Proteomic analysis of human omental adipose tissue in the polycystic ovary syndrome using two-dimensional difference gel electrophoresis and mass spectrometry

Marta Cortón¹, José I. Botella-Carretero²,³, Juan A. López⁴, Emilio Camafeita⁴, José L. San Millán²,³, Héctor F. Escobar-Morreale²,³ and Belén Peral¹,⁵

¹Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, E-28029 Madrid, Spain; ²Department of Endocrinology, Hospital Universitario Ramón y Cajal and Universidad de Alcalá, E-28034 Madrid, Spain; ³Department of Molecular Genetics, Hospital Universitario Ramón y Cajal and Universidad de Alcalá, E-28034 Madrid, Spain; ⁴Unidad de Proteómica, Centro Nacional de Investigaciones Cardiovasculares, E-28029 Madrid, Spain
⁵Correspondence address. E-mail: bperal@iib.uam.es

BACKGROUND: Our aim was to study the protein expression profiles of omental adipose tissue biopsies obtained from morbidly obese women with or without polycystic ovary syndrome (PCOS) at the time of bariatric surgery to evaluate the possible involvement of visceral adiposity in the development of PCOS. METHODS: Ten PCOS patients and nine control samples were included. We used two-dimensional difference gel electrophoresis (2D-DIGE) followed by in-gel digestion, and mass spectrometry (MS) of selected protein spots. RESULTS: The 2D-DIGE technology allowed the analysis of ~1840 protein spots in the comparative study of control and patient proteomes, revealing 15 statistically significant spot changes (>2-fold, P < 0.05). Unambiguous protein identification was achieved for 9 of these 15 spots by MS. This preliminary study revealed differences in expression of proteins that may be involved in lipid and glucose metabolism, oxidative stress processes and adipocyte differentiation; they include proapolipoprotein Apo-A1, annexin V, glutathione S-transferase M3 (GSTM3), triosephosphate isomerase, peroxiredoxin 2 isoform a, actin and adipocyte plasma membrane-associated protein. The most relevant finding was an increase of GSTM3 in the omental fat of PCOS patients confirming previous studies conducted by our group. CONCLUSIONS: Proteomic analysis of omental fat reveals differential expression of several proteins in PCOS patients and non-hyperandrogenic women presenting with morbid obesity. The application of this novel methodology adds further evidence to support the role of visceral adiposity in the pathogenesis of PCOS.

Keywords: ovary; polycystic; proteomics; hyperandrogenism; visceral adipose tissue

Introduction

The polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 6–7% of premenopausal women (Asunción et al., 2000; Diamanti-Kandarakis and Christakou, 2006). PCOS is characterized by hyperandrogenism and ovarian dysfunction (Azziz et al., 2006), and appears to be a complex disorder in terms of inheritance, resulting from the interaction of protective and predisposing genomic variants with a very important environmental influence, including diet and lifestyle (Escobar-Morreale et al., 2005b).

The primary defect in PCOS appears to be an exaggerated androgen secretion, by ovarian theca cells (Nelson et al., 2001) and possibly by the adrenals, upon which several factors act triggering the development of the PCOS phenotype. Among these factors, abdominal adiposity and/or obesity play a major role in many PCOS patients, in part because of the induction of insulin resistance and hyperinsulinemia, and hyperinsulinemia meanwhile facilitates androgen secretion in the ovaries and adrenals (Gambineri and Pasquali, 2006).

PCOS patients are frequently obese (Gambineri and Pasquali, 2006). As many as 42% of women with PCOS were overweight or obese in population-based studies conducted in the USA (Azziz et al., 2004), and, conversely, up to 28% of overweight or obese women seeking medical advice for weight loss present with PCOS (Alvarez-Blasco et al., 2006). Furthermore, recent data from our group suggest that obesity is the leading factor in the development of the PCOS associated with morbid obesity, because the marked and sustained weight loss achieved after bariatric surgery actually resolved the PCOS in such patients (Escobar-Morreale et al., 2005a).

This is not really surprising considering that adipose tissue is no longer considered as a mere energy storage depot, but an...
extremely active endocrine organ that secretes hormones, growth factors, adipokines and other molecules, and participates in a large number of physiological processes that are involved in the maintenance of energy homeostasis of the body (Rosenbaum et al., 1997). Furthermore, the hyperandrogenism characteristic of PCOS patients may influence the pathophysiology of adipose tissue, because androgen excess facilitates the deposition of abdominal visceral fat in human and animal models (Xita and Tsatsoulis, 2006). It is noteworthy that abdominal adiposity may develop even in lean PCOS patients (Carmina et al., 2007), and we have proposed that the PCOS phenotype may be maintained in affected women by a vicious circle of androgen excess facilitating abdominal adiposity, insulin resistance and further androgen excess (Escobar-Morreale and Millan, 2007). For this reason, we considered it of interest to study the gene and protein expression profiles of abdominal adipose tissue in PCOS patients.

