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

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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 (Corton *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 (Corton *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 recoursed 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) (Corton *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 (Corton *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/m^2] and nine non-hyperandrogenic women (age 38.2 ± 6.2 year; BMI 50.8 ± 6.2 kg/m^2). 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 $\geq$ 40 kg/m^2 or BMI $\geq$ 35 kg/m^2 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 −80ºC 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 200 000*g* 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 determined using the RD/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). For protein detection in Western blot analysis, 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), supplemented with protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 0.002 g/l aprotinin and 0.002 g/l leupeptin). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 18 000*g* for 1.5 h (4ºC). Protein concentration was determined 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 minimally 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 normalized 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 mA/IPG 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 Ettan 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 automatically 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 performed using flexAnalysis software (Bruker-Daltonics). MALDI-MS and MS/MS data were combined through MS BioTools program (Bruker-Daltonics) to search the NCBInr database using Mascot software (Matrix Science, London, UK) (Perkins *et al*., 1999).

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 peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used as secondary antibody. The specific proteins were then visualized by enhanced chemiluminescence kit (GE Healthcare). Quantification 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 $\alpha = 0.05$ 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 $\alpha = 0.05$ 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

Table I: Clinical and biochemical characteristics of the PCOS patients and non-hyperandrogenic controls from whom the omental biopsies used for different techniques were obtained.

	Controls ($n = 9$)	PCOS ($n = 10$)	*P*-value
Waist to hip ratio	0.83 ± 0.11	0.82 ± 0.07	0.824
Hirsutism score	1.3 ± 1.5	7.8 ± 6.2	0.010
Systolic blood pressure (mm Hg)	134.4 ± 12.9	124.6 ± 13.3	0.136
Diastolic blood pressure (mm Hg)	74.4 ± 7.8	71.8 ± 12.4	0.617
Mean blood pressure (mm Hg)	94.4 ± 8.2	89.4 ± 11.6	0.320
Total cholesterol (mg/dl)	178.0 ± 30.9	174.8 ± 33.1	0.834
HDL—cholesterol (mg/dl)	43.9 ± 9.5	37.0 ± 8.7	0.128
LDL—cholesterol (mg/dl)	104.8 ± 23.0	113.1 ± 24.9	0.471
Triglycerides (mg/dl)	129.0 ± 85.0	132.1 ± 40.8	0.922
Total testosterone (ng/dl)	45.7 ± 18.4	61.3 ± 32.6	0.212
Free testosterone (ng/dl)	0.84 ± 0.33	1.36 ± 0.65	0.043
SHBG (μg/dl)	299 ± 90	196 ± 110	0.042
Dehydroepiandrosterone-sulfate (ng/ml)	1025 ± 546	1864 ± 1030	0.058
17-hydroxyprogesterone (ng/ml)	0.86 ± 0.61	0.69 ± 0.41	0.486
Androstendione (ng/ml)	2.1 ± 0.4	3.4 ± 1.6	0.030
Fasting glucose (mg/dl)	97.4 ± 24.9	92.3 ± 9.1	0.549
Fasting insulin (μU/ml)	18.7 ± 16.7	24.9 ± 13.0	0.378
Glucose/insulin ratio	0.06 ± 0.03	0.04 ± 0.02	0.071

Data are means ± SD.
LDL, low-density lipoprotein. To convert to SI units, multiply, cholesterol by 0.0259 (result in mmol/l), triglycerides by 0.0113 (result in mmol/l), total testosterone by 0.03467 (result in nmol/l), free testosterone by 34.67 (result in pmol/l), SHBG by 0.111 (result in nmol/l), dehydroepiandrosterone-sulfate by 0.002714 (result in μmol/l), 17-hydroxyprogesterone by 3.026 (result in nmol/l), androstenedione by 3.49 (result in nmol/l), glucose by 0.0555 (result in mmol/l) and insulin by 6.945 (result in pmol/l).

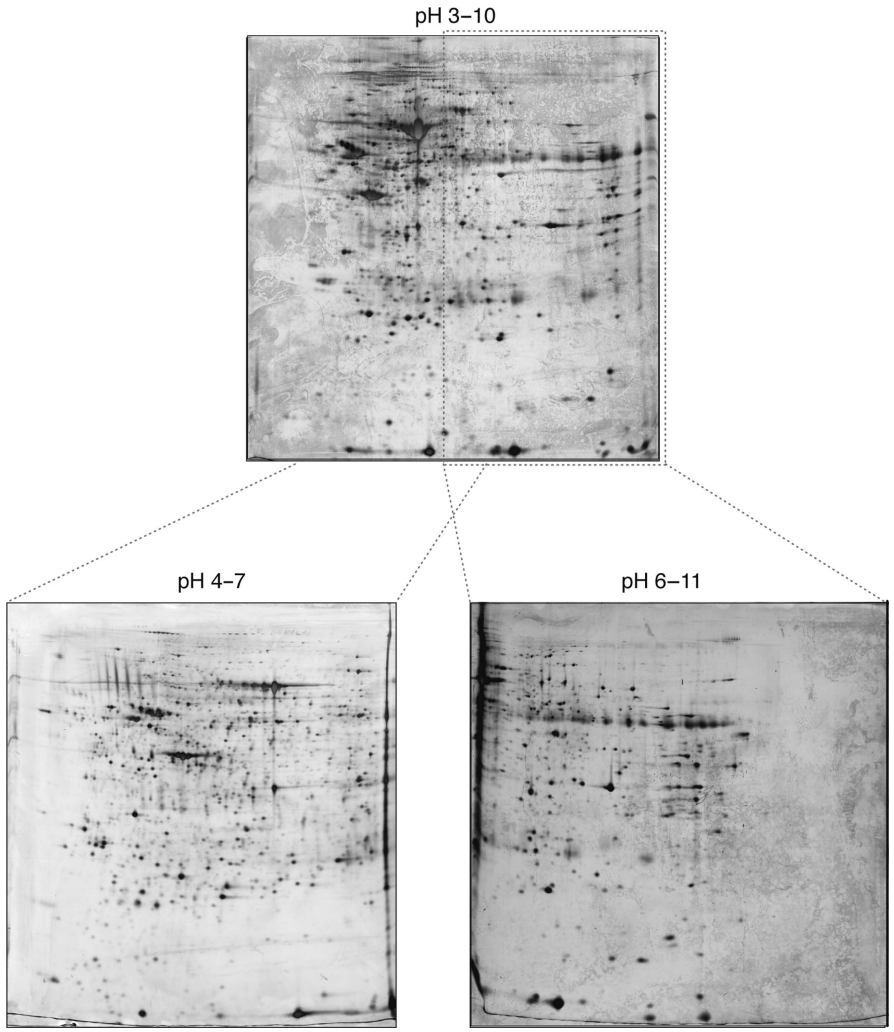

Figure 1: Protein pattern from human omental adipose tissue focused in three pH ranges

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.

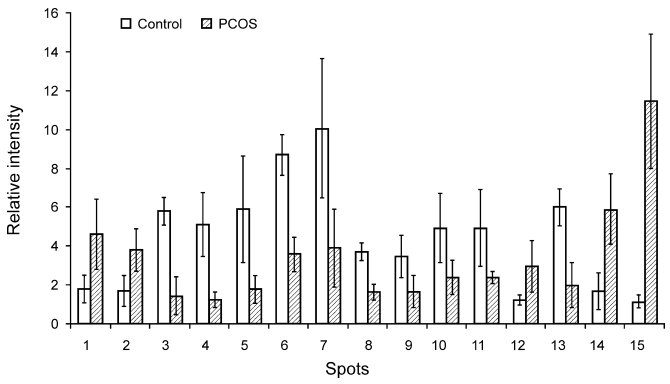

Figure 2: Relative intensity between the PCOS patients and control samples of the 15 spots showing significant differences in 2D-DIGE Ten of these were down-regulated and five were up-regulated in the PCOS group when compared with control samples. Spots number 1 to 9 are the same as the spots represented in Fig. 3A, whereas spots 10 to 15 could not be further identified by MS. Data are expressed as means $\pm$ SD

