustered into two main branches, one of overexpressed genes (six clusters) and another of underexpressed genes four clusters). Different Affymetrix probe sets for the same gene, as well as functionally related

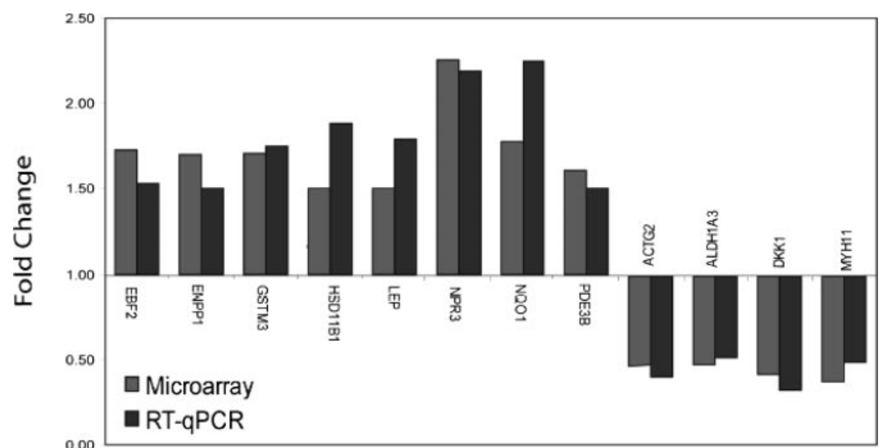

FIG. 1. Comparison of gene expression by analyses of microarray and RT-qPCR for 12 genes with altered expression in PCOS patients compared with nonhyperandrogenic women. Data are presented as fold change values from significance analysis of microarrays and ΔΔCt methods.

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TABLE 4. List of the genes showing differential expression in omental fat of PCOS patients that contained putative androgen response elements in their promoter regions

Gene	Matrix similarity	Sequence[a]	Strand	Matrix
Up-regulated genes				
ALOX15	0.91	accccacctgctGTTCtct	−	V$GREF/ARE0.2
ARTS-1	0.91	ctttcactttcgGTCCtgg	−	V$GREF/ARE0.2
FLJ14054	0.9	cgcaagctgcctGTCCcag	+	V$GREF/ARE0.2
FLRT2	0.93	taatagcatgatGTCCttt	+	V$GREF/ARE0.2
LEP	0.9	gaaatccttgatGTCCctc	−	V$GREF/ARE0.2
LRAP	0.96	ctagtacttactGTACtag	+	V$GREF/ARE0.2
LRAP	0.99	ctttcactttctGTTCtaa	−	V$GREF/ARE0.2
PCYOX1	0.9	gaggccttacaGTTGtcg	+	V$GREF/ARE0.2
PIK3R1	0.9	ggcatgcgtgatGTCCtac	+	V$GREF/ARE0.2
PIK3R1	0.97	attggactgcctGTTCtag	+	V$GREF/ARE0.2
PIK3R1	0.89	ttgtggctctgtGTACtct	+	V$GREF/ARE0.2
SCD	0.96	cgcacgcatcctGTTCagg	−	V$GREF/ARE0.2
SYNC	0.94	gatggacttgcGTACctc	−	V$GREF/ARE0.2
TCF4	0.9	taaaaacttacaGTGCtgg	+	V$GREF/ARE0.2
TGFBR3	0.91	tcccaactccatGTCCtca	−	V$GREF/ARE0.2
TGFBR3	0.91	gaagggcctcctGTGCtct	+	V$GREF/ARE0.2
UPK3B	0.81	ccggagcatggTGTTgtgt	+	V$GREF/ARE0.1
Down-regulated genes				
ACTA2	0.93	ttctcccatgctGTTCtca	−	V$GREF/ARE0.2
ACTA2	0.94	ctgccctttctGTTCtca	+	V$GREF/ARE0.2
CD44	0.9	atctttccttaGTCCttc	−	V$GREF/ARE0.2
FCGR1A	0.83	caagaaccacaTGTTgtct	−	V$GREF/ARE0.1
MYH11	0.9	ctgagacttggtGTCCata	−	V$GREF/ARE0.2
PLN	0.89	cattaacactttGTGCttc	−	V$GREF/ARE0.2

The search for androgen response elements was conducted using the MatInspector software (23) using the V$ARE0.01 and V$ARE0.02 matrixes contained in the MatBase version 6.1 library. Matrix similarity values above 0.80 are considered optimal, as perfect matches result in values of 1.00.

