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Clinical Research Article

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Insulin-Mediated Substrate Use in Women With Different Phenotypes of PCOS: the Role of Androgens

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Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; FFA, free fatty acid; FSH, follicle-stimulating hormone; Gnonox, nonoxidative glucose metabolism; Gox, glucose oxidation; HDL, high-density lipoprotein; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LH, luteinizing hormone; Lox, lipid oxidation; PCOm, polycystic ovarian morphology; PCOS, polycystic ovary syndrome; PKA-HSL, protein kinase A–hormone-sensitive lipase complex; SHBG, sex hormone–binding globulin; WHR, waist to hip ratio.

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Abstract

Context: Few studies have explored in vivo insulin action on substrate use in women with PCOS. In particular, no data are available in women with different PCOS phenotypes.
Objective: The aim of the study was to evaluate insulin action on glucose (Gox) and lipid (Lox) oxidation, nonoxidative glucose metabolism (Gnonox), and serum free fatty acids (FFAs) in different PCOS phenotypes.
Methods: Participants included 187 nondiabetic women with PCOS diagnosed according to the Rotterdam criteria. Data from a historical sample of 20 healthy women were used as reference values. Whole-body substrate use data were obtained by the hyperinsulinemic euglycemic clamp associated with indirect calorimetry. Serum androgens were assessed by liquid chromatography–mass spectrometry and equilibrium dialysis.
Results: During hyperinsulinemia, the increase of Gox (ΔGox), Gnonox, as well as the suppression of Lox (ΔLox) and serum FFA (Δ% FFA) were altered in each PCOS phenotype. Moreover, Gnonox and Δ% FFA were lower in women with the classic phenotype than in those with the ovulatory or the normoandrogenic phenotypes, and ΔGox was lower in women with the classic than in those with the ovulatory phenotype. In multivariable

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analysis fat mass and free testosterone were independent predictors of ΔGox, Gnonox, and Δ% FFA, whereas only fat mass predicted ΔLox.
Conclusion: In women with PCOS, regardless of phenotype, insulin-mediated substrate use is impaired. This phenomenon is greater in individuals with the classic phenotype. Free testosterone plays an independent role in insulin action abnormalities in glucose and lipid metabolism.

Key Words: glucose oxidation, lipid oxidation, lipolysis, insulin sensitivity, PCOS phenotypes, androgens

Insulin resistance is a common finding in women with polycystic ovary syndrome (PCOS). When evaluated by gold-standard methodologies, this metabolic abnormality affects about 70% to 75% of patients, involving the large majority of overweight-obese women and more than half of normal-weight women (1, 2).

By using the hyperinsulinemic euglycemic clamp, insulin action on glucose metabolism is directly measured as the amount of glucose metabolized by the whole body, with skeletal muscle being the predominant tissue in hyperinsulinemic conditions (3). Interestingly, when the clamp is combined with calorimetry, it is possible to estimate separately in vivo insulin action both on the oxidative (Gox) and nonoxidative (Gnonox) glucose metabolism, as well as on lipid oxidation (Lox) (4). Moreover, the assessment of serum free fatty acid (FFA), at baseline and during hyperinsulinemia, gives information on insulin inhibition of lipolysis, that is, on adipose tissue insulin resistance.

Available data on substrate use in women with PCOS are limited, and they mainly refer to obese individuals with both hyperandrogenism and oligoanovulation (ie, the classic phenotype of PCOS) (5, 6). A reduced insulin action in Gox, Lox, and Gnonox was found in some studies that included obese women with PCOS, compared with obese controls. However, conflicting results have been reported when both obese and lean participants were investigated (7-9). In these studies, the impairment of insulin action has been attributed either to obesity (8) or to PCOS status (9). It is noteworthy that other major clinical characteristics of these women, such as hyperandrogenemia, were generally not taken into account when analyzing these parameters.

Interestingly, divergence in whole-body glucose metabolism between the different phenotypes of PCOS has been documented and suggests that not all women with PCOS are exposed to the same metabolic risk (10). In particular, women with both hyperandrogenism and oligoanovulation (ie, with the classic phenotype of PCOS) showed a more severe insulin resistance on whole-body glucose use than women without oligoanovulation (the ovulatory phenotype) or without hyperandrogenism (the normoandrogenic phenotype).

To better define the metabolic risk of women with PCOS, it would be useful to assess insulin action on substrate use in women with different PCOS phenotypes. To the best of our knowledge, no data on this issue are available yet.

The aim of this study was to evaluate insulin action on Gox, Gnonox, Lox, as well as on serum FFA concentration in a well-characterized cohort of women with PCOS, including individuals with different phenotypes of the syndrome, and to establish the potential role of excess body fat and excess androgen in these alterations.

Materials and Methods

Participants

A total of 187 White women with PCOS with normal glucose tolerance were included in the present retrospective study. All were individuals who had undergone a hyperinsulinemic euglycemic clamp procedure associated with indirect calorimetry, and all were among the women recruited to the Verona PCOS Pathophysiology and Phenotype (Verona 3P) Study (10). All these women were referred to the outpatient clinic of the Division of Endocrinology, Diabetes and Metabolism of the Verona Hospital (Italy), for hirsutism and/or menstrual abnormalities. PCOS was diagnosed according to the Rotterdam workshop criteria, that is, the presence of at least 2 of the 3 following features: clinical and/or biochemical hyperandrogenism, chronic oligoanovulation, and polycystic ovarian morphology (PCOm), after exclusion of secondary causes (11). Clinical hyperandrogenism was defined by the presence of hirsutism (modified Ferriman-Gallwey score ≥ 8), and biochemical hyperandrogenism by increased free testosterone levels, according to the Androgen Excess and PCOS Society consensus statement on PCOS (12). Chronic oligoanovulation was diagnosed by the presence of either oligomenorrhea (≤ 8 cycles/year) or, in women with regular menses, by luteal-phase serum progesterone of less than 12 nmol/L during 2 consecutive menstrual cycles. PCOm was diagnosed according to the Rotterdam workshop recommendations (11), whenever possible by a transvaginal approach. To rule

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out secondary causes, serum thyrotropin, prolactin, and 17-hydroxyprogesterone were assessed in all participants, and other investigations were carried out when appropriate on clinical grounds.

