

Insulin Resistance, Hyperinsulinemia, and Mitochondria Dysfunction in Nonobese Girls With Polycystic Ovarian Syndrome

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Objective: Obese girls with polycystic ovarian syndrome (PCOS) have decreased insulin sensitivity (IS), muscle mitochondrial dysfunction and increased liver fat, which may contribute to their increased risk for type 2 diabetes. Less is known regarding normal-weight girls with PCOS.

Methods: Normal-weight girls with PCOS [n = 18, age 15.9 ± 1.8 years, body mass index (BMI) percentile 68 ± 18] and normal-weight controls (NWC; n = 20; age 15.0 ± 2.1 years, BMI percentile 60 ± 21) were studied. Tissue-specific IS was assessed with a four-phase hyperinsulinemic-euglycemic clamp with isotope tracers and a 2-hour oral glucose tolerance test (OGTT). Hepatic fat was determined using magnetic resonance imaging. Postexercise muscle mitochondrial function was assessed with ³¹P MR spectroscopy.

Results: Both groups had similar demographics, anthropomorphics, physical attributes, habitual physical activity levels and fasting laboratory values, except for increased total testosterone and DHEAS in PCOS. Clamp-assessed peripheral IS was lower in PCOS (10.4 ± 2.4 mg/kg/min vs 12.7 ± 2.1; P = 0.024). The 120-minute OGTT insulin and glucose concentrations were higher in PCOS (114 IU/mL ± 26 vs 41 ± 25, P = <0.001 and 119 ± 22 mg/dL vs 85 ± 23, P = 0.01, respectively). Muscle mitochondrial ADP and phosphocreatine time constants were slower in PCOS. Despite a higher percentage liver fat in PCOS, hepatic IS was similar between groups, as was adipose IS.

Conclusions: Normal-weight girls with PCOS have decreased peripheral IS and muscle mitochondrial dysfunction, abnormal glucose disposal, relative postprandial hyperinsulinemia, and increased hepatic fat compared to NWC. Despite a normal BMI, multiple aspects of metabolism appear altered in normal-weight girls with PCOS.

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Abbreviations: 3DPAR, 3-day activity recall questionnaire; ADP, adenosine 5'-diphosphate; AMH, anti-Müllerian hormone; BMI, body mass index; FAI, free androgen index; FFA, free fatty acid; GIR, glucose infusion rate; HOMA, homeostatic model assessment; HS, hepatic steatosis; IC₅₀, 50% inhibitory concentration; IGT, impaired glucose tolerance; IS, insulin sensitivity; MCR, mean clearance rate; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NIH, National Institutes of Health; NWC, normal-weight control; OGTT, oral glucose tolerance test; ³¹P, phosphorus-31; PCOS, polycystic ovarian syndrome; PCr, phosphocreatine; Ra, rate of appearance; SHBG, sex hormone-binding globulin; VO_{2peak}, peak volume of oxygen.

Freeform/Key Words: PCOS, insulin sensitivity, mitochondrial function, hepatic fat, adolescence

Polycystic ovarian syndrome (PCOS), characterized by hyperandrogenism, oligo-ovulation, and polycystic ovaries, is a common endocrine disorder affecting $\geq 10\%$ of reproductive-age women [1, 2]. Metabolic syndrome components, including lower insulin sensitivity (IS) and related impaired glucose tolerance (IGT), type 2 diabetes mellitus, nonalcoholic fatty liver disease, and cardiovascular disease are more common in the obese PCOS population and develop in adolescence [2–6]. Many of these comorbidities lead to decreased longevity; thus, appropriate management of PCOS is necessary to prevent future life-threatening complications [5]. However, the risk of developing cardiometabolic disease in normal-weight women with PCOS is less clear, making it challenging to develop appropriate screening and prevention strategies. Further understanding of the role of obesity in PCOS-related comorbidities is needed to optimize prevention strategies within the full range of body mass indexes (BMIs).