[a] *Uppercase letters* represent core sequence.

genes, appeared together in the same cluster, which gave us an estimation of the reliability of the expression patterns found.

Assuming that coexpressed genes may be coregulated, the TransFAT tool was used to search for common *cis*-regulatory elements. As shown in Fig. 2, we found some significant associations between putative TFBSs and groups of coexpressed genes. We considered only those TFBSs that are significantly overrepresented in most of the genes from a particular gene cluster. A significant overrepresentation of TFBSs previously involved in the pathogenesis of type 2 diabetes was found, including hepatic nuclear factor 4 (HNF4), forkhead box I, and forkhead box A2 (FOXA2) (26, 27). Several gene clusters manifested overrepresentation of transcriptional effectors of Wnt/β-catenin signaling pathway, such as TCF4 in cluster B, or lymphoid enhancer-binding factor 1 in cluster J. We have also observed significant overrepresentation of myelocytomatosis viral oncogene homolog binding sites for all three genes in cluster D and for a very homogeneous subgroup of genes from cluster F.

Discussion

Our present results revealed abnormal expression in omental adipose tissue of genes encoding certain components of several biological pathways, described herein, that appear to be specific of PCOS because patients and nonhyperandrogenic controls presented with metabolic comorbidities with similar frequencies.

Abnormalities in insulin signaling pathway

Several genes involved in insulin resistance signaling pathway presented altered expression in the omental adipose tissue from our PCOS patients, in conceptual agreement with the defective *in vitro* insulin-mediated glucose uptake by PCOS adipocytes reported earlier (28, 29).

Overexpression of ENPP1 (also termed PC-1) might contribute to the defective insulin signaling in PCOS patients, because ENPP1 is a negative regulator of insulin receptor tyrosine kinase activity and interacts with the insulin receptor (IR) α-subunit leading to inhibition of its β-subunit (30). Accordingly, increased expression of ENPP1 in adipose tissue and muscle is associated with reduced insulin action in nondiabetic insulin-resistant subjects (31). However, it should be highlighted that the search for association with genomic variation within ENPP1 in PCOS patients has yielded conflicting results (32, 33).

Overexpression of PI3KR1, encoding for the regulatory p85α subunit of phosphatydylinositol-3-kinase, could be involved in the development of insulin resistance in our PCOS patients. Mice lacking p85α have improved sensitivity to insulin (34), and an increase in p85 expression without changes in that of the catalytic subunit of phosphatydylinositol-3-kinase leads to a reduced insulin sensitivity in skeletal muscle from insulin-resistant individuals (35).

The actin cytoskeleton is essential in regulating the traffic of the glucose transporter 4 vesicles to the plasma membrane. Several of the genes dysregulated in our study might contribute to insulin resistance by interfering with this mechanism, including the overexpression of RHOQ, PKN2, and

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TABLE 5. GO terms differentially represented in PCOS-dysregulated genes

GO Term	Found[a]	Total[b]	χ^2	*P* value
PCOS up-regulated processes				
GO biological process				
Nitric oxide biosynthesis	2	19	90.86	<0.001
Response to toxin	2	22	77.95	<0.001
Xenobiotic metabolism	2	55	28.93	<0.001
Response to chemical substance	3	314	7.73	0.013
Fatty acid biosynthesis	2	80	18.76	0.002
Electron transport	2	457	4	0.021
GO molecular function				
Cytochrome b5 reductase	2	9	198.1	<0.001
NAD(P)H dehydrogenase (quinone) activity	2	50	450.0	<0.001
Membrane alanyl aminopeptidase	2	20	87.0	<0.001
Protein binding, bridging	2	109	13.0	<0.001
Iron ion binding	2	94	15.6	<0.001
Oxidoreductase activity	6	815	10.4	<0.001
Receptor signaling protein activity	2	261	3.6	<0.001
Extracellular matrix structural component	2	151	8.4	0.001
Growth factor activity	2	246	4.0	0.013
GO cellular component				
Extracellular space	4	437	7.9	0.006
Extracellular matrix	4	494	6.4	0.007
Membrane	17	5839	1.3	0.047
PCOS down-regulated processes				
GO biological process				
Cell-cell adhesion	6	248	5.0	<0.001
Cell recognition	5	27	476.7	<0.001
Antigen presentation, via MHC class II	9	33	137.45	<0.001
Immune response	7	1144	11.47	<0.001
Development	11	2805	7.25	<0.001
GO molecular function				
Hyaluronic acid binding	6	32	602.3	<0.001
IgG binding	2	10	105.02	<0.001
MHC class II receptor activity	3	36	130.58	<0.001
Sugar binding	7	189	129	<0.001
Motor activity	5	203	58.19	<0.001
Actin binding	3	377	7.87	0.001
Calcium ion binding	5	879	7.5	0.001
GO cellular component				
Extracellular matrix	8	494	6.4	<0.001
Actin filament	2	37	56.0	0.047