No patient was suffering from other diseases or was taking medications that could potentially interfere with the evaluations carried out in the study. In particular, no patients had received oral contraceptives, insulin-sensitizing agents, antiandrogens, or glucocorticoids in the 6 months prior to the study.

A sample of 20 healthy women, who had previously undergone a hyperinsulinemic euglycemic clamp procedure associated with indirect calorimetry, served to generate reference values on the effect of insulin on substrate use.

All these control participants were White, normal-weight, healthy women, with normal glucose tolerance, regular and ovulatory menstrual cycles, and absence of clinical signs of hyperandrogenism or PCOm. These individuals were recruited through advertisements at the local university, nursing school, and Verona City Hospital.

All participants gave their informed written consent before the study, which was conducted in accordance with the Declaration of Helsinki and approved by the institutional ethics committee.

Protocol

All participants underwent a complete physical examination, including assessment of height, body weight, waist and hip circumferences, and blood pressure. Body fat and fat-free mass were measured by bioelectrical impedance (BIA 103, Akern) (13). Hirsutism was quantified by the modified Ferriman-Gallwey score (14).

In the early follicular phase of a spontaneous menstrual cycle, or, in women with severe menstrual alterations, after at least 3 months of amenorrhea, a fasting venous blood sample was drawn for hormonal and metabolic assessment. The hormonal profile comprised gonadotropins, sex hormone–binding globulin (SHBG), total and free testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS). The metabolic assessment included lipid profile, uric acid, and glucose and insulin response to an oral glucose tolerance test, which was performed according to the World Health Organization procedure (15).

On a separate day, a hyperinsulinemic euglycemic clamp procedure was carried out to assess whole-body insulin-stimulated glucose use (16). In the basal, noninsulin-stimulated period, and under steady-state hyperinsulinemic conditions, indirect calorimetry was performed to measure O_2 consumption and CO_2 production, in order to quantify substrate oxidation (4).

Briefly, after overnight fasting, intravenous catheters were inserted in an antecubital vein of one arm for infusions and in a dorsal hand vein for arterialized blood sampling. After baseline sampling, a primed, continuous insulin infusion (Humulin R, Eli Lilly & Co) was started and maintained for 2 to 3 hours, at a constant rate of 80 $mU/m^2 \cdot min$. Euglycemia (serum glucose concentrations at ~ 90 mg/dL) was concurrently maintained throughout the clamp by a variable infusion of 20% dextrose, adjusted by monitoring plasma glucose levels in arterialized venous blood every 5 to 10 minutes. Sampling for assessing serum insulin and FFAs was made for 30 minutes at baseline and, during the last 30 minutes of the clamp, at 10-minute intervals.

We have previously found that, in nondiabetic hyperandrogenic individuals and control women, endogenous glucose production was negligible at this insulin infusion rate (17). Therefore, the amount of glucose infused (M clamp) can be considered equivalent to whole-body insulin-stimulated glucose use.

Gox and Lox rates were calculated by gas exchanges measured over 30 minutes, both at baseline and during the steady-state period of the clamp, by indirect calorimetry, using a Quark RMR instrument (COSMED) equipped with a ventilated hood. Gnonox was calculated as the difference between whole-body glucose use (M clamp) and Gox clamp during the steady-state period of the clamp.

Because muscle is responsible for most insulin-stimulated glucose metabolism (3), the glucose infusion rate during the steady-state period of the clamp, as well as the derived substrate use measures, were normalized for fat-free mass. In addition, because there were differences among PCOS patients in the serum insulin concentrations reached during the clamp, and because glucose metabolism is proportional to insulin levels, these parameters were normalized for the mean insulin concentrations reached during the steady-state period of the clamp (18). Finally, insulin-mediated suppression of serum FFA, an index of adipose tissue insulin sensitivity (19), was calculated as the percentage of reduction (Δ%) of FFA concentrations during the steady-state period of the clamp, and this value as well was adjusted for serum insulin concentrations.

Assays

Plasma glucose was assayed by a Glucose Analyzer (YSI 2300 STAT PLUS) and serum insulin was assayed by an immunoradiometric method (Biosource). Serum FFAs were measured by an enzymatic colorimetric assay (Wako Chemicals GmbH). Total and high-density lipoprotein (HDL) cholesterol, triglycerides, and uric acid

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were assayed by an automated analyzer according to standard laboratory procedures (Dimension Vista 1500, Siemens).

Serum total testosterone and androstenedione were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS), using a Micromass Quattro Premier XE Mass Spectrometer from Waters Corporation, as previously described (20). In all women, free testosterone was calculated with the Vermeulen formula, using total testosterone measured by LC-MS/MS and SHBG assayed by an immunoradiometric method (Orion Diagnostica).

In 156 PCOS patients, free testosterone fraction was also measured by the equilibrium dialysis, as previously described (20).

Calculations

Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in meters squared (m^2), and waist to hip ratio (WHR) as waist circumference in centimeters (cm) divided by hip circumference in centimeters (cm).

Gox and Lox were calculated using standard formulas (4). In these analyses the protein oxidation rate was assumed to be equal to 31.2 mg/min, the mean value previously obtained in our laboratory by measuring nitrogen urinary excretion rate in a sample of PCOS patients who underwent the same protocol.

Insulin-induced variations of substrate use (Δ) were calculated as follows:

ΔGox: Gox clamp – Gox basal;

ΔLox: Lox basal – Lox clamp;

Gnonox: M clamp – Gox clamp;

Δ% FFA: (FFA basal – FFA clamp) × 100/FFA basal.

Statistical Analysis

Continuous variables were summarized by means and SD, and categorical variables were expressed as percentages.

Normality of the distribution of the variables was assessed by the Shapiro-Wilk test, and skewed variables were log- or square root–transformed before analysis. As not all variables were normalized after transformation, to compare women with the different phenotypes of PCOS and reference values obtained in healthy controls, the *t* test for unpaired data, one-way analysis of variance or nonparametric tests (Mann-Whitney or Kruskal-Wallis) were used, as appropriate.

Univariate associations between insulin-induced variations of substrate use and each variable of interest were assessed by Spearman rank correlations.

To assess predictors of substrate use, multiple regression analysis was performed, using alternatively ΔGox, Gnonox, ΔLox, or Δ% FFA as the dependent variable, and age, fat mass (or, alternatively, BMI or waist circumference) and serum free testosterone (or alternatively other androgens) as independent variables.

P values less than .05 were considered statistically significant. Analyses were carried out using STATA version 12.1 (StataCorp).

Results

Among all women with PCOS included in the study, 165 individuals (88.2%) had clinical and/or biochemical hyperandrogenism, 152 (81.3%) had chronic anovulation, and 167 (89.3%) had PCOm. According to the combination of these features in each individual, 120 (72.7%) women had the complete PCOS phenotype (ie, clinical and/or biochemical hyperandrogenism, chronic oligoanovulation, and PCOm), 14 (8.5%) had the classic phenotype (hyperandrogenism and oligoanovulation, without PCOm), 35 (18.7%) had the ovulatory phenotype (clinical and/or biochemical hyperandrogenism and PCOm), and 18 (9.6%) had the normoandrogenic phenotype (chronic oligoanovulation and PCOm). As subgroups of women with both hyperandrogenism and oligoanovulation had similar characteristics, independently of the presence or the absence of PCOm, and those without PCOm were a small number, in subsequent analyses these 2 subgroups were combined under the term *classic phenotype*.

Table 1 shows the main clinical, hormonal, and metabolic characteristics of these women, as a whole and subdivided according to their PCOS phenotypes. In the whole cohort of women with PCOS, mean BMI was in the overweight range (28.6 ± 7.6), with a prevalent central fat distribution (waist circumference = 91.2 ± 17.8 cm). As expected, these women had increased levels of free testosterone and low levels of SHBG. The groups of women with different phenotypes significantly differed in terms of fat excess and distribution. In particular, BMI and fat mass were significantly higher in women with the classic phenotype than in those with the ovulatory or the normoandrogenic phenotypes, whereas women with both the classic and the ovulatory phenotypes had higher waist circumference and WHR than normoandrogenic women. As expected, owing to the criteria used to define the phenotypes, hirsutism scores and serum free testosterone were higher in the classic and ovulatory phenotypes than in the normoandrogenic one. Similarly, total testosterone and androstenedione were higher in women with the classic phenotype than in those with the ovulatory or the normoandrogenic phenotypes. Serum SHBG was lower in women with the classic than in

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Table 1. Main characteristics of women with polycystic ovary syndrome as a whole and subdivided according to phenotype (mean ± SD)

	Reference intervals	All PCOS N = 187	Classic PCOS N = 134	Ovulatory PCOS N = 35	Normoandrogenic PCOS N = 18	P^a between phenotypes
Age, y	–	23.6 ± 5.3	23.3 ± 5.4	23.9 ± 5.5	24.9 ± 4.5	.32
BMI	< 25	28.6 ± 7.6	29.8 ± 7.9[d,g]	26.9 ± 5.9	23.1 ± 4.7	**.01**
Waist circumference, cm	< 80	91.2 ± 17.8	93.9 ± 18.3[g]	88.1 ± 14.6[f]	77.9 ± 11.5	**.001**
WHR	< 0.80	0.85 ± 0.10	0.85 ± 0.10[g]	0.84 ± 0.08[f]	0.78 ± 0.07	**.001**
Fat mass, kg	–	27.4 ± 14.1	29.4 ± 14.7[d,g]	23.8 ± 12.0	18.7 ± 7.1	**.03**
Fat-free mass, kg	–	49.4 ± 8.2	50.6 ± 8.7[g]	48.3 ± 6.4[f]	42.7 ± 3.3	**.001**
Ferriman-Gallwey score	< 8	9.3 ± 6.8	10.0 ± 7.1[g]	9.8 ± 5.7[g]	3.3 ± 1.8	**< .001**
Free testosterone, ng/dL[b]	≤ 0.46	0.71 ± 0.33	0.79 ± 0.33[e,g]	0.57 ± 0.25	0.42 ± 0.10	**.001**
Free testosterone, ng/dL[c]	≤ 0.49	0.74 ± 0.36	0.83 ± 0.36[e,g]	0.63 ± 0.28[f]	0.41 ± 0.08	**.001**
Total testosterone, ng/dL	≤ 41	39.0 ± 16.2	41.8 ± 16.6[e,g]	32.8 ± 14.1	29.7 ± 9.8	**.03**
Androstenedione, ng/dL	≤ 240	170.8 ± 66.0	180.9 ± 68.8[e,f]	142.8 ± 50.8	147.9 ± 49.4	**.003**
DHEAS, µmol/L	< 10.8	6.0 ± 2.4	6.1 ± 2.4	6.2 ± 2.6	5.1 ± 1.6	.22
LH/FSH	< 2.0	1.9 ± 1.2	2.0 ± 1.2[d]	1.4 ± 0.9	2.0 ± 1.3	**.03**
SHBG, nmol/L	39-121	36.2 ± 20.6	33.5 ± 18.9[g]	39.9 ± 25.4	49.0 ± 17.3	**.001**
Total cholesterol, mg/dL	< 200	164.0 ± 31.4	165.1 ± 31.2	159.4 ± 35.1	164.7 ± 26.6	.41
HDL cholesterol, mg/dL	> 50	51.4 ± 14.0	50.2 ± 14.4[f]	53.1 ± 12.1	57.3 ± 13.5	**.04**
Triglycerides, mg/dL	< 150	85.3 ± 56.7	89.5 ± 59.8	77.2 ± 46.7	69.8 ± 48.3	.06
Uric acid, mg/dL	≤ 5.9	4.3 ± 1.2	4.5 ± 1.2[f]	4.0 ± 1.2	3.8 ± 0.8	**.04**
Systolic blood pressure, mm Hg	< 130	118.7 ± 13.7	118.6 ± 14.6	122.3 ± 10.3[f]	112.7 ± 10.2	**.02**
Diastolic blood pressure, mm Hg	< 85	75.4 ± 10.6	75.3 ± 11.1	76.9 ± 10.1	72.9 ± 7.5	.13

Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; HDL, high-density lipoprotein; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; SHBG, sex hormone–binding globulin; WHR, waist to hip ratio.