Genomic and proteomic profiling provide tools to efficiently ascertain differences in the expression of thousands of genes or proteins in complex diseases. We have recently used genome-wide expression profiling of PCOS adipose tissue by means of DNA microarrays (Corto´n et al., 2007). Our results suggested that the contribution of abdominal obesity to the pathogenesis of PCOS is not limited to the facilitation of insulin resistance described earlier, but also involves other several biological pathways. This genomic study revealed changes in the expression patterns of genes encoding for components of several biological pathways related to insulin and Wnt signaling, inflammation, immune function, lipid metabolism and oxidative stress (Corto´n et al., 2007). Gene ontology (GO) annotations and detailed analysis of the altered pathways highlighted the role of oxidative stress in PCOS omental fat. It is noteworthy that the GO analysis has revealed a significant overrepresentation of GO terms related to ‘oxidoreductase activity and toxicity’ including ‘response to toxin and nicotinamide adenine dinucleotide (phosphate) dehydrogenase (quinone) activity’, and ‘oxidoreductase activity’ in PCOS patients compared with control subjects. Moreover, we observed an increase of glutathione S-transferase M3 (GSTM3) gene that was also confirmed by quantitative RT–PCR. Interestingly, the protein encoded by this gene, an antioxidant enzyme involved in the degradation of cytotoxic products in the cell, was also found to be overexpressed in the proteomic comparative analysis as described in this report and confirmed by Western blot.

DNA arrays measure only changes at the mRNA level, whereas biological functions are mainly exerted by proteins. Therefore, gene expression may not accurately reflect biological function because differences in translational regulation and post-translational events also influence the proteome. Therefore to address the role of omental adipose tissue in PCOS, we have carried out a comparative proteomic study using high resolution two-dimensional gel electrophoresis (2-DE). To circumvent the problems associated with making comparisons across gels (Duncan and Hunsucker, 2005), regarding reproducibility and experimental variability, we have recurred to fluorescence 2D-DIGE, a technology that provides an increase in analytical precision, dynamic range and sensitivity allowing a reproducible and reliable comparative analysis of samples (Alban et al., 2003).

A proteomic approach has been recently applied to the study of PCOS focusing on ovarian tissue using conventional 2-DE and MALDI-TOF (Ma et al., 2007). In addition, serum samples from PCOS patients have been analyzed using a pre-fractionation method together with 2-DE and MALDI-TOF (Matharoo-Ball et al., 2007). The SELDI technology has been used to produce differential protein profiles in serum samples from PCOS patients (Zhao et al., 2005, 2007); however, these profiling studies need further efforts to identify and validate protein candidates associated with expression differences.

We here describe the first proteomic analysis of the role of omental fat in PCOS. Despite the fact that proteomic-based approaches have been widely used to study different human tissues in a variety of pathologies, to our knowledge, no reports have been published using this approach in human omental adipose tissue. To date, only two studies have investigated the proteome of human adipose tissue. One report from our group presented, for the first time, a protocol for the extraction, separation and identification of human adipose tissue proteins by conventional 2-DE followed by mass spectrometry (MS) (Corto´n et al., 2004). In another work, Celis et al. (2005) reported the identification of proteins from mammary adipose tissue in breast cancer patients.

To provide new insights into the role that visceral adipose tissue plays in the pathophysiology of PCOS in morbidly obese women, we have conducted a comparative proteomic study using biopsies obtained during bariatric surgery in these patients. Interestingly, our present results confirm the relevance of oxidative stress processes in PCOS, in agreement with those obtained using genomic strategies (Corto´n et al., 2007).

Materials and Methods

Subjects

Nineteen morbidly obese premenopausal women submitted for bariatric surgery were included in the present study: ten PCOS patients [age 31.6 ± 7.5 year; body mass index (BMI) 54.3 ± 10.1 kg/m²] and nine non-hyperandrogenic women [age 38.2 ± 6.2 year; BMI 50.8 ± 6.2 kg/m²]. These women were selected from a larger sample of consecutive morbidly obese patients only on the basis of availability of omental fat samples obtained during surgery. The study was conducted according to the recommendations of the Declaration of Helsinki and was approved by the ethics committees of Hospital Ramón y Cajal (Madrid). Signed informed consent was obtained from all subjects.