The insulin resistance associated with PCOS has been widely studied in adult women; however, the role of obesity *per se* is still unclear. Several studies of peripheral IS measured using the reference standard euglycemic hyperinsulinemic clamp showed that adults with PCOS and a BMI ranging from normal to obese had insulin resistance compared with non-PCOS adults with a similar BMI [7, 8]. At the tissue level, insulin resistance is present in the liver, adipose, and muscle tissues in women with PCOS [9–11]. Furthermore, it has been reported that nearly 50% to 70% of adults with PCOS and obesity have hepatic steatosis (HS), an early manifestation of nonalcoholic fatty liver disease. In contrast, only 20% to 30% of obese women without PCOS have HS, and HS in normal-weight adult women is rare [12, 13]. In contrast, when IS was assessed via a 2-hour oral glucose tolerance test (OGTT), no evidence of insulin resistance was found in normal-weight women with PCOS [14]. Additionally, several studies have shown that only obese women with PCOS have an increased risk of post-OGTT hyperglycemia, suggesting that obesity is required for OGTT differences to manifest [10, 15]. Thus, additional reference standard data are needed from normal-weight women with PCOS.

Although PCOS in adults has been extensively studied, findings beyond the clinical measures in the pediatric PCOS population have been limited, especially in normal-weight girls. We, and others, have previously shown substantial insulin resistance in obese girls with PCOS compared with obese girls without PCOS [3–5], including lower peripheral, hepatic, and adipose IS [3–5, 16]. Furthermore, compensatory hyperinsulinemia in the fasting and postprandial state has also been shown to correlate with obesity in PCOS adolescents [16]. We have documented that nearly 50% of PCOS girls with a BMI ≥ 95 th percentile have HS [3] and that muscle mitochondrial function, examined using phosphorus-31 magnetic resonance spectroscopy (^{31}P -MRS) after near-maximal isometric calf exercise, was lower in obese PCOS girls than in obese control girls [4]. IGT, defined as a 2-hour OGTT glucose level > 140 mg/dL, is present in girls with PCOS, regardless of their weight [17]; however, more rigorous studies are lacking. Thus, it is clear that cardiometabolic disease involving multiple tissues is already established in obese girls with PCOS.

Detailed studies of the risk factors for cardiometabolic disease in normal-weight girls with PCOS have not been reported. Also, the findings from adult women might be biased by the longer duration of disease exposure. Thus, the aim of the present study was to deeply phenotype normal-weight girls with and without PCOS by assessing tissue-specific IS using the reference-standard clamp method, muscle mitochondrial function, and hepatic fat.

1. Methods

A. Participant Population

The girls were from the normal-weight groups in the RESISTANT (resistance to insulin in type 1 and type 2 diabetes) study and AIRS (androgens and insulin resistance study) [3, 4, 18].

The inclusion criteria were female sex, nonobese BMI (≤ 90 th percentile), age 12 to 20 years, and sedentary status (< 3 h/wk of exercise). Sedentary status was confirmed using a 3-day activity recall questionnaire (3DPAR) and the use of an accelerometer for 7 days, because activity can independently affect the measurements of IS. The exclusion criteria were diabetes mellitus, alanine transferase > 80 IU/mL, blood pressure $> 140/90$ mm Hg, hemoglobin < 9 mg/dL, serum creatinine > 1.5 mg/dL, smoking, medications affecting IS, antihypertensive medications, statins, pregnancy, and breastfeeding. PCOS was defined using the National Institutes of Health (NIH) criteria of oligomenorrhea and clinical and/or biochemical evidence of hyperandrogenism and ≥ 2 years after menarche [19, 20]. PCO morphology was not assessed owing to the lack of normative standards for youth [20]. The University of Colorado Anschutz Medical Campus institutional review board and the Children's Hospital of Colorado scientific advisory review committee approved the present study. All participants aged 18 to 20 years provided written informed consent, and the parents and participants provided consent and assent, respectively, for all participants aged < 18 years.

B. Overall Study Design

The participants completed two study visits. The first visit included medical history, physical examination, qualification laboratory tests, and a 2-hour 75-g OGTT. Tanner staging was performed by a pediatric endocrinologist (M.C.G. or K.J.N.). Before the second visit, the participants ate a 3-day clinical and translational research center metabolic kitchen-provided isocaloric diet (55% carbohydrate, 15% protein, 30% fat) and avoided exercising. All participants were in the follicular phase of the menstrual cycle, if possible, which was verified by the serum progesterone concentration. The second visit was scheduled for 2 days, with day 1 consisting of dual-energy x-ray absorptiometry for body composition; abdominal magnetic resonance imaging (MRI); in-MRI exercise testing with ^{31}P -MRS; and bicycle ergometer cardiopulmonary fitness testing. The next morning, fasting metabolic and hormonal laboratory test results were collected, and a four-phase hyperinsulinemic euglycemic clamp test with isotope tracers was performed.