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

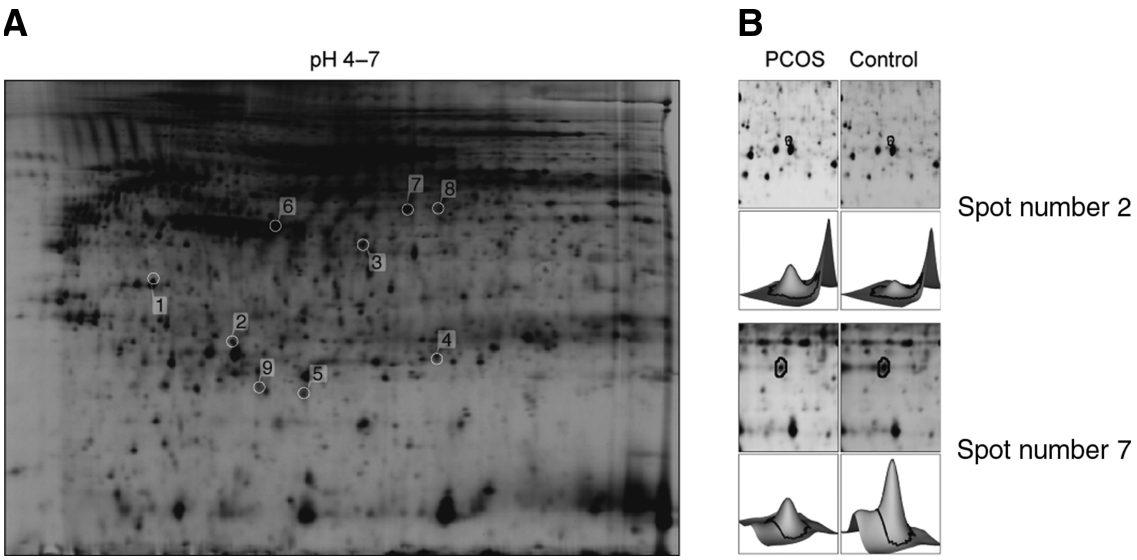

Figure 3: 2D-DIGE of fat proteome
(**A**) Spot map corresponding to the mixed internal standard, which is common to all the gels analyzed. pH 4–7 immobilized pH gradient strips were used for isoelectric focusing and 12% SDS–PAGE for the second dimension. Numbered spots indicate proteins identified by MALDI-TOF/TOF that showed significant differential expression between PCOS and non-PCOS samples and correlate with those in Table II. (**B**) Graphical representation of differential protein expression for two selected spots as revealed by the DeCyder software

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). GSTs are also involved in diverse signaling pathways related to adipocyte differentiation. For example, they contribute to the formation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). An important function of 15d-PGJ_2 is to serve as an activating ligand for the proadipogenic transcription factor, peroxisome proliferator-activated receptor γ (PPARγ) (Jowsey *et al*., 2003).

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

Spot Number[a]	Fold change[b]	*P*-value[c]	Protein name	Accession number[d]	MW/pI[e]	Mascot score[f]	Coverage[g]	Matched peptides[h]	Biological function
1	2.71	0.046	Annexin V	gi|999937	36/4.7	161	29	10	Phospholipid binding
2	2.43	0.00004	GSTM3	gi|14250650	27/5.4	177	37	9	Glutathione metabolism
3	−4.39	0.048	Albumin	gi|27692693	49/6	119	21	8	Transport
4	−4.01	0.025	Triosephosphate isomerase[i]	gi|17389815	27/6.5	194	28	6	Glucose metabolism
5	−3.12	0.037	Peroxiredoxin 2 isoform a[j]	gi|32189392	22/5.7	170	38	5	Antioxidant activity
6	−2.58	0.048	Actin, beta	gi|15277503	40/5.8	109	19	6	Cytoskeleton
7	−2.56	0.0081	APMAP	gi|24308201	48/5.8	359	38	17	Adipocyte differentiation
8	−2.26	0.0025	Albumin	gi|27692693	49/6	100	15	6	Transport
9	−2.13	0.025	Proapolipoprotein ApoA1	gi|178775	29/5.3	287	63	18	Cholesterol transport

[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 KEGGLGPLNIPLLADVTR peptide with a Mascot score of 162.