[a]Statistically significant *P* values are shown in bold type.

[b]Free testosterone calculated by the Vermeulen formula.

[c]Free testosterone measured by equilibrium dialysis (available in 156 women with PCOS).

[d]*P* less than .05.

[e]*P* less than .01 vs ovulatory PCOS.

[f]*P* less than .05.

[g]*P* less than .01 vs normoandrogenic PCOS.

those with the normoandrogenic phenotype. In addition, HDL cholesterol was significantly lower and uric acid higher in women with the classic than in those with the normoandrogenic phenotype.

Table 2 and Fig. 1 show the glucose metabolism parameters derived from combining the hyperinsulinemic euglycemic clamp and indirect calorimetry procedure in women with PCOS, subdivided according to phenotype. The findings were also compared with data previously obtained in healthy controls.

Plasma glucose levels were similar in women with the different phenotypes of PCOS and controls, whereas serum insulin levels significantly differed between groups, both at basal and during the steady-state period of the clamp (Table 2). In particular, at baseline serum insulin concentrations were significantly higher in women with the classic phenotype than in those with the ovulatory or the normoandrogenic phenotypes and controls, whereas normoandrogenic PCOS women and controls had similar values. However, insulin concentrations reached during the steady-state period of the clamp were higher in each phenotype of PCOS than in controls, as well as in women with the classic vs those with the ovulatory or the normoandrogenic phenotypes.

Whole-body glucose use (M clamp value) was significantly lower in each PCOS phenotype than in healthy controls. Moreover, it was lower in women with the classic phenotype than in those with the normoandrogenic ($P = .003$) or the ovulatory phenotype, though in this latter comparison the difference was of borderline significance ($P = .064$).

At baseline the Gox and Lox rate did not show differences between each PCOS phenotype and control, whereas women with the ovulatory phenotype had significantly higher serum FFA levels as compared to normoandrogenic PCOS women and controls.

Changes of Gox, Lox, Gnonox, and FFA induced by hyperinsulinemia during the clamp are reported as box plots in Fig. 1. The increase of Gox and the reduction of Lox induced by hyperinsulinemia were significantly impaired in

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Table 2. Parameters of glucose and lipid metabolism at baseline and during use of the hyperinsulinemic euglycemic clamp

	Classic PCOS N = 134	Ovulatory PCOS N = 34	Normoandrogenic PCOS N = 18	Controls N = 20	*P*[a]
Glucose basal, mg/dL	84.5 ± 8.5	82.5 ± 7.8	84.3 ± 10.6	85.3 ± 5.6	.32
Glucose clamp, mg/dL	89.4 ± 6.4	89.7 ± 6.4	89.0 ± 5.4	88.7 ± 6.6	.86
Insulin basal, mU/L	15.1 ± 12.3[b,e,g]	11.1 ± 6.6[f]	7.0 ± 5.1	6.4 ± 3.7	**< .001**
Insulin clamp, mU/L	223.4 ± 60.5[c,d,g]	189.7 ± 46.6[g]	197.7 ± 32.8[g]	143.8 ± 34.8	**< .001**
M clamp, mg/kgFFM × min	9.2 ± 3.3[e,g]	10.4 ± 3.3[g]	11.7 ± 3.6[f]	14.0 ± 2.7	**< .001**
Gox basal, mg/kgFFM × min	0.82 ± 1.81	0.75 ± 1.13	0.58 ± 1.41	1.53 ± 0.87	.08
Lox basal, mg/kgFFM × min	1.62 ± 0.51	1.72 ± 0.61	1.76 ± 0.67	1.55 ± 0.43	.73
Free fatty acid basal, µmol/L	606.9 ± 173.6	646.7 ± 254.6[d,f]	515.1 ± 168.2	528.3 ± 196.8	**.05**

Abbreviations: FFM, fat-free mass; Gox, glucose oxidation; Lox, lipid oxidation; PCOS, polycystic ovary syndrome.
[a]Statistically significant *P* values are shown in bold type.
[b]*P* less than .05 and
[c]*P* less than .01 vs ovulatory PCOS.
[d]*P* less than .05 and
[e]*P* less than .01 vs normoandrogenic PCOS.
[f]*P* less than .05 and
[g]*P* less than .01 vs controls.