C. Protocol for Tracer Infusion and Four-Phase Hyperinsulinemic Euglycemic Clamp

Fasting blood samples were taken at 6 AM to measure the concentrations of glucose, insulin, glycerol, and free fatty acids (FFAs) and the background enrichment of glucose and glycerol. Next, a 4.5-mg/kg ($6,6\text{-}^2\text{H}_2$) glucose (Isotec, Inc., Miamisburg, OH) prime, followed by a continuous infusion at the rate of 0.04 mg/kg/min ($6,6\text{-}^2\text{H}_2$) glucose was paired with a primed and then a constant infusion of $^2\text{H}_5$ glycerol (prime, 1.6 $\mu\text{mol/kg}$; constant infusion rate, 0.11 $\mu\text{mol/kg/min}$) [3]. The basal phase was from the start of the tracers (6 AM) until the start of insulin 2 hours later. Insulin was infused for each of the 90-minute clamp phases (10 $\text{mU/m}^2/\text{min}$, 16 $\text{mU/m}^2/\text{min}$, and 80 $\text{mU/m}^2/\text{min}$), with a prime administered at the beginning of each dose change. Also, 20% dextrose "spiked" with [$6,6\text{-}^2\text{H}_2$] glucose was infused at a variable rate to maintain the blood glucose level at 95 mg/dL, measured every 5 minutes using a YSI analyzer (YSI instruments, Yellow Springs, OH). Serum measurements, including glucose, FFA, glycerol, and insulin, in addition to glucose and glycerol tracer enrichments, were taken every 10 minutes during the last 30 minutes of each of the four clamp phases, and the steady-state glucose infusion rate (GIR) was determined from the last 30 minutes of each phase [3, 21].

D. Protocol to Evaluate Physical Activity and Maximal Exercise Capacity

The participants wore an ambulatory actigraph GT3x accelerometer (Actigraph Corp., Pensacola, FL) for 7 days before the overnight visit to track their habitual physical activity. The data were corrected for wear time and categorized into specific activity levels: sedentary, light, lifestyle, moderate, vigorous, and very vigorous according to standard cutpoints [22]. Habitual activity was quantified in METs (metabolic equivalents of task) using a 3DPAR

questionnaire [23]. A graded cycle ergometer (Lode, Groningen, Netherlands) protocol to exhaustion was used to determine the peak volume of oxygen ($\text{VO}_{2\text{peak}}$), as previously described [24, 25]. All subjects achieved a peak respiratory exchange ratio of >1.1 .

E. Protocol to Evaluate Diet

The customary macronutrient pattern was ascertained using a food frequency questionnaire at screening, modified to incorporate common food choices among ethnically and regionally diverse youth aged 10 to 19 years participating in another large childhood diabetes study, SEARCH for Diabetes in Youth [26].

F. Protocol for MRI and MRS

A 3 Tesla magnet (GE Healthcare, Milwaukee, WI), running version 15M4 software, was used for MRI and MRS. Abdominal MRI was performed to obtain the measures of hepatic, visceral, and subcutaneous fat [27–29]. Hepatic fat was quantified using a modification of the Dixon method [30] involving a multibreath-hold double gradient echo T1-weighted sequence, as previously described [25, 31–33].

Phosphate spectra from the posterior calf were collected at rest, during isometric plantarflexion exercise at 70% of the maximum volitional contraction for 90 seconds, and during 5 minutes of recovery. A custom-built, magnetic resonance-compatible plantarflexion device was used for the exercise testing, as previously described, and the force was monitored throughout the exercise session [32, 33]. A custom-built 12-cm phosphate coil was used for data collection (Clinical MR Solutions, Brookfield, WI). Quality control measures included the exercise force generated per muscle area sampled (kg/cm^2). The spectroscopy data were analyzed with time domain fitting using the jMRUI software package [34, 35] and AMARES (advanced method of accurate, robust and efficient spectral fitting), a nonlinear least-square-fitting algorithm using previously built knowledge files [36], with curve fitting of time constant data, as previously described [4, 32, 33, 37]. The outcomes of interest were the time constants for adenosine 5'-diphosphate (ADP), phosphocreatine (PCr), calculated rate of oxidative phosphorylation, anaerobic glycolysis, creatine kinase reaction, and maximal mitochondrial capacity.

G. Body Composition Determination

Body composition (fat and fat-free mass) was assessed using standard dual-energy x-ray absorptiometry (Hologic, Waltham, MA) methods [38].

H. Protocol for Laboratory Analysis

Analