Metabolic Heterogeneity in Polycystic Ovary Syndrome Is Determined by Obesity: Plasma Metabolomic Approach Using GC-MS

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BACKGROUND: Abdominal adiposity and obesity influence the association of polycystic ovary syndrome (PCOS) with insulin resistance and diabetes. We aimed to characterize the intermediate metabolism phenotypes associated with PCOS and obesity.

METHODS: We applied a nontargeted GC-MS metabolomic approach to plasma samples from 36 patients with PCOS and 39 control women without androgen excess, matched for age, body mass index, and frequency of obesity.

RESULTS: Patients with PCOS were hyperinsulinemic and insulin resistant compared with the controls. The increase in plasma long-chain fatty acids, such as linoleic and oleic acid, and glycerol in the obese patients with PCOS suggests increased lipolysis, possibly secondary to impaired insulin action at adipose tissue. Conversely, nonobese patients with PCOS showed a metabolic profile consisting of suppression of lipolysis and increased glucose utilization (increased lactic acid concentrations) in peripheral tissues, and PCOS patients as a whole showed decreased 2-ketoisocaproic and alanine concentrations, suggesting utilization of branched-chain amino acids for protein synthesis and not for gluconeogenesis. These metabolic processes required effective insulin signaling; therefore, insulin resistance was not universal in all tissues of these women, and different mechanisms possibly contributed to their hyperinsulinemia. PCOS was also associated with decreased α-tocopherol and cholesterol concentrations irrespective of obesity.

CONCLUSIONS: Substantial metabolic heterogeneity, strongly influenced by obesity, underlies PCOS. The possibility that hyperinsulinemia may occur in the absence of universal insulin resistance in nonobese women with PCOS should be considered when designing diagnostic and therapeutic strategies for the management of this prevalent disorder.

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Polycystic ovary syndrome (PCOS) is the most prevalent endocrine disorder in women of fertile age (1). PCOS is characterized by androgen excess and is associated with metabolic disorders such as obesity, insulin resistance, and diabetes (2–4). The occurrence of disorders of glucose tolerance and diabetes in women with PCOS appears to be mainly dependent on the association with insulin resistance, with obesity playing a major triggering role (5). Whether the increased risk of diabetes pertains to all patients of PCOS irrespective of obesity, or only to obese patients and nonobese patients with additional risk factors, is still matter of debate (6, 7).

The application of hypothesis-free genomic and proteomic techniques to the study of PCOS confirmed the involvement of insulin resistance and low-grade chronic inflammation in its pathogenesis (8), but also revealed the participation of metabolic pathways related to iron metabolism, Wnt signaling, oxidative stress, immune function, and lipid metabolism in the pathogenesis of this prevalent disorder (8–11).

Recently introduced metabolomics aim at the identification and quantification of all metabolites in biological systems (12). Metabolites include a wide array of compound classes such as amino acids, lipids,
organic acids, nucleotides, and others which are quite diverse in their physical and chemical composition and occur in a wide range of concentrations (12). Compared with genomics, transcriptomics, and proteomics, metabolomics provides data that are the most predictive of the phenotype (12), because the metabolome is the net result of genomic, transcriptomic, and proteomic variability, and of their interaction with the environment (13).

Nontargeted hypothesis-free metabolomic approaches intend to compare as many metabolites as possible of different samples, reporting those metabolites that quantitatively differ based on statistical analysis (13). As with other “-omics” techniques, nontargeted metabolomic profiling does not rely on previous knowledge in the field and might unravel novel mechanisms of disease and even therapeutic targets that may have been lost by classic targeted approaches.

To provide new insights on the impact that obesity exerts on the metabolic derangements frequently associated with PCOS, we characterized the intermediate metabolism phenotypes associated with PCOS and obesity in premenopausal women by applying a nontargeted GC–MS–based metabolomic approach to plasma samples.

Materials and Methods

SUBJECTS

We selected plasma samples from 36 patients with PCOS and 39 control women who had no evidence of androgen excess or ovulatory dysfunction. We selected both groups from our historical database of patients and controls using age and body mass index (BMI) as sole criteria, to avoid any selection bias related to prior knowledge of their metabolic or hormonal characteristics. The selection process aimed to have similar age, BMI, and frequency of obesity, as defined by a BMI ≥30 kg/m², in both groups.

The diagnosis of PCOS relied on the presence of clinical and/or biochemical hyperandrogenism, oligoovulation, and exclusion of secondary etiologies as reported (14). Evidence of oligoovulation was provided by serum luteal phase progesterone measurements <4 µg/L (12.7 pmol/L) in patients with regular menses, oligomenorrhea (>6 cycles longer than 36 days in the previous year), or amenorrhea (absence of menstruation for 3 consecutive months). Although ovarian morphology was not analyzed, by having hyperandrogenism and oligoovulation, all patients fulfilled all the current definitions of PCOS (15–17), and PCOS was ruled out reliably in the controls because all these women did not have hyperandrogenism and showed regular ovulatory menstrual cycles.

None of the women had a personal history of hypertension, diabetes mellitus, or cardiovascular events or had received treatment with oral contraceptives, antiandrogens, insulin sensitizers, iron supplements, or drugs that might interfere with blood pressure regulation, lipid profile, or carbohydrate metabolism for the previous 6 months. We obtained written informed consent from all the participants and approval from the local Ethics Committee.

After a 3-day 300-g carbohydrate diet and 12-h overnight fasting, we obtained serum and plasma samples during the follicular phase of the menstrual cycle or during amenorrhea. We obtained plasma from whole-blood samples collected in tubes containing EDTA as anticoagulant. For the present study, we stored plasma samples at −80 °C until analyzed by GC–MS. The technical characteristics of the assays used for plasma glucose and lipids and serum hormone measurements have been described (14, 18). The composite insulin sensitivity index (19) was determined from the glucose and insulin measurements obtained during a standard oral glucose tolerance test.

SAMPLE PREPARATION

We performed GC–MS analysis according to Agilent’s specifications (20). Plasma aliquots (100 µL) were thawed to 4 °C. Samples were briefly vortex-mixed to rehomogenize. Each aliquot was supplemented with 20 µL internal standard solution (1 µg/µL succinic-d4 acid; Sigma-Aldrich Química SA). Proteins were then precipitated by the addition of 900 µL cold methanol/water (8:1 vol/ vol) followed by 4 min ultrasonication and 10 s vortex-mixing. Aliquots were subsequently maintained on ice for 10 min. After centrifugation for 10 min (19 000 g, 4 °C), 2 replicates of 200 µL each of the supernatant were transferred to a GC autosampler vial, and 20 µL myristic-d27 acid (Sigma Aldrich) was added to each aliquot and used as internal standard for retention time lock (RTL system provided in Agilent’s ChemStation Software, Agilent Technologies). Finally, the mixture was lyophilized overnight using a Lyotrap freeze–dryer (LTE Scientific).

The first step of the derivatization was methoxi- mation to prevent ring formation and to stabilize carbonyl moieties. We incubated the lyophilized plasma residues with 50 µL methoxyamine in pyridine (0.3 µg/µL) for 16 h at room temperature. To increase the volatility of the compounds, in the next step we silylated the samples using 30 µL N-methyl-N-(trimethylsilyl)trifluoroacetamide with 1% trimethyl-chlorosilane (Thermo Fisher Scientific) for 1 h at room temperature.
NONTARGETED GC-MS ANALYSIS

We analyzed the derivatized plasma samples with a GC-MS system that consisted of a HP6890 gas chromatograph coupled to a quadrupole HP5973 mass spectrometric detector (Agilent Technologies). The detector had a resolving power of unit mass resolution over mass range m/z 5–500 and mass accuracy of ± 0.2u. The system was equipped with electron impact ionization, an autosampler (7683 series), an injector (7683B series), and the ChemStation software (G1712DA, rev. 02.00). We used the retention time locking software (RTL, Agilent Technologies) to stabilize retention times from run to run.

The head pressure was adjusted in the constant pressure mode to the locked retention time of 16.727 min (17.5 μL) of the reference compound myristic-d27 acid. Derivatized sample (1 μL each) was injected in the gas chromatograph system with a split inlet (split ratio 5:1) equipped with a J&W Scientific DB 5-MS+DG stationary phase column (30 mm × 0.25 mm i.d., 0.1 μm film, Agilent Technologies) coupled to the HP5973 mass selective detector. The column temperature was maintained at 60 °C for 1 min, then increased to 325 °C at a rate of 10 °C/min, and finally held at this temperature for 10 min. The detector was operated in the scan mode with electron impact ionization (70 eV). Mass spectra were recorded after a solvent delay of 4 min with 2.46 scans/s (mass scanning range of m/z 50–600, threshold abundance value of 50 counts). The source temperature and quadrupole temperature were 230 °C and 150 °C, respectively.