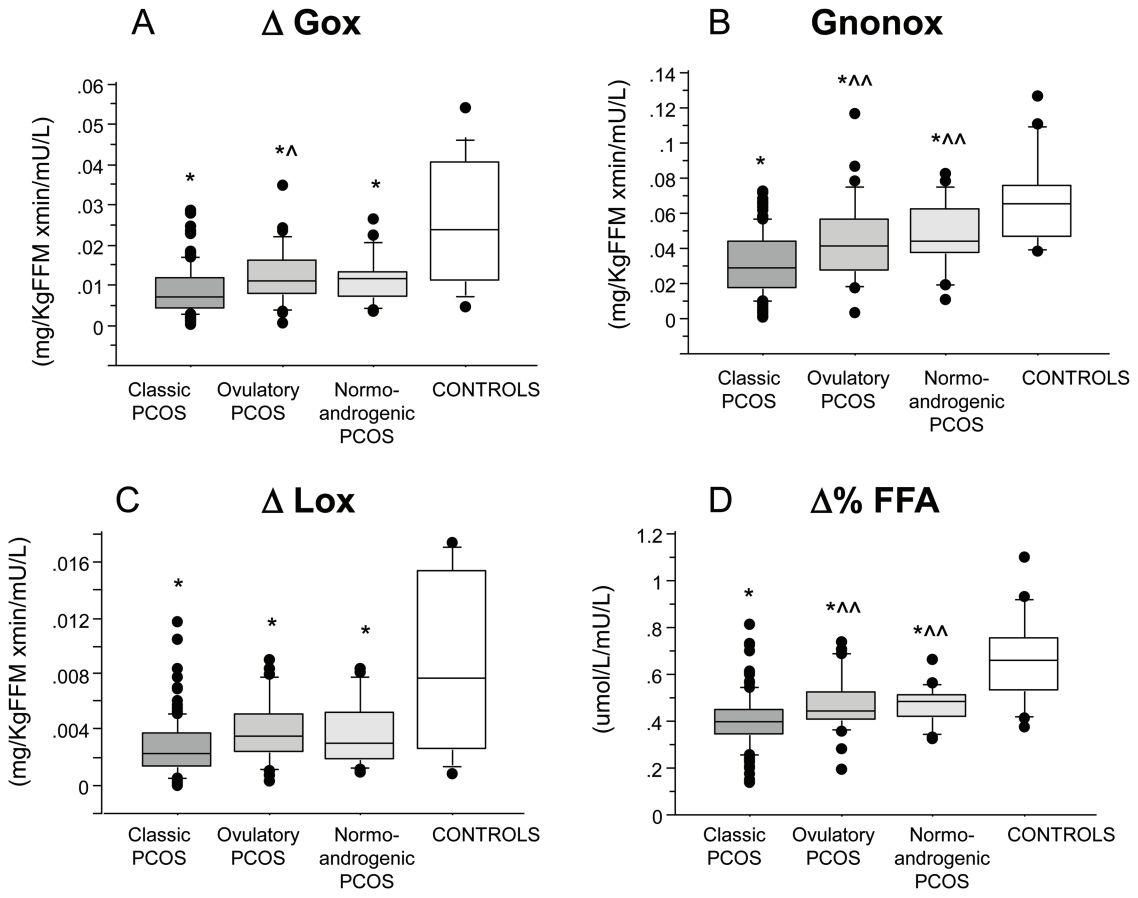

Figure 1. Box plots of insulin-mediated substrate use (Δglucose oxidation [Gox], nonoxidative glucose metabolism [Gnonox], Δlipid oxidation [Lox], Δ% free fatty acid [FFA]) in women with polycystic ovary syndrome (PCOS) subdivided according to clinical phenotype (gray bars) and in healthy controls (white bars). ^*P* less than .05 and ^^*P* less than or equal to .01 vs classic PCOS; **P* less than or equal to .002 vs controls.

every PCOS phenotype as compared to controls; the impairment of Gox was also more pronounced in the classic than in the ovulatory phenotype (see Fig. 1A), whereas there were no differences between PCOS phenotypes in terms of lipid oxidation (see Fig. 1C). Gnonox (see Fig. 1B) was also significantly lower in all PCOS subgroups than in controls. This impairment was more pronounced in the classic than in the ovulatory and the normoandrogenic

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phenotypes. Similarly, the percentage of serum FFA reduction (see Fig. 1D) induced by hyperinsulinemia during the clamp procedure was lower in every PCOS phenotype than in controls, and in the classic phenotype as compared with the ovulatory and the normoandrogenic phenotypes. These differences were confirmed after the inclusion of BMI as a covariate.

Table 3 reports simple correlations between insulin-induced changes in substrate use and several clinical and endocrine characteristics of patients. ΔGox, ΔLox, ΔGnonox, and Δ% FFA showed significant inverse relationships with indexes of adiposity (BMI, waist circumference, WHR, and fat mass). Significant inverse relationships were also found between each of these 4 parameters and either free testosterone (Fig. 2) or total testosterone, whereas no relationships were found with androstenedione, DHEAS, LH/FSH ratio, and clinical hirsutism (Ferriman-Gallwey score). Conversely, a significant direct relationship was observed between these parameters and SHBG. Significant associations were also observed between insulin-induced variations of Gox, Gnonox, Lox, and FFAs and several metabolic variables: There were direct relationships with insulin sensitivity (M clamp) and HDL cholesterol, whereas there were inverse relationships with fasting insulin, triglycerides, uric acid, and systolic and diastolic blood pressure. Finally, significant inverse associations were also observed between ΔGox and Gnonox and fasting glucose, and between ΔGox and ΔLox and total cholesterol.

To identify the independent predictors of insulin action on substrate use, multiple regression analyses were conducted, including age, fat mass, and free testosterone as independent variables (Table 4). All 3 variables independently predicted Gnonox and Δ% FFA, with models explaining 41% and 27% of variance, respectively. Fat mass and free testosterone also independently predicted ΔGox (explained variance 23%), whereas only fat mass was a predictor of ΔLox (explained variance 16%).

Table 3. Correlations between insulin-induced variations of substrate use during use of hyperinsulinemic euglycemic clamp and clinical, metabolic, and hormonal parameters

	ΔGox		Gnonox		ΔLox		Δ% FFA	
	ρ	*P*[a]	ρ	*P*[a]	ρ	*P*[a]	ρ	*P*[a]
Age	0.112	.13	0.321	**< .001**	0.101	.17	0.268	**< .001**
BMI	–0.532	**< .001**	–0.673	**< .001**	–0.445	**< .001**	–0.556	**< .001**
Waist circumference	–0.504	**< .001**	–0.587	**< .001**	–0.426	**< .001**	–0.485	**< .001**
WHR	–0.398	**< .001**	–0.391	**< .001**	–0.344	**< .001**	–0.253	**< .002**
Fat mass	–0.527	**< .001**	–0.637	**< .001**	–0.434	**< .001**	–0.538	**< .001**
Fat-free mass	–0.415	**< .001**	–0.513	**< .001**	–0.364	**< .001**	0.408	**< .001**
Ferriman-Gallwey score	–0.034	.66	–0.088	.25	–0.035	.65	0.051	.54
Free testosterone[b]	–0.404	**< .001**	–0.532	**< .001**	–0.301	**< .001**	–0.423	**< .001**
Free testosterone[c]	–0.397	**< .001**	–0.520	**< .001**	–0.269	**.001**	–0.422	**.001**
Total testosterone	–0.174	**.02**	–0.166	**.03**	–0.149	.05	–0.174	**.03**
Androstenedione	–0.042	.57	–0.121	.10	–0.054	.48	–0.135	.09
DHEAS	–0.059	.43	–0.073	.33	0.037	.63	–0.080	.31
LH/FSH	–0.073	.34	–0.023	.76	0.058	.45	–0.078	.34
SHBG	0.415	**< .001**	0.635	**< .001**	0.308	**< .001**	0.481	**< .001**
Fasting glucose	–0.165	**.03**	–0.278	**< .001**	–0.012	.87	–0.112	.16
Fasting insulin	–0.532	**< .001**	–0.687	**< .001**	–0.404	**< .001**	–0.537	**< .001**
M clamp	0.561	**< .001**	0.874	**< .001**	0.416	**< .001**	0.365	**< .001**
Total cholesterol	–0.235	**.002**	–0.140	.06	–0.181	**.02**	–0.119	.13
HDL cholesterol	0.372	**<.001**	0.522	**< .001**	0.303	**< .001**	0.349	**< .001**
Triglycerides	–0.394	**<.001**	–0.498	**< .001**	–0.270	**< .001**	–0.354	**< .001**
Uric acid	–0.322	**<.001**	–0.324	**< .001**	–0.285	**< .001**	–0.230	**.005**
Systolic blood pressure	–0.300	**<.001**	–0.314	**< .001**	–0.214	**.005**	–0.269	**< .001**
Diastolic blood pressure	–0.289	**<.001**	–0.284	**< .001**	–0.198	**.009**	–0.233	**.003**

Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; FFA, free fatty acid; FSH, follicle-stimulating hormone; Gnonox, nonoxidative glucose metabolism; Gox, glucose oxidation; HDL, high-density lipoprotein; LH, luteinizing hormone; Lox, lipid oxidation; PCOS, polycystic ovary syndrome; SHBG, sex hormone–binding globulin; WHR, waist to hip ratio.

[a]Statistically significant *P* values are shown in bold type.

[b]Free testosterone calculated by the Vermeulen formula.

[c]Free testosterone measured by equilibrium dialysis (available in 156 women with PCOS).

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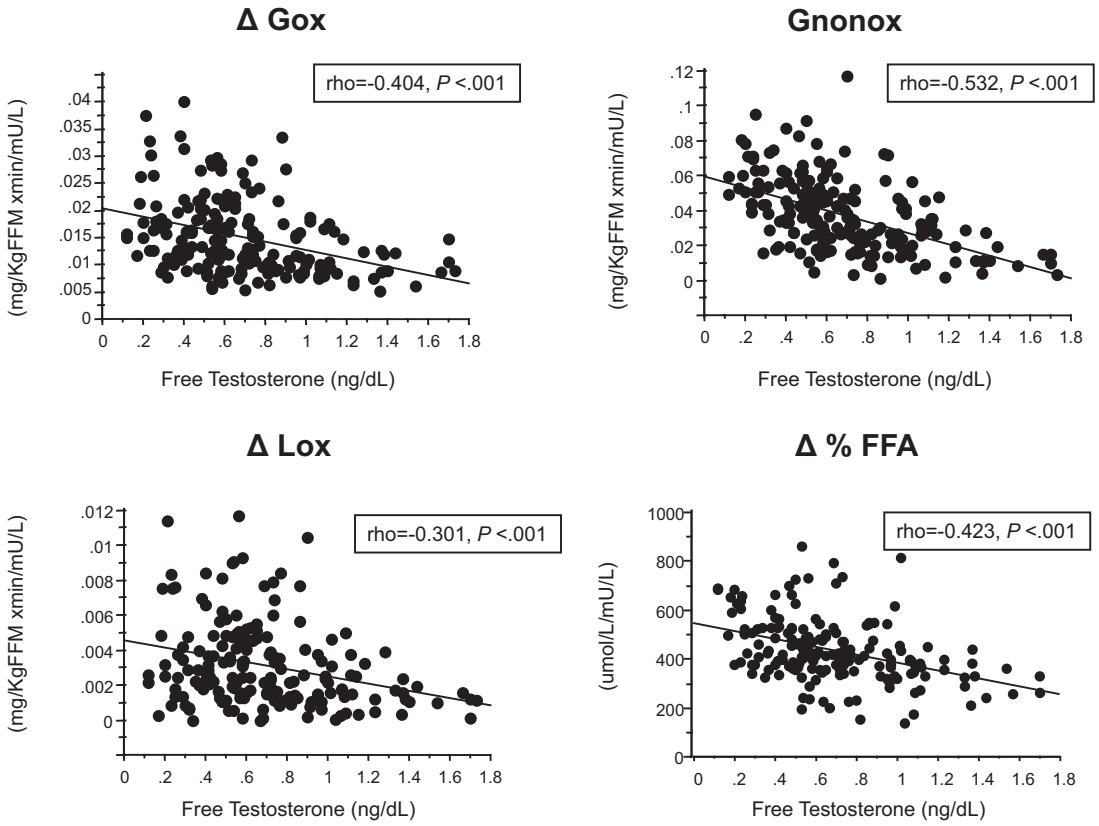

Figure 2. Relationships between insulin-mediated substrate use (Δglucose oxidation [Gox], nonoxidative glucose metabolism [Gnonox], Δlipid oxidation [Lox], Δ% free fatty acid [FFA]) and free testosterone serum levels in women with polycystic ovary syndrome (PCOS) included in the study.

Table 4. Predictors of insulin-induced substrate use in multiple regression analysis

Dependent variables, explained variance	ΔGox, 22.8%		Gnonox, 40.8%		ΔLox, 15.5%		Δ% FFA, 26.6%	
	Stand. coeff. β	*P*[a]	Stand. coeff. β	*P*[a]	Stand. coeff. β	*P*[a]	Stand. coeff. β	*P*[a]
Independent variables								
Age	.085	.21	.214	**< .001**	.086	.23	.156	**.03**
Fat mass	–.320	**< .001**	–.441	**< .001**	–.289	**< .001**	–.293	**< .001**
Free testosterone[b]	–.203	**.01**	–.198	**.004**	–.127	.12	–.236	**.004**

Abbreviations: FFA, free fatty acid; Gnonox, nonoxidative glucose metabolism; Gox, glucose oxidation; Lox, lipid oxidation; Stand. coeff., standard coefficient.
[a]Statistically significant *P* values are shown in bold type.
[b]Free testosterone calculated by the Vermeulen formula.