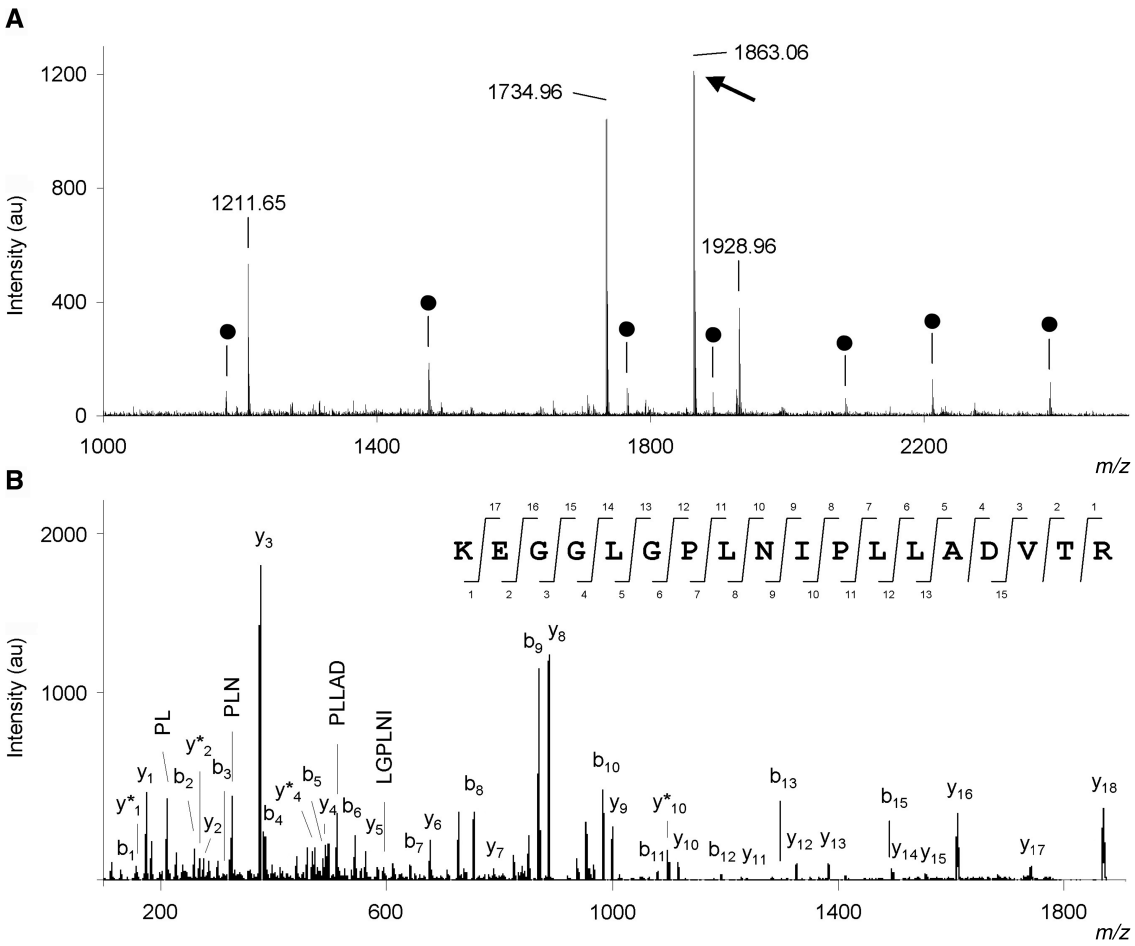

Figure 4: MALDI peptide mass fingerprinting spectra
(**A**) MALDI peptide mass fingerprinting spectrum from spot 5 is shown. Relevant mass signals employed for database searching have been labeled, and known trypsin and keratin peptide signals have been marked with a black dot. The precursor ion selected for subsequent MS/MS measurement is indicated by an arrow. (**B**) MALDI peptide fragmentation fingerprinting spectrum from the above precursor ion at $m/z =$ 1863.06. Ions ascribed to the main fragmentation series, y (C-terminal series) and b (N-terminal series), employed for database searching, have been labeled, and the sequence matched is displayed. C-terminal fragment ions corresponding to the loss of ammonia (-17 amu) are indicated by an asterisk, and identified internal fragments are labeled with their amino acid sequence

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 H_2O_2, regulating the levels of H_2O_2, 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 H_2O_2 in PCOS fat cells, thus damaging the DNA. It has been suggested that DNA damage induced by H_2O_2 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

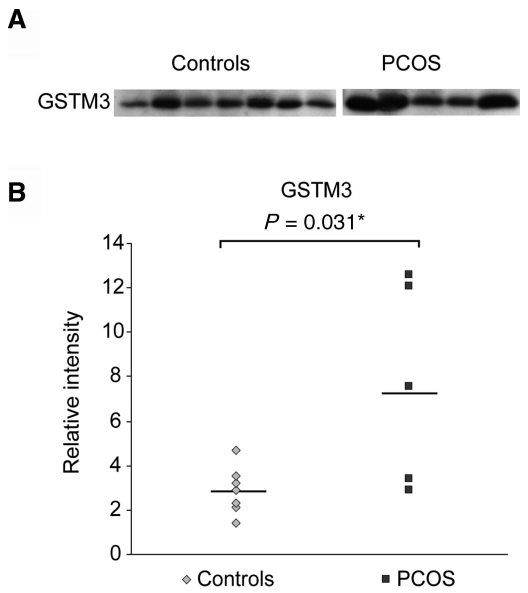

Figure 5: Western blot analysis for GSTM3 protein
(**A**) Results obtained using protein extracts from five PCOS omental biopsies and seven control samples. (**B**) Graphical representation of the average relative intensity values for control and patient samples. The median expression in each group is indicated by a horizontal line and the *P*-value obtained from Mann–Whitney analysis is indicated

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*., 2006). 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 (Pompella *et al*., 2003), whereas the overabundance of ROS contributes to insulin resistance (Rudich *et al*., 1998; Tirosh *et al*., 1999) and oxidative stress in a circular process. The underexpression of Prx2 in PCOS adipose tissue contributes to this scenario, because H_2O_2 might not be properly eliminated, therefore augmenting ROS levels in the fat cells.

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 preadipocytes 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 antiandrogenic 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

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 (Albrektsen *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 pI 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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