We used QC samples consisting of pooled plasma to check the GC-MS system for reproducibility. QC samples were injected periodically after 12 study samples. The reproducibility of our QC sample peak areas in terms of % CV was 29% for cholesterol, 6% for glucose, and 14% for glycerol, which were within the acceptable range of <30% CV (21). To check the reproducibility of the ionization efficiency in the plasma precipitate, we performed 5 repeated GC/MS injections of the same plasma precipitate supplemented to a final concentration of 3 g/L myristic acid d27. We obtained a 5% CV of the area of the representative ion (m/z 312 at retention time = 16.727 min). Moreover, injecting another plasma precipitate 6 times provided a 15% CV for a set of 10 known metabolites spanning the entire chromatogram. In addition, before analyzing the samples, 3 blank runs injecting hexane were performed daily to check for carryover effects. We carefully randomized the samples to ensure that potential experimental drifts affected the experimental groups to the same extent.

NONTARGETED GC-MS DATA ANALYSIS

Raw GC/MS files were exported in the platform-independent netCDF format (*.cdf). We used the XCMS software (version 1.6.1) running on R 2.9.2 (R Project for Statistical Computing, http://www.r-project.org/) for automatic peak detection and peak alignment. Mass spectral features expressed as mass-to-charge ratio retention time (m/zRT) pairs were imported into Matlab (TheMathWorks). Features that were present in both plasma aliquots of ≥80% of the samples of at least 1 of the experimental groups were normalized to the internal standard succinic-d4 acid. Subsequently, we performed statistical analysis of the 2952 normalized m/zRT features obtained from XCMS. Only those fragments showing statistically significant differences among the groups of women were used to annotate peaks. Groups of features sharing the same retention time that turned out to be statistically significant and that presented with a high degree of correlation were considered representative of a single metabolite. For metabolite annotation, AMDIS (Automated Mass Spectral Deconvolution and Identification System) (National Institute of Standards and Technology) was run for peak deconvolution, and both the Fiehn GC/MS Metabolomics RTL Library and NIST mass spectral databases were used for tentative identification (match with library >65%).

TARGETED GS-MS ANALYSIS

A subset of metabolites showing differences among groups in our nontargeted experiment were further quantified for confirmatory purposes using the standard addition method. For this confirmatory experiment, we used 40 plasma samples randomly selected from those of the 36 patients and 39 controls included in the nontargeted analysis. These samples corresponded to 20 patients with PCOS and 20 controls. In both groups, 10 women were nonobese and 10 were obese. Quantifications were performed on the same GC-MS single-quadrupole instrument and chromatographic column used for the nontargeted analysis, but we modified and optimized slightly the chromatographic conditions to target the specific compounds. The mass spectrometer was operated in single ion monitoring mode. We quantified the metabolites according to their corresponding target ion and identified them according to their qualifier ions and retention times (see Supplemental Data and Supplemental Table 1, which accompany the online version of this article at http://www.clinchem.org/content/vol58/issue6, for description of the targeted quantitative method and QC parameters).
Table 1. Clinical and hormonal characteristics of patients with PCOS compared with control women who had no evidence of hyperandrogenism or ovulatory dysfunction.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 39)</th>
<th>PCOS (n = 36)</th>
<th>P value\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonobese (n = 20)</td>
<td>Obese (n = 19)</td>
<td>Nonobese (n = 18)</td>
</tr>
<tr>
<td>Age, years</td>
<td>25 (22–29)</td>
<td>28 (25–31)</td>
<td>25 (22–28)</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
<td>23.1 (21.3–24.8)</td>
<td>35.6 (33.7–37.6)</td>
<td>23.8 (22.0–25.5)</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.75 (0.72–0.77)</td>
<td>0.79 (0.76–0.82)</td>
<td>0.76 (0.73–0.80)</td>
</tr>
<tr>
<td>Frequency of smokers, n (%)</td>
<td>6 (30)</td>
<td>9 (47)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>0.8 (0–2)</td>
<td>9 (7–11)</td>
<td>11 (8–14)</td>
</tr>
<tr>
<td>Free testosterone, ng/dL</td>
<td>0.6 (0.5–0.7)</td>
<td>0.8 (0.7–0.9)</td>
<td>0.8 (0.7–0.9)</td>
</tr>
<tr>
<td>Sex hormone-binding globulin, (\mu g/dL)</td>
<td>441 (333–540)</td>
<td>279 (225–333)</td>
<td>351 (270–441)</td>
</tr>
<tr>
<td>Dehydroepiandrosterone-sulfate, ng/mL</td>
<td>1511 (1142–1879)</td>
<td>1658 (1253–2027)</td>
<td>1990 (1511–2506)</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>92 (88–95)</td>
<td>94 (92–97)</td>
<td>88 (83–92)</td>
</tr>
<tr>
<td>Fasting insulin, (\mu U/mL)</td>
<td>4.8 (3.3–6.3)</td>
<td>10.7 (8.4–13.4)</td>
<td>8.5 (6.0–11.1)</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>8.7 (6.8–10.6)</td>
<td>4.8 (3.3–6.3)</td>
<td>5.7 (4.0–7.4)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>166 (139–178)</td>
<td>181 (162–201)</td>
<td>166 (151–185)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>50 (42–54)</td>
<td>46 (39–50)</td>
<td>58 (42–73)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>100 (89–116)</td>
<td>116 (100–131)</td>
<td>97 (81–108)</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>62 (53–71)</td>
<td>88 (71–115)</td>
<td>62 (53–80)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are shown as mean (95% CI) unless noted otherwise.

\textsuperscript{b} Differences in continuous variables among groups were analyzed by 2-way ANOVA, and categorical data were analyzed by \(\chi^2\) tests.
STATISTICAL ANALYSIS

After confirming normality of dependent variables by the Kolmogorov–Smirnov test, we analyzed continuous clinical variables by 2-way ANOVA and categorical variables by χ² tests. We used 2-way ANOVA to analyze the influence of PCOS status and obesity and the interaction between both independent variables on the m/zRT intensities obtained by XCMS software, normalized to internal standard succinic acid-d₄. GC-MS variables were rank transformed before submitting them to 2-way ANOVA, since most of them did not follow a normal distribution. As in previous studies (22), we did not apply any false-discovery rate adjustment to P values because this approach may be too stringent for GC-MS–based nontargeted metabolomics. This adjustment depends on the number of univariate test comparisons equaling the number of mass ions in our dataset. However, the number of mass ions is not equivalent to the number of metabolites because several mass ions represent the same metabolite, the electron impact ionization mode leads to a high degree of fragmentation in the detector, and the same metabolite can be represented by different derivatization species. Therefore, being dependent on the P values of the comparisons of the 2952 mass ions analyzed and not on the number of metabolites potentially detected in our samples, application of a false-discovery rate may increase the false-negative rate, missing differences actually present in the population (22).

Two-way ANOVA was also used for the analysis of the effects of PCOS, obesity, and their interaction on the results derived from targeted quantifications. Finally, because 2-way ANOVA incorporates multiple comparisons among groups into its calculations, we set the level of statistical significance at α = 0.05.

Results

As expected, we found no differences in age and BMI among patients with PCOS and controls. When compared with the controls, however, patients with PCOS presented with increased waist-to-hip ratio, hirsutism score, free testosterone, dehydroepiandrosterone sulfate and fasting insulin concentrations, and decreased fasting glucose and insulin sensitivity index values (Table 1, online Supplemental Table 2), irrespective of the occurrence of obesity.

When considered as a whole, obese women presented with increased BMI and waist-to-hip ratio, free testosterone concentrations, fasting insulin, LDL cholesterol and triglyceride concentrations, reduced sex hormone–binding globulin and HDL cholesterol concentrations, and decreased insulin sensitivity index compared with their nonobese counterparts (Table 1, online Supplemental Table 2). We found no statistically significant interaction between PCOS status and obesity, indicating that the effects of PCOS were common to nonobese and obese women and, vice versa, the effects of obesity were the same in the patients with PCOS and in the controls (Table 1, online Supplemental Table 2).