The diagnosis of PCOS was established by the presence of oligo-ovulation, clinical and/or biochemical hyperandrogenism, and exclusion of hyperprolactinemia, non-classic congenital adrenal hyperplasia and androgen-secreting tumors (Zawadzki and Dunaif, 1992; Azziz et al., 2006). Hirsutism was quantified by the modified Ferriman–Gallwey score (Hatch et al., 1981). Evidence for oligo-ovulation 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 days. None of the patients and controls was being treated with insulin sensitizers, statins or hormonal therapy.
The methods used to study the patients and controls, and to establish
the diagnosis of PCOS, have been described earlier (Corton et al.,
2007). Aliquots of several of the omental biopsies used here have
been used previously for genomic techniques (Corton et al., 2007).
The indication for bariatric surgery was morbid obesity in all patients,
as defined by a BMI ≥ 40 kg/m² or BMI ≥ 35 kg/m² in the presence
of significant comorbidity (National Institutes of Health Consensus
Development Panel, 1991). During surgery, biopsies of omental adipose
tissue were obtained, washed in chilled 9 g/l NaCl solution,
partitioned into pieces and immediately frozen in liquid nitrogen
and stored at −80°C until protein extraction. The surgeon aimed to
obtain the samples from similar anatomical locations in all the
subjects.

Sample preparation
Proteins were extracted from omental adipose tissue by using a
Polytron PT-1200C homogenizer (Kinematica AG, Lucerne,
Switzerland) directly in lysis buffer (8.4 mol/l urea, 2.4 mol/l thiourea, 50 g/l 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 50 mmol/l DTT). The suspension was shaken for 1 h at
room temperature and centrifuged at 200 000g for 1.5 h, according
to the procedure developed by our group (Corton et al., 2004). For
2D-DIGE, interfering components were removed using the 2D
Clean Up Kit (GE Healthcare, Chalfont St Giles, UK), and
proteins were diluted in 7 mol/l urea, 2 mol/l thiourea, 40 g/l 3--
[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate and
30 mmol/l Tris–HCl pH 8.5. The protein concentration was deter-
mined using the RC/DC Protein Assay (Bio-Rad Laboratories,
Hercules, CA, USA). For protein detection in Western blot analyses,
adipose tissue was homogenized in radioimmuno precipitation assay
(RIPA) buffer (1 g/l SDS, 5 g/l sodium deoxycholate, 10 g/l Nonidet P-40, 150 mmol/l NaCl, 50 mmol/l Tris–HCl, pH 8.0), sup-
plemented with protease inhibitors (1 mmol/l phenylmethylsulfonyl
fluoride, 0.002 g/l aprotinin and 0.002 g/l leupeptin). Cellular
debis and lipids were eliminated by centrifugation of the solubilized
samples at 18 000g for 1.5 h (4°C). Protein concentration was deter-
mained by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Conventional 2-DE
Protein extracts (100–150 μg) were diluted in the rehydration solution
[7 mol/l urea, 2 mol/l thiourea, 20 g/l 3-[3-cholamidopropyl]
dimethylammonio]-1-propanesulfonate and 0.8% (v/v) IPG buffer
adding 50 mmol/l DTT. For IPG strips of pH 4–7, proteins were
applied by in-gel rehydration, while for IPG strips 6–11 and 3–10,
proteins were applied by cup-loading to strips previously hydrated
in the rehydration solution containing 100 mmol/l hydroxyethyl disulfide,
as previously described (Corton et al., 2004). Proteins were separated
in the second dimension using 12.5% Tris–glycine gels in a Protean II
XL system (Bio-Rad Laboratories). 2-DE gels were silver stained using
a protocol compatible with MS (Shevchenko et al., 1996).

2D-DIGE separation
Proteins were labeled according to the manufacturer (GE Healthcare).
Briefly, 50 μg of each PCOS and control protein extracts were mini-

mally labeled with 400 pmol of the N-hydroxysuccinimide esters of
Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min.

An internal standard, containing equal amounts of each cell lysate,
was labeled with Cy2 fluorescent dye and used in the whole set of
experiments to ensure that every protein expressed in all five PCOS
and control samples was present in the standard protein mixture.
Since the same internal standard is run among all gels, it can be nor-
malized and matched across the gels, which dramatically decreases
gel-to-gel variation. The labeling reaction was quenched with 1 μl
of 10 mmol/l lysine on ice for 10 min and in the dark.

The PCOS, control and internal standard protein samples were
mixed adequately and run in a single gel (150 μg total protein). The
proteins were separated in the first dimension with 24 cm immobilized
pH gradient strips pH 4–7 at 0.05 mm/strip in the IPGphor IEF II
System (GE Healthcare) following a voltage increase in four steps:
300 V for 3 h, linear gradient to 1000 V in 4 h, linear gradient to
8000 V in 2 h and 8000 V until steady state. After the first dimension,
the strips were equilibrated and separated on 12% Tris–glycine gels
using an Etan Dalt Six device (GE Healthcare). The gels were scanned
with a Typhoon 9400 scanner (GE Healthcare) using appropriate
wavelengths and filters for Cy2, Cy3 and Cy5 dyes. Image
analysis was performed with DeCyder version 5.1 software (GE
Healthcare).

In-gel digestion
To visualize protein spots, 2D-DIGE gels were silver stained. The
silver-stained spots were excised manually and then digested auto-
matically with modified porcine trypsin (sequencing grade;
Promega, Madison, WI, USA) at a final concentration of 8 mg/l
in 50 mmol/l ammonium bicarbonate using a Proteineer DP protein
digestion station (Bruker-Daltonics, Bremen, Germany) according
to the protocol of Shevchenko et al. (1996). Peptide extraction was

carried out with 5 g/l trifluoroacetic acid (99.5% purity; Sigma
Chemical).

Matrix-assisted laser desorption/ionization tandem MS
MALDI-MS(/MS) and database searching
An aliquot of the digestion solution was mixed with an aliquot of
α-cyano-4-hydroxycinnamic acid (Bruker-Daltonics) in 33% aqueous
acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited
onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics) and
allowed to dry at room temperature. MALDI-MS(/MS) data were
obtained using an Ultraflex time-of-flight (TOF) mass spectrometer
equipped with a LIFT-MS/MS device (Bruker-Daltonics) (Suckau
et al., 2003). Detailed analysis of peptide mass mapping data was per-
duced using flexAnalysis software (Bruker-Daltonics). MALDI-MS
and MS/MS data were combined through MS BioTools program
(Bruker-Daltonics) to search the NCBI nr database using Mascot soft-

Western blot analysis
Biopsies were obtained from five PCOS patients other than the five
patients whose samples were used in 2D-DIGE and seven controls,
four of which were different from the control samples used in
2D-DIGE. RIPA protein extracts (15 μg) were run on 12% SDS–
PAGE and transferred to polyvinylidene fluoride membranes
(Millipore, Billerica, MA, USA) by conventional procedures. Equal
protein loading and transferring were checked by Ponceau red stain.
Membranes were immunoblotted with the rabbit anti-human
GSTM3 polyclonal antibody (at a dilution of 1:2000, kindly donated
by Dr John Hayes). Anti-rabbit IgG coupled to horse-radish peroxi-
dase (Nordic Immunological Laboratories, Tilburg, The Netherlands)
was used as secondary antibody. The specific proteins were then visu-
alized by enhanced chemiluminescence kit (GE Healthcare). Quantifi-
cation of protein expression was performed using Quantity One
Software (Bio-Rad).

Statistical analysis
Differences in clinical and hormonal variables between PCOS patients
and non-hyperandrogenic controls were evaluated by the unpaired
t-test and the results were expressed as means ± standard deviation (SD). The equality of the variances was estimated by Levene's test and the results of the t-test were interpreted accordingly, setting α = 0.05 as the level of statistical significance.

Relative protein quantification across all diseased and control samples was performed using DeCyder software, to co-detect and quantify the spots on a given gel in terms of the ratios of the Cy3 and Cy5 sample volumes to the standard Cy2 volume, and to match the spots and standardize the ratios across the gels accounting for the observed differences in the Cy2 sample volumes on the gels. This software provides two choices for determining if a protein is differentially expressed between two groups: one is based on the fold change calculated as the ratio of the average standardized abundances corresponding to the two groups of samples, which was set at greater than 2-fold threshold; and the second is based on the P-value from the t-test, setting α = 0.05 as the level of statistical significance.

### Results

A description of the clinical, metabolic and hormonal profiles of patients and controls is shown in Table I. As expected, PCOS patients presented with an increased hirsutism score and serum free testosterone and androstenedione levels, and decreased SHBG concentrations. No other statistically significant differences were observed in measurements including clinical variables, lipid profiles and indexes of insulin resistance.

As a first approach, whole fat samples from obese women were extracted and separated by 2-DE gels using different pH range in the isoelectric focusing, to optimize 2-DE experimental conditions in terms of resolution and focusing capacity. Representative 2-DE separations are shown in Fig. 1 for different pH ranges. Using the PDQuest software (Bio-Rad Laboratories), we found that the 4–7 pH range resolved 1100 proteins, whereas the 6–11 and 3–10 pH ranges resolved 500 and 600 proteins, respectively. Therefore, the comparative proteomic analysis of omental adipose tissue was carried out using the pH 4–7 range.