The inclusion in the model of BMI or waist circumference, instead of fat mass, gave similar results (data not shown). Similarly, inclusion in the models of free testosterone measured by equilibrium dialysis, instead of calculated free testosterone, did not change the results, although the sample size was lower.

Discussion

In the present study, by using the hyperinsulinemic euglycemic clamp technique combined with indirect calorimetry, we have found that women with every clinical phenotype of PCOS show an impairment in insulin-induced substrate use: Women with each phenotype had impaired insulin-stimulated oxidative and nonoxidative glucose metabolism, as well as impaired Lox and insulin-induced suppression of lipolysis, as compared with healthy controls. However, in women with both hyperandrogenism and oligoanovulation, the classic phenotype of PCOS, the impairment of insulin action on these metabolic pathways was greater than in women with nonclassic phenotypes. Interestingly, serum free testosterone was an independent predictor of the impaired insulin action in all these metabolic aspects.

The hyperinsulinemic euglycemic clamp technique is considered the best available standard for the measurement

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of in vivo insulin action (18). In this methodology, exogenous insulin is administered at a primed-constant rate in order to maintain a predefined hyperinsulinemic plateau, whereas plasma glucose is clamped at the normal fasting concentrations through a concurrent infusion of exogenous glucose. In this way, insulin action is assessed under comparable and constant conditions of plasma insulin and plasma glucose levels. In hyperinsulinemic conditions determining a complete suppression of endogenous glucose production, the exogenous glucose infusion rate equals the amount of glucose used by all the tissues in the body and thus provides a quantitation of overall insulin sensitivity. In addition, when indirect calorimetry is performed during the clamp procedure, the effect of insulin on the pattern of substrate oxidation can be measured and it is also possible to measure the effect of insulin on other processes modulated by this hormone, such as lipolysis.

To the best of our knowledge, no data are available on insulin-induced substrate use in women with the different PCOS phenotypes. Gox, Gnonox, Lox, and serum FFAs were previously investigated in women with PCOS, focusing on the impact of obesity or PCOS status per se on these features. In general, these studies recruited women with the classic phenotype of PCOS, and most of the research reported an impairment of insulin action on these aspects in obese patients, as compared with BMI-matched controls (5-9, 21). However, results in lean patients were discordant (7-9, 21).

Glintborg and colleagues and Kim et al, in 2 small studies examining 25 obese women and 21 obese adolescents with PCOS, respectively, found reduced insulin stimulation of Gox and Gnonox, with reduced suppression of Lox, as compared with obese controls (5, 6). In contrast, Broskey et al did not find PCOS-related Gnonox alterations, as these values were similar in obese PCOS women and obese controls, although lower than in lean controls (21).

Morin-Papunen and colleagues found impaired insulin action on Gox, Gnonox, and Lox in obese but not in lean PCOS individuals vs their respective controls (7). Adamska et al (8) reported that PCOS and obesity both were independently associated with an impaired effect of insulin on Gnonox, whereas alterations in insulin-stimulated Gox and Lox were associated with obesity but not PCOS. Finally, Svendsen et al (9) found that PCOS status was associated with lower insulin-stimulated Gox and Gnonox, independently of obesity, that however synergistically contributed to insulin-stimulated Gox impairment. None of these studies focused on differences between PCOS phenotypes. Our findings of reduced insulin action both on glucose and lipid metabolism in women with PCOS, regardless of their phenotype, suggest a specific defect(s) associated with the PCOS status in the metabolic pathways underlying insulin action. However, the presence and/or severity of other factors, such as adiposity and androgen excess, may contribute to differences between phenotypes.

In a hyperinsulinemic condition, skeletal muscle is the main tissue metabolizing glucose and, therefore, the hypothesis of a PCOS-specific molecular defect in muscle has been investigated (22). The earliest hypothesis of a serine kinase defect in a proximal step of insulin signaling (23) was not consistently confirmed by subsequent studies, which suggested either a defect of insulin signaling located at protein kinase B and its substrates (24) or alterations of other glucose-regulating pathways (25, 26). Interestingly, a recent study, carried out in skeletal muscle of lean, insulin-resistant PCOS women with impaired insulin-stimulated Gox, did not find alterations in proximal insulin signaling cascade, but a reduced expression and activity of adenosine monophosphate–activated protein kinase, a key regulator of metabolism and glucose uptake in muscle (25). The authors also reported reduced insulin-stimulated metabolic flexibility in these women, which was associated with reduced activity of pyruvate dehydrogenase, a key enzyme in the oxidative pathway. Moreover, a higher content of intramuscular lipids was found and it has been demonstrated that lipid accumulation, either within the muscle cells or between fibers, may contribute to skeletal muscle insulin resistance (25).

Also mitochondrial dysfunction may potentially account for impaired glucose use in the oxidative pathway in muscle of women with PCOS (27). Using ^{31}P-magnetic resonance spectroscopy, Cree-Green and colleagues (28) found that mitochondrial oxidative phosphorylation was lower in hyperandrogenic obese adolescents than in age- and BMI-matched controls. Interestingly, in multivariable analysis, insulin resistance was predicted by serum androgens, oxidative phosphorylation, and FFA concentrations during hyperinsulinemia.

The expression of key genes for oxidative phosphorylation was reported to be reduced in skeletal muscle of women with PCOS (27), suggesting that mitochondrial dysfunction may contribute to PCOS insulin resistance. Interestingly, in a recent study, Nilsson et al (26) identified PCOS-specific changes in muscle DNA methylation, suggesting that women with PCOS may have epigenetic and transcriptional changes in skeletal muscle that, at least in part, may explain the metabolic abnormalities of these women.

Very few studies have investigated the molecular mechanisms underlying the Gnonox in skeletal muscle of women with PCOS. Glintborg et al (29) found impaired activation of muscle glycogen synthase, a key enzyme in this process, in 24 obese women with PCOS during

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clamp-induced hyperinsulinemia, as compared to controls; notably, this defect was only partly reversed by 16 weeks of pioglitazone treatment.

All these studies were conducted in relatively small groups of women with PCOS, and there has been no comparison between women with different phenotypes nor exploration of the contribution of specific factors such as androgen excess.