Table 2 summarizes detailed peak annotations and assignment and univariate statistical analysis derived from the nontargeted GS-MS study of the interplay between PCOS and obesity. 2-Ketoisocaproic acid, α-tocopherol, alanine, and cholesterol were lower in patients with PCOS compared with controls irrespective of obesity (Fig. 1A, Table 2). Conversely, citramalic acid, glycine, gluconic acid lactone, phenylalanine, and palmitoleic acid were either higher or lower in nonobese vs obese women irrespective of PCOS (Fig. 1B, Table 2). Obesity influenced glycerol, lactic acid, and oleic acid, but this influence was different in the PCOS and control groups (Fig. 2, Table 2).

We found statistically significant interactions between PCOS and obesity in the plasma abundance of 3 metabolites also affected by obesity (lactic acid, glyc erol, and oleic acid). Three metabolites, adipic acid, linoleic acid, and glycine, were affected by the interaction between obesity and PCOS but showed no overall significant effects for any of these independent variables (Fig. 2, Table 2). Of the 6 metabolites showing statistically significant interactions among PCOS and obesity, glycerol, adipic, linoleic, and glycine acid were reduced in nonobese PCOS patients and increased in obese PCOS patients compared with their nonobese and obese counterparts (Fig. 2). Considering PCOS patients and controls as a whole, lactic acid was increased in nonobese women compared with obese women, yet this difference was caused by an increase in nonobese PCOS patients (Fig. 2). Conversely, oleic acid was increased in obese women compared with nonobese women considering PCOS patients and controls as a whole, but oleic acid concentrations decreased in nonobese PCOS patients and increased in obese PCOS patients compared with nonobese and obese controls, respectively (Fig. 2).

Finally, the targeted GS-MS quantification conducted in a subset of plasma samples from 20 patients with PCOS and 20 controls confirmed the effects of obesity on palmitoleic acid (P = 0.0022), linoleic acid (P = 0.0001), and oleic acid (P = 0.0007). On the other hand, the interactions among obesity and PCOS for adipic and oleic acid were not confirmed by this targeted analysis, yet these interactions were close to reaching statistical significance (P = 0.055) even after decreasing sample size by half.
Discussion

Our results indicate substantial metabolic heterogeneity in PCOS, modulated mostly by its association with obesity. The increase in plasma long-chain fatty acids, such as linoleic and oleic acid, and glycerol found in the obese patients with PCOS, as well as the increase in oleic and palmitoleic acid found in obese women irrespective of PCOS, suggests increased lipolysis possibly secondary to impaired insulin action at adipose tissue (23, 24). The increase in circulating free fatty acids may contribute further to the insulin resistance associated with both obesity and PCOS (25), and to the association of PCOS and obesity with the metabolic syndrome and nonalcoholic fatty liver disease (26).

These findings agree with the metabolic signature previously associated with obesity that also includes increased circulating branched-chain amino acids (BCAAs) (27). Peripheral insulin resistance leads to an increase in circulating BCAA concentrations because their utilization in tissues such as muscle requires conserved insulin signaling. In a situation of insulin resistance, BCAAs are used for gluconeogenesis through pyruvate transamination into alanine, thereby contributing to glucose intolerance (27, 28). The decreased glycine levels in our obese patients may also indicate increased utilization for gluconeogenesis. Furthermore, an increase in BCAA catabolism may explain the increase in phenylalanine concentrations in our obese women, because large neutral amino acids such as phenylalanine compete with BCAAs for transport into mammalian cells (29).

Yet the changes observed in the metabolic profile of our women with PCOS, especially in the nonobese

### Table 2. Results of nontargeted GC-MS approach to the study of the interplay between obesity and PCOS in premenopausal women. 

<table>
<thead>
<tr>
<th>Comparison by metabolite</th>
<th>Fold change</th>
<th>Quantitative ion, m/z</th>
<th>Retention time, min</th>
<th>Fiehn/NIST match, % probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls vs PCOS patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Ketoisocaproic acid</td>
<td>↑ 1.5</td>
<td>216</td>
<td>9.15</td>
<td>90</td>
</tr>
<tr>
<td>Alanine</td>
<td>↑ 1.3</td>
<td>188</td>
<td>11.2</td>
<td>97</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>↑ 1.6</td>
<td>502</td>
<td>27.45</td>
<td>88</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>↑ 1.5</td>
<td>458</td>
<td>27.56</td>
<td>97</td>
</tr>
<tr>
<td>Nonobese vs obese women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>↓ 1.2</td>
<td>311</td>
<td>18.72</td>
<td>87</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>↓ 1.2</td>
<td>120</td>
<td>13.58</td>
<td>77</td>
</tr>
<tr>
<td>Glycine</td>
<td>↑ 1.2</td>
<td>248</td>
<td>10.48</td>
<td>77</td>
</tr>
<tr>
<td>Citramalic acid</td>
<td>↓ 1.3</td>
<td>247</td>
<td>12.72</td>
<td>71</td>
</tr>
<tr>
<td>Gluconic acid lactone</td>
<td>↓ 1.2</td>
<td>217</td>
<td>17.0</td>
<td>69</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>↓ 1.0</td>
<td>191</td>
<td>7.01</td>
<td>77</td>
</tr>
<tr>
<td>Glycerol</td>
<td>↑ 1.4</td>
<td>205</td>
<td>10.05</td>
<td>91</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>↓ 1.4</td>
<td>339</td>
<td>20.53</td>
<td>99</td>
</tr>
<tr>
<td>Interaction between PCOS and obesity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>–</td>
<td>191</td>
<td>7.01</td>
<td>99</td>
</tr>
<tr>
<td>Glycric acid</td>
<td>–</td>
<td>292</td>
<td>10.85</td>
<td>95</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>–</td>
<td>275</td>
<td>13.05</td>
<td>72</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>205</td>
<td>10.05</td>
<td>91</td>
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<tr>
<td>Linoleic acid</td>
<td>–</td>
<td>337</td>
<td>20.43</td>
<td>96</td>
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<tr>
<td>Oleic acid</td>
<td>–</td>
<td>339</td>
<td>20.53</td>
<td>99</td>
</tr>
</tbody>
</table>

*Metabolites showing statistically significant differences after GC-MS raw data, normalized to internal standard succinic-d4 acid, and rank transformed. Metabolites were submitted to 2-way ANOVA introducing PCOS status and obesity as independent variables (see also Figs. 1 and 2).

*Identification was unequivocally confirmed by comparison of retention times and spectral data to the corresponding pure standard compounds. Fold changes are also presented (↑ indicates increased relative areas of the quantitative ion with respect to its corresponding control group; ↓ indicates decreased relative areas). We used AMDIS and both NIST and Fiehn libraries to identify the most likely metabolites from the features differentially present as a function of PCOS status, obesity, or their interaction.
sample, strongly suggest that insulin resistance is not universal in these patients and that the increase in serum insulin concentrations may result from entirely different mechanisms. First, the changes in glycerol and long-chain fatty acids in nonobese women with PCOS were the opposite of those found in the obese PCOS patients, since these metabolites were decreased in nonobese patients with PCOS compared with the

Fig. 1. Metabolites showing differences in the m/zRT intensities normalized to internal standard succinic-d4 acid. The box plot includes the median (horizontal line) and the interquartile range, and the whiskers indicate the minimum and maximum data values, unless outliers are present, in which case the whiskers extend to a maximum of 1.5 times the interquartile range. (A), Differences between patients with polycystic ovary syndrome (PCOS) and controls, irrespective of obesity. (B), Differences between nonobese and obese women, irrespective of PCOS status. *P < 0.05; †P < 0.01; ‡P < 0.005.
nonobese controls, suggesting suppression of lipolysis. This finding requires conserved insulin sensitivity in adipose tissue, and possibly the presence of increased insulin concentrations, although an impairment in catecholamine-induced lipolysis in subcutaneous tissue is also characteristic of nonobese women with PCOS (23, 30, 31) and might have contributed to the reduced lipolysis suggested by our present results. Second, the decrease in 2-ketoisocaproic acid concentrations in patients with PCOS suggests decreased transamination of leucine in the first step of BCAA catabolism—which is common to leucine, isoleucine, and valine—instead of the increased transamination previously suggested as a metabolic footprint of obesity and diabetes (27, 28). Under normal conditions, alanine arising from BCAA nitrogen accounts for 25% of gluconeogenesis from amino acids (32). These data, together with the decreased alanine concentrations found in patients with PCOS, suggest that BCAAs are being used for protein synthesis in these women, and not for gluconeogenesis as happens in obesity and diabetes (27, 28). However, the actual meaning of change in alanine concentrations is ambiguous because a decrease in plasma alanine concentration has been also associated with increased gluconeogenesis in diabetic db−/db− mice (33).