We also tested the reproducibility of 4–7 pH range 2-DE gels comparing the variation within different gels in the same group of individuals; the analysis of 1100 common spots revealed a coefficient of variation of ~40% between same-group gels. It is well known that variability between gels, mostly associated with the experimental procedure utilized, is a serious shortcoming of conventional 2-DE (Alban et al., 2003). To circumvent these inconveniences, 2D-DIGE was employed. This technique enables multiple samples to be analyzed on the same gel, and the inclusion of an internal standard of pooled samples on all gels allows for improved inter-gel alignment of gel features, and relative quantification of spot volumes (Alban et al., 2003). This approach reduced the coefficient of variation to 17.6%, notably lower than that of conventional methods.

By using 2D-DIGE, we analyzed the fat proteome from a group of 10 individuals, five PCOS women and five non-PCOS controls. To avoid labeling bias arising from the varying fluorescence properties of gels at different wavelengths, protein extracts were labeled using dye-swapping with either Cy3 or Cy5 fluorescent dyes, so that a given condition can be labeled with both dyes, and then each Cy3/Cy5-labeled sample pair was mixed with a Cy2-labeled internal standard onto each gel. After 2-DE, the Cy2, Cy3 and Cy5 channels were individually imaged from each of the five gels using mutually exclusive excitation and emission wavelengths. Image analysis performed with DeCyder software permitted the detection of ~1840 spots per gel. Statistical analyses were performed setting the threshold for differentially expressed proteins at greater than 2-fold and P < 0.05. The software revealed significant changes in the abundance of 15 protein spots between PCOS and control samples: 10 of them were down-regulated, and five spots were up-regulated, in PCOS omental adipose tissue with respect to that of non-hyperandrogenic women (Fig. 2).

These 15 spots were excised, digested in-gel with trypsin and analyzed by MALDI-TOF/TOF. Nine proteins could be identified by peptide mass fingerprinting and/or peptide fragmentation fingerprinting followed by a database search. Figure 3 shows the position of these nine spots in a selected 2D-DIGE gel and Table II displays detailed information about the corresponding proteins identified. The six remaining spots could not be identified probably due to the low protein amount in the spot, since only trypsin autolysis and keratin background peaks showed in the corresponding MALDI mass spectra. These results were confirmed when the 15 spots showing significant differences between the two conditions were excised from different gels and subjected to digestion and MALDI-TOF/TOF analysis. As an example, Fig. 4 shows the MALDI mass spectra that permitted the identification of the
Protein in spot 5 as peroxiredoxin 2 isoform a upon database searching and sequence confirmation of the peptide at $m/z = 1863.06$ by MALDI-MS/MS fragmentation analysis.

Proteomic analysis revealed altered expression levels of several proteins in PCOS omental adipose tissue in comparison with those of non-hyperandrogenic obese women. The PCOS samples showed up-regulation of GSTM3 that we confirmed by Western blot in one-dimensional gels. Results validating the overexpression of GSTM3 in PCOS samples are shown in Fig. 5. Annexin V was also overexpressed in PCOS samples compared with those of non-hyperandrogenic women, whereas albumin, triosephosphate isomerase I (TPI1), peroxiredoxin 2 isoform a, actin beta, adipocyte plasma membrane-associated protein (APMAP) and proapolipoprotein ApoA1 were underexpressed (Table II).

**Discussion**

Our present proteomic analysis of omental adipose tissue provides further evidence supporting the concept that the participation of visceral adiposity in the pathogenesis of PCOS is not limited to the well-known involvement of insulin resistance, hyperinsulinemia and facilitation of androgen excess (Gambineri and Pasquali, 2006). On the contrary, the differences we have found in protein expression in the omental fat of morbidly obese women presenting with or without PCOS at the time of...
bariatric surgery suggest the participation of several other mechanisms, in agreement with our recent results from applying genomic techniques to similar samples (Corton et al., 2007).

The most relevant finding has been the involvement of oxidative stress and toxicity processes in PCOS with the identification of two dysregulated proteins in PCOS adipose tissue, GSTM3 and peroxiredoxin 2 (Prx2). A condition of ‘oxidative stress’ damages components of the cell membranes, proteins or genetic material, by ‘oxidizing’ them. Increased oxidative stress has been suggested to occur in many disease processes including disorders related to insulin resistance such as PCOS which is due, in part, to the hyperglycemia and free fatty acids that induce an over-production of reactive oxygen species (ROS) (Evans et al., 2002).