The results of the present study clearly demonstrate that women with both hyperandrogenism and oligoanovulation are those with greater impairment of insulin action on both the glucose and lipid metabolism, and that these individuals are also those with worse metabolic profile among individuals with PCOS. These women significantly differed in terms of fat mass and serum androgens as compared with women with the other phenotypes of PCOS, suggesting that these factors may have a role in the metabolic abnormalities of these women. Consistently, multivariable analysis showed an independent effect both of free testosterone and fat mass on Gox, Gnonox, and lipolysis in these women.

The potential role of androgens in skeletal muscle insulin resistance is suggested by several experimental evidences. Supraphysiological androgen administration both to transgender men and cisgender women showed rapid onset of insulin resistance in multistep clamp studies (30, 31). We have previously found that 3 to 4 months of antiandrogen treatment ameliorated insulin sensitivity in hyperandrogenic women, with a parallel increase of Gox and Gnonox, although posttreatment values did not reach those of control participants (17). Data in animal models also support this hypothesis (32).

In adipose tissue, lipolysis (ie, hydrolysis of triglycerides into glycerol and fatty acids) and subsequent oxidation of fatty acids are fundamental pathways for generating substrates for energy production. These pathways are especially active in the fasting state and are inhibited in the postprandial hyperinsulinemic state. In women with PCOS an impaired insulin suppression of Lox has been reported in obese (5-8) but not in lean individuals (7). As regards lipolysis, Morin-Papunen et al (7) did not find significant differences in FFA concentrations during hyperinsulinemia in either obese or lean PCOS women vs BMI-matched controls. Glintborg and colleagues (6) found impaired suppression of FFAs during hyperinsulinemic conditions in obese women with PCOS. Kim et al, who evaluated lipolysis in obese adolescent girls with PCOS, reported impaired suppression during glucose clamp-induced hyperinsulinemia (5).

These in vivo data suggest the presence of adipose tissue insulin resistance in women with PCOS. As regards the molecular mechanisms, a decrease in maximal insulin-stimulated glucose transport in the adipocytes of women with PCOS was reported by some authors (33, 34), but not confirmed by others (35). However, glucose transporter 4 expression was found to be significantly decreased in PCOS adipocytes, providing a possible mechanism for a decrease in insulin-stimulated glucose uptake. Interestingly, Ek and colleagues documented resistance to catecholamine lipolytic activity in subcutaneous fat cells of lean PCOS women, due to a decreased amount of β2-adrenergic receptors and reduced function of the protein kinase A–hormone-sensitive lipase complex (PKA-HSL). These defects may cause low in vivo lipolytic activity and enlarged subcutaneous fat cell size, promoting later development of obesity (36). In visceral adipose tissue of lean women with PCOS, the same authors (37) reported increased lipolytic activity due to alterations of the stoichiometric properties of the PKA-HSL complex. This unique defect may favor the release of fatty acids from the visceral fat depot toward the liver, thereby inducing insulin resistance in the liver.

Androgens have a potentially relevant effect on adipose tissue insulin resistance. In cultured subcutaneous adipocytes, insulin-induced glucose uptake was lowered by testosterone in a dose-dependent manner (38). Notably, this effect was reversed by androgen receptor blockade, proving that it is mediated by androgen receptor activation. Very interesting data have recently been reported by O'Reilly et al using the in vivo microdialysis technique in subcutaneous adipose tissue. These authors reported that PCOS women had increased RNA expression, in this tissue, of AKR1C3, the key enzyme synthesizing testosterone from its precursor androstenedione (39). Interestingly, PCOS women, as compared to controls, had increased testosterone levels both in serum and, to a greater extent, in adipose tissue, supporting a key role of this tissue in the peripheral synthesis of activated androgens. In addition, this study showed that androgens determined activation of lipogenesis and reduction of lipolysis and fatty acid oxidation in subcutaneous adipocytes. These alterations favor lipid accumulation and increased fat mass, and may potentially contribute to the whole-body insulin resistance of PCOS women.

We have previously analyzed the relationships between body fat, hyperandrogenemia, and insulin resistance in these women. In the multivariable analysis body fat and free testosterone were both independent predictors of insulin sensitivity, but only insulin sensitivity, not body fat, was an independent predictor of serum androgens (40). This suggests that adiposity may play a major role in determining insulin resistance in PCOS women as well, whereas the association between body fat and hyperandrogenism could be mainly indirect.

More information on the pathophysiology of insulin action on substrate use in PCOS could be derived from the study of the molecular mechanisms potentially underlying these processes, in biopsies of relevant tissues

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and/or in cell cultures, as well as via in vivo metabolomics analyses. In addition, longitudinal studies could explore changes in substrate use after treatments influencing potential pathogenic factors of the syndrome, such as those with antiandrogen drugs or insulin sensitizers. Also, studies examining the effect of weight loss, obtained by diet, bariatric surgery, or glucagon-like peptide-1 agonists, might provide intriguing information on the role of fat mass in these phenomena.

The major strengths of this study are the large sample of PCOS women investigated and the careful and comprehensive characterization of participants by state-of-the-art methods, in particular the hyperinsulinemic euglycemic clamp associated with indirect calorimetry, to evaluate insulin sensitivity and substrate use, and LC-MS/MS and equilibrium dialysis to measure serum androgen concentrations.

There are also limitations to this study. First, the sample size of healthy controls was relatively small. However, it should be considered that performing a hyperinsulinemic euglycemic clamp associated with indirect calorimetry procedure is a complex task and it is extremely difficult to collect a large number of healthy volunteers. In addition, the number of women with the normoandrogenic phenotype of PCOS was also small. This could have limited the possibility of recognizing some differences in the comparison between these individuals and those with the ovulatory phenotype. A second limitation is that this is an observational study, and cause-effect relationships cannot be firmly established.

In conclusion, in women with PCOS, regardless of their phenotype, insulin-mediated substrate use was significantly impaired as compared to healthy controls. Moreover, women with hyperandrogenism and oligoanovulation (ie, with the classic/complete phenotype) showed greater abnormalities in insulin-induced use of substrates than women with other phenotypes. Androgen excess, as well as adiposity, may play an independent role in impaired insulin action in both glucose and lipid metabolism.

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Data Availability: Some or all data sets generated during and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

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