Although a certain degree of insulin resistance was present in our patients with PCOS because these women remained normoglycemic in the presence of hyperinsulinemia, these results suggest that peripheral insulin resistance did not dominate the picture in a significant number of PCOS patients, especially in the nonobese. Suppression of lipolysis in adipose tissue and protein synthesis in tissues such as muscle requires conserved insulin signaling (34). The finding of increased plasma lactic acid concentrations in our nonobese patients with PCOS further suggests insulin-stimulated glucose uptake and consumption in the muscle of these patients. In conceptual agreement, oocytes from nonobese patients with PCOS show increased glucose and pyruvate consumption during overnight in vitro maturation, a characteristic that is not present in oocytes when patients are treated with the insulin-lowering drug metformin (35). These re-
results indicate that there is no resistance to the actions of insulin in PCOS oocytes, and that their increased glucose consumption may result from exposure to increased insulin concentrations.

We may also speculate that the putative increase in BCAA usage with respect to that of nonhyperandrogenic women may be related to the anabolic effects of androgens on protein synthesis, for example in muscle and bone mass (36), coupled to the increased circulating insulin concentrations that characterize both lean and obese women with PCOS (2). Of note, a large body of evidence supports the possibility of insulin action varying between metabolic responses and between target tissues in women with PCOS (25, 37, 38), in agreement with our results.

For this explanation to be plausible, the increased insulin concentrations of lean women with PCOS must not result only from peripheral insulin resistance. There are scientifically sound data supporting this possibility: on the one hand, euglycemic-hyperinsulinemic clamp studies demonstrated that in nonobese women with PCOS a reduced hepatic clearance of insulin, not just peripheral insulin resistance, is a major contributor to their increased circulating insulin concentrations, whereas obese patients with PCOS presented with peripheral insulin resistance (39); on the other hand, myotubes from patients with PCOS may show normal insulin sensitivity (38). Therefore, an increase in insulin concentrations coupled to the lack of resistance for some insulin actions or lack of insulin resistance in some nonperipheral target tissues, may explain an increase in utilization of BCAAs and glucose in muscle and perhaps in other tissues, as suggested by the metabolic profile of our nonobese patients with PCOS.

The decreased concentrations of the lipid-soluble vitamin α-tocopherol could contribute to the increased oxidative stress previously associated with PCOS (40). Conversely, the finding of decreased free cholesterol concentrations in our patients with PCOS is relatively unexpected since we were not able corroborate any change in plasma lipid concentrations by classic analytical procedures in these women. Furthermore, we have no clear explanation for the changes in other metabolites, such as citramalic, adipic acid, gluconic acid lactone, or glyceric acid, although the last 2 metabolites (which are products of glucose and glycerol oxidation, respectively) may represent artifacts resulting from the high temperatures reached during gas chromatography.

Our experimental approach is not free from limitations. First, we did not apply a false-discovery rate despite conducting multiple separate tests and therefore, having set the level of statistical significance at $\alpha = 0.05$, as many as 5% of the differences in normalized m/zRT features that led to the identification of metabolites may represent false-positive results. Second, we confirmed only some of the results using targeted GC-MS–based quantification, yet the sample size in this confirmatory experiment was reduced to almost half. For these reasons, our present results should be considered preliminary until confirmed in other series of patients.

In summary, our results indicate substantial metabolic heterogeneity in PCOS, suggesting that insulin resistance and hyperinsulinemia contribute to the pathogenesis of this disorder, but that the latter is not necessarily secondary to the former. Nonobese women with PCOS present a metabolic footprint compatible with a predominant effect of hyperinsulinemia in the absence of peripheral insulin resistance at adipose tissue and muscle. This might be related to the previously described decrease in hepatic clearance of insulin in such women (39) and to the heterogeneity in insulin action between metabolic responses and between target tissues characteristic of women with PCOS (37, 38). Yet obese women with PCOS present with a metabolic profile in which the consequences of insulin resistance, possibly related to abdominal adiposity (2), predominate.

However, the most plausible scenario is that patients with PCOS may present throughout a whole spectrum of relative contributions of both pathogenetic mechanisms to hyperinsulinemia, with obesity playing a definite role as inductor of insulin resistance. The possibility that hyperinsulinemia may occur in the absence of universal insulin resistance in some women with PCOS, especially when abdominal adiposity and or obesity are not marked, should be considered when designing diagnostic and therapeutic strategies for the management of this prevalent disorder.
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