In PCOS omental fat, the expression level of GSTM3 was increased compared with controls. This protein belongs to the multigene family of the GSTs, in particular to the Mu class of cytosolic GSTs, which catalyze the conjugation of glutathione with compounds containing an electrophilic center and can also exert peroxidase, isomerase and thiol transferase functions (Mannervik, 1985). This protein is an antioxidant enzyme involved in the degradation of cytotoxic products in the cell and, among other functions, GSTs are implicated in the biosynthesis of leukotrienes, prostaglandins, testosterone and progesterone, and perform a cytoprotective function through detoxification of lipid peroxidation products in adipocytes (Jowsey et al., 2003).

Table II: Proteins identified by MALDI-TOF/TOF exhibiting significant changes in PCOS patients as compared with non-hyperandrogenic women.

<table>
<thead>
<tr>
<th>Spot Numbera</th>
<th>Fold changeb</th>
<th>P-valuec</th>
<th>Protein name</th>
<th>Accession numberd</th>
<th>MW/pIe</th>
<th>Mascot scoref</th>
<th>Coverageg</th>
<th>Matched peptidesh</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.71</td>
<td>0.046</td>
<td>Annexin V</td>
<td>gi</td>
<td>999937</td>
<td>36/4.7</td>
<td>161</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2.43</td>
<td>0.00004</td>
<td>GSTM3</td>
<td>gi</td>
<td>14250650</td>
<td>27/5.4</td>
<td>177</td>
<td>37</td>
<td>9</td>
</tr>
<tr>
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<td>0.048</td>
<td>Albumin</td>
<td>gi</td>
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<td>49/6</td>
<td>119</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
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<td>0.025</td>
<td>Triosephosphate isomerase1</td>
<td>gi</td>
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<td>194</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
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<td>0.037</td>
<td>Peroxiredoxin 2 isoform 2</td>
<td>gi</td>
<td>32189392</td>
<td>22/5.7</td>
<td>170</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
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<td>Actin, beta</td>
<td>gi</td>
<td>15277503</td>
<td>40/5.8</td>
<td>109</td>
<td>19</td>
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<td>gi</td>
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<td>100</td>
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<td>6</td>
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<tr>
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<td>0.025</td>
<td>Proapolipoprotein ApoA1</td>
<td>gi</td>
<td>178775</td>
<td>29/5.3</td>
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</tr>
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*a*Spot number in 2-DE gel in Fig. 3A. *b*Average volume ratio (PCOS versus control). *c*Student t-test P-value. *d*Protein accession number from NCBI database. *e*Theoretical molecular weight in kDa and theoretical pI. *f*Protein candidate score as provided by Mascot. *g*Protein sequence coverage in percentage. *h*Number of matched peptides. *i*Identification was confirmed by MS/MS analysis of DCGATWVVLGHSER peptide with a Mascot score of 96. *j*Identification was confirmed by MS/MS analysis of KEGGLPLNIPLADVTR peptide with a Mascot score of 162.
Our present proteomic results have validated the overexpression of the GSTM3 gene in PCOS omental fat as previously revealed by two different transcript profiling strategies such as DNA microarrays and quantitative RT–PCR (Corton et al., 2007). This genomic study identified other dysregulated genes involved in depleting and/or generating toxic products such as NQO1, ALDH1A3, PCYOX1, ALOX15 and DNAJB14. Furthermore, the classification of dysregulated genes into GO categories revealed a significant overrepresentation of GO terms related to ‘oxidoreductase activity and toxicity’ in PCOS patients compared with non-PCOS controls (Corton et al., 2007).

Prx2 was found to be down-regulated in PCOS omental fat. This protein belongs to a family of antioxidant enzymes that reduce hydrogen peroxide and alkyl hydroperoxides, playing an antioxidant protective role in cells through its peroxidase activity (Chae et al., 1993). Prx2 eliminates endogenous H₂O₂, regulating the levels of H₂O₂, an intracellular signaling molecule, i.e. common to many cytokine-induced signal transduction pathways. The down-regulation of this enzyme could reflect an increase in the concentration of H₂O₂ in PCOS fat cells, thus damaging the DNA. It has been suggested that DNA damage induced by H₂O₂ may explain the increased endometrial cancer susceptibility in PCOS women (Dinger et al., 2005). In addition, it has been recently reported that Prx2 plays an important role in the regulation of pro-inflammatory responses to lipopolysaccharide through the involvement of endogenous ROS signaling (Yang et al., 2007). The altered expression of Prx2 protein in PCOS omental fat may contribute to the inflammatory state characteristic of PCOS. Furthermore, peroxiredoxin 2 is encoded by the PRXD2 gene located at 19p13.2, which is a susceptibility region for PCOS (Urbanek et al., 2005), although there is a relatively large distance of four megabases between the PRXD2 gene and the D19S884 marker showing linkage with PCOS in previous studies (Urbanek et al., 2005).