Only GO categories with FDR-adjusted *P* value < 0.05 in χ^2 test were considered as significantly over- or underrepresented between genes with altered expression in PCOS compared with nonhyperandrogenic samples. NAD(P)H, Nicotinamide adenine dinucleotide (phosphate); MHC, major histocompatibility class.

[a] Number of dysregulated probe sets related to the GO term.

[b] Number of probe sets in the HG-U133A chip related to the GO term.

EBF2 (36–38) and the underexpression of two isoforms of actin (ACTG2 and ACTA2) and one isoform of myosin (MYH11). Moreover, the genes encoding MYH11, ACTG2, and ACTA2 contain putative binding sites for the FOXA2 transcription factor (Fig. 2). Considering that the compensatory hyperinsulinemia attributable to insulin resistance may induce insulin-mediated phosphorylation of FOXA2, resulting in its nuclear exclusion and inactivation of its transcriptional activity (39), we speculate that inhibition of FOXA2 activity in PCOS might be related to the decreased expression of the cytoskeleton proteins found in our series, closing a vicious circle of insulin resistance, hyperinsulinemia, decreased glucose transporter 4 translocation into the cell membrane, and further insulin resistance. Yet also, PKN2 encodes a serine/threonine kinase and might be involved in the serine phosphorylation of the IR and IR substrate proteins that have been proposed to impair insulin action in PCOS (40–42).

Furthermore, RHOQ, EBF2, and PKN2 share the same pattern of expression and present the same putative TFBS for the hepatic nuclear factor 4 transcription factor previously related to type 2 diabetes (26), suggesting a common involvement of these genes in the mechanism of insulin resistance in omental adipose tissue in PCOS.

Abnormalities in genes related to lipid metabolism

PDE3B, which encodes the major phosphodiesterase isoform in adipocytes and plays a pivotal role in the antilipolytic action of insulin, and NPR3, encoding the natriuretic peptide receptor C, which might decrease the lipolytic effect induced by natriuretic peptides when activated (43), are overexpressed in our PCOS patients. These antilipolytic genes might represent a compensatory mechanism against increased catecholamine-induced lipolysis, which increases the release of free fatty acids favoring insulin resistance, observed in visceral fat cells from PCOS women (44).