Supporting the evidence for altered oxidative stress processes in our genomic and proteomic studies, it has been recently reported that the excessive generation of ROS in response to hyperglycemia is increased in PCOS independently.
of obesity and that this could contribute to the pro-inflammatory state that induces insulin resistance and hyperandrogenism in women with this disorder (Gonzalez et al., 2004). Women with PCOS have been reported to be more likely to experience oxidative stress than healthy women (Sabuncu et al., 2001). In this study, an increased oxidant status was found in women with PCOS, and this was related to central obesity, age, blood pressure, serum glucose, insulin and triglyceride levels and insulin resistance; moreover, their antioxidant status was found to be insufficient, thus suggesting that oxidative stress might contribute to the increased risk of cardiovascular disease in women with PCOS (Sabuncu et al., 2001). Furthermore, other studies have also revealed that oxidative stress promotes increased inflammation and induces insulin resistance (Ogihara et al., 2004), and may interfere with reproductive function (Iborra et al., 2005).

Taken together, these findings reveal the important role played by oxidative stress processes in PCOS, where the balance between ROS and antioxidants is disrupted toward an overabundance of ROS. In PCOS adipocytes, the overexpression of GSTM3 may respond to an augment of oxidized cytotoxic products that have to be eliminated by conjugation with glutathione. In a severe oxidant status, the content of glutathione is diminished, as well as the cell defenses against ROS with glutathione. In a severe oxidant status, the content of glutathione is diminished, as well as the cell defenses against ROS with glutathione.

Furthermore, the increased protein expression of annexin V in adipose tissue may be a compensatory mechanism against the oxidative stress induced by chronic inflammation. Annexin V reduces the activation of Jak2 and Stat1α in response to interferon-γ by forming a stable complex with the R2 subunit of the human interferon-γ receptor (Leon et al., 2006) and, in concert with other pro-inflammatory cytokines, interferon-γ is the most important trigger for the formation and release of ROS (Schroecksnadel et al., 2006). Additionally, given its role as a marker of apoptosis, the increased annexin V protein expression in omental adipose tissue may simply reflect the apoptosis of adipocytes and pre-adipocytes induced by the tumor necrosis factor alpha (TNF-α) secreted by these cells as a result of the inflammatory process associated with obesity (Prins et al., 1997).

Of note, the chronic inflammatory milieu associated with PCOS (Escobar-Morreale et al., 2005b) and with obesity (Fernandez-Real and Ricart, 2003) may also underlie the reduced protein expression of APMAP in PCOS omental tissue. Although the precise biological function of this glycosylated membrane protein is unknown at present, it is considered a marker of adipocyte differentiation, that is present mostly in mature adipocytes (Albrektsen et al., 2001). At least partly, the actions of the TNF-α secreted by adipocytes limit the increase in fat mass characteristic of obesity by inducing adipocyte dedifferentiation, and impairing preadipocyte differentiation (Prins et al., 1997). The decrease in APMAP expression in our PCOS samples may result from such effects of TNF-α, especially considering that administration of TNF-α reduces APMAP mRNA in adipocytes in vitro (Albrektsen et al., 2001).

Proapolipoprotein A-1 also showed a decreased expression in PCOS omental adipose tissue. Apolipoprotein A-1 is the major constituent of high-density lipoprotein (HDL) cholesterol and participates in cholesterol metabolism by extracting free cholesterol from peripheral tissues, thus exerting a cardioprotective effect by preventing lipid accumulation in arterial walls (Mooradian et al., 2006). Decreased serum HDL-cholesterol levels is the one of the most common abnormalities observed in the lipid profile of PCOS patients (Talbott et al., 1995; Rajkhowa et al., 1997), suggesting an important role in the development of cardiovascular disease in PCOS patients (Yilmaz et al., 2005). Interestingly, it has been estimated that myocardial infarction is seven times more likely in patients with PCOS than in women with normal ovaries (Dahlgren et al., 1992). The treatment with insulin sensitizers (Diamanti-Kandarakis et al., 2000) or anti-androgenic oral contraceptives (Luque-Ramirez et al., 2007) result in an increase in ApoA1 and HDL-cholesterol levels, improving other parameters related with insulin, glucose and lipid metabolism, and diminishing the overall cardiovascular risk associated to PCOS.

It is noteworthy that the expression of ApoA1 gene is precisely regulated by various transcription factors at the transcriptional and post-transcriptional level (Mooradian et al., 2006), among them FoxA/HNF-3 and HNF-4 transcription factors. Significant overrepresentation of putative binding sites for these transcription factors was found in groups of co-expressed genes differentially dysregulated in the genomic

**Figure 5:** Western blot analysis for GSTM3 protein. (A) Results obtained using protein extracts from five PCOS omental biopsies and seven control samples. (B) Graphical representation of the average relative intensity values for control and patient samples. The median expression in each group is indicated by a horizontal line and the P-value obtained from Mann–Whitney analysis is indicated.
study performed in PCOS (Corton et al., 2007); however, ApoA1 gene was not revealed as differentially expressed in the transcription profiling study. Despite all these findings, it is not known whether underexpression of ApoA1 protein in PCOS is mediated through obesity per se or is independent of BMI and is thus the result of other metabolic factors.

The involvement of cytoskeleton proteins in the pathogenesis of PCOS previously suggested by our recent genomic study (Corton et al., 2007) is also supported by the present proteomic findings. Actin protein content was statistically reduced in PCOS patients with respect to control subjects, and this finding may be related to the insulin resistance of PCOS patients considering that the actin cytoskeleton plays an important role in regulating the insulin-mediated traffic of glucose transporter 4 vesicles to the adipocyte plasma membrane, a process involving also Rho GTPases (Chiang et al., 2001) such as RHOQ, which is differentially expressed in PCOS omental fat (Corton et al., 2007). Actin is not only a cytoskeleton component, but also participates in gene expression regulation. Thus, actin has been recently reported to regulate the expression of c-fos serum response element-binding transcription factor (SRF), a nuclear protein involved in cell cycle regulation, apoptosis, cell growth and cell differentiation (Vartiainen et al., 2007).

Furthermore, the down-regulation in PCOS adipose tissue of TPI1 may contribute to cytoskeleton dysregulation: TPI1, an enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehydes-3-phosphate, completing the preparatory phase of glycolysis, interacts with Rho in the regulation of intracellular sodium, probably through Na,K-ATPase activation, by providing glycolytic ATP that fuels energy for membrane functions (Jung et al., 2002). Also, TPI1 is associated with the plasma membrane and binds indirectly to structural proteins such as actin and microtubules (Jung et al., 2002). Therefore, down-regulation of TPI1 may interfere with several functions of adipose tissue cells. On the contrary, we have no reasonable explanation for the reduced albumin content of the omental samples from PCOS patients when compared with that of controls.

The described proteins (or genes) have been identified as expressed in human and/or mouse adipose tissue in previous studies involving fat tissue (Albreksten et al., 2001; Lanne et al., 2001; Corton et al., 2004, 2007; Celis et al., 2005). Most of these differential proteins are not adipose tissue specific; however, it must be taken into account that in white adipose tissue, the major organ for storage of triacylglycerols in mammals, a great number of proteins expressed are not only involved in lipid metabolism, but also in many other processes such as satiety, bone function and reproduction.

Although our present results may provide the basis for a better understanding of the role that visceral adiposity plays in the pathogenesis of the PCOS associated with morbid obesity, we must also acknowledge certain limitations of our experimental design. First, we have used whole adipose tissue biopsies and, therefore, the differences in protein expression found here do not result only from those present in adipocytes but also from other cell types including inflammatory cells and blood vessels. However, this approach has probably permitted the detection of differences pertaining to inflammatory processes that result from the secretion of cytokines and other molecules by immune cells within the stroma of visceral fat. Second, the small number of samples used in this study is an important shortcoming that reduces the statistical power of the analysis. We chose to analyze a small number of subjects in order to keep homogeneity in terms of obesity-related phenotypes such as type 2 diabetes, dyslipidemia and cardiovascular disease. Third, the present results were obtained in women with morbid obesity, and must not be extrapolated to women presenting with milder grades of obesity. It must also be noted that 2-DE presents some limitations such as poor representation of low-abundant, extreme pl and molecular weight and very hydrophobic proteins, and therefore some differentially expressed proteins (e.g. regulatory proteins) involved in the pathogenesis of the disorder may be missed. In addition, our study focused on the 4—7 pH range, obviating regulated proteins with a more alkaline or acidic isoelectric point.

In summary, our present proteomic approach reveals differences between PCOS patients and non-hyperandrogenic women in the expression of several proteins that are involved in lipid and glucose metabolism, inflammation and oxidative stress processes. This work constitutes one of the few proteomic studies reported in PCOS, as well as the first analysis of human visceral adipose tissue. It is noteworthy that the present proteomic results complement those of the genomic studies previously reported by our group, and therefore may contribute to explain the role of abdominal adiposity in the pathogenesis of PCOS. Further characterization of the genes and proteins differentially expressed in the omental fat of morbidly obese PCOS patients may contribute to a better understanding of the pathophysiology of this common disorder.

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