Yet also, lipogenic enzymes such as SCD and ALOX15, a

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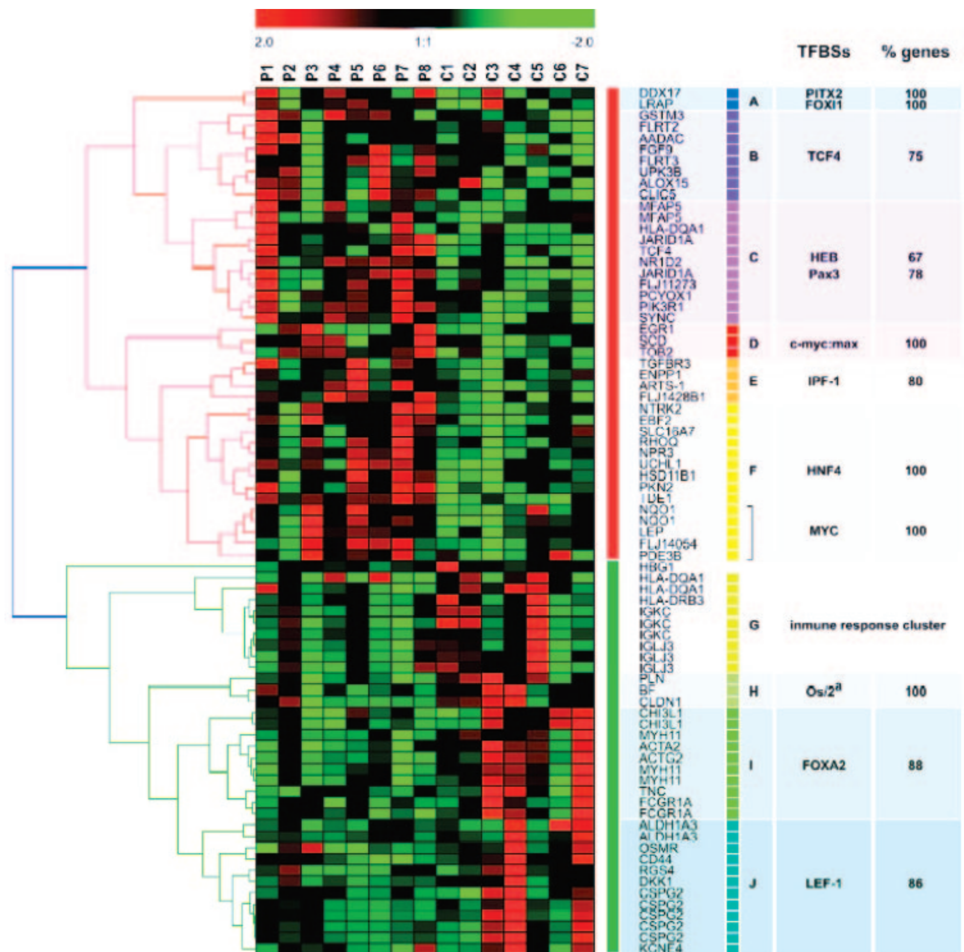

FIG. 2. Hierarchical average linkage clustering of dysregulated genes between PCOS (P1–8) and control (C1–7) omental adipose samples. Genes are grouped according to the similarity of their expression patterns across all samples, using the Pearson's correlation as metric distance. The data set includes 78 Affymetrix probe sets previously selected as significantly over- or underexpressed in PCOS samples as described in *Results*. Each *row* represents a single probe set, and each *column* represents an experimental sample. Each *cell* in the matrix represents the $\log_2$ ratio of the abundance of a transcript to the mean abundance across all experiments. Increasing *green* intensities denote genes that decrease in expression, and increasing *red* intensities denote genes that increase in expression in PCOS samples compared with control samples. *Colored bars* (A–J) indicate the gene clusters obtained. On the *right* side of the figure, putative TFBSs with significant overrepresentation in each set of coexpressed genes are shown ($P < 0.05$ in Fisher's test). We have considered only TFBSs present in a high percentage of the genes (as indicated in "% genes" column). [a] Not significant ($P = 0.06$).

lipoxygenase, were overexpressed in PCOS omental fat, possibly contributing to insulin resistance and, because lipoxygenases catalyze the oxidation of several fatty acids leading to different oxidized lipids with inflammatory functions as leukotrienes, to the chronic inflammatory milieu of PCOS. In conceptual agreement, scd1$^{-/-}$ knockout mice show improved insulin signaling in adipose tissue and skeletal muscle (45) and inhibition of lipoxygenases improves insulin action in rat models of insulin resistance and type 2 diabetes (46).

Abnormalities in genes related to the metabolic syndrome

The expression of *HSD11B1* is increased in the omental tissue of our PCOS patients. This gene encodes for type 1 11β-hydroxysteroid-dehydrogenase, which catalyzes the conversion of inactive cortisone into active cortisol. By increasing cortisol levels in adipose tissue, increased *HSD11B1* expression has been proposed to play a role in insulin-resistant disorders such as the metabolic syndrome and PCOS (47). Conflicting results have been reported regarding the involvement of common polymorphisms in *HSD11B1* in the pathogenesis of PCOS (48, 49), suggesting that *HSD11B1* overexpression might not be directly related to gene variants.

The overexpression of the leptin gene in omental adipose tissue from our PCOS patients may be secondary to the hyperinsulinemia and putatively increased local cortisol concentrations described (50), contributing to the increase in serum leptin levels in PCOS patients reported in some previous studies (51).

Evidence for altered oxidative stress processes

Increased oxidant stress occurs in insulin-resistant processes caused by hyperglycemia and elevated free fatty acids, resulting in the generation of reactive oxygen species. Exaggerated generation of the latter in response to hyperglycemia has been demonstrated in PCOS (52), together with augmentation of oxidant status and a moderated increase in some antioxidants (53). We have found overexpression of genes involved in oxidative stress and toxicity processes such as NQO1, GSTM3, PCYOX1, ALOX15, and DNAJB14 and underexpression of ALDH1A3. The altered expression of genes involved in depleting (GSTM3, NQO1, ALDH1A3) and/or generating toxic products (PCYOX1) reflect the induction or repression of biological pathways as compensatory mechanisms to reduce the increased oxidant stress in PCOS adipocytes.

Abnormalities in the Wnt-signaling pathway

DKK1, a negative regulator of this pathway, is underexpressed, and the TCF4 gene, which encodes for a Wnt transcriptional effector, is overexpressed in PCOS. We also iden-

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tified several groups of coexpressed genes showing a significant overrepresentation for putative TFBSs of this pathway (Fig. 2). Wnt signaling is involved in gender differentiation, folliculogenesis, and ovulation (54), as well as in adipogenesis (55). We hypothesize that the Wnt/β-catenin signaling pathway could be disturbed in PCOS adipose tissue, as has been shown for the ovary in previous PCOS gene expression studies (56, 57).

Immunological function

The expression of various genes belonging to the immune system, such as class II major histocompatibility complex molecules, several IgG chains, the BF complement factor, FCGR1A, and ARST-1, showed altered expression patterns in PCOS patients. This particular result might be related to differences in the accumulation of macrophages in the adipose tissue during the development of obesity, yet a previous study using microarray analysis reported a similar pattern of gene dysregulation in the ovaries of PCOS women (57). Whether these findings are related to the proinflammatory background contributing to insulin resistance and PCOS remain to be established.

Limitations

The small sample size analyzed possibly contributed to the lack of statistically significant differences in indexes of insulin resistance between patients and controls and explained why we could not match a subgroup of PCOS patients and controls for insulin resistance to delineate with precision the influence of hyperandrogenism on gene expression. Therefore, we cannot conclude whether the dysregulation of the expression of some of the genes described is a direct consequence of androgen excess on adipose tissue or a primary omental defect that could influence the pathophysiology of hyperandrogenism in PCOS patients. Also, it should be highlighted that the present results were obtained in women with morbid obesity and cannot be extrapolated to women presenting with milder grades of obesity.

Summary and conclusions

Our present results based on microarray analysis revealed differences in the expression pattern of 63 genes, supported by several normalization and statistical approaches, and by the use of RT-qPCR.

Of note, the genes dysregulated in PCOS omental fat are not only restricted to those involved in insulin signaling and resistance but also include other functional pathways related to, among others, Wnt signaling, inflammation, immune function, and oxidative stress.

The involvement of several biological pathways suggests that the participation of visceral adipose tissue in the pathogenesis of PCOS and its metabolic complications is not limited to the well-known influence of obesity on insulin resistance and the contribution of hyperinsulinism to ovarian hyperandrogenism, yet actually involves several different pathogenic mechanisms and biological pathways.

Although the use of human samples in microarray studies is limited by the high variability among individuals resulting from the genetic heterogeneity of human beings, and although gene expression analyses should be complemented by functional studies and/or proteomic techniques currently ongoing, our present results demonstrate up- and down-regulation of genes encoding molecules previously proposed to influence the pathogenesis of PCOS, insulin resistance, and the metabolic syndrome and also a considerable number of dysregulated genes that, from now on, based on our findings, should be considered as candidate genes for the etiology of PCOS.

Hopefully, the complete characterization of these genes, their proteins, and the biological pathways differentially involved in the relationship between visceral obesity and PCOS will contribute to a better understanding of the pathophysiology of this prevalent disorder.

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Address all correspondence to: Héctor F. Escobar-Morreale, M.D., Ph.D., Department of Endocrinology, Hospital Ramón y Cajal & University of Alcalá, Carretera de Colmenar km 9′1, E-28034 Madrid, Spain. E-mail: hescobarm.hrc@salud.madrid.org. Address all requests for reprints to: Belén Peral, Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas & Universidad Autónoma de Madrid, Arturo Duperier, 4, E-28029 Madrid, Spain. E-mail: bperal@iib.uam.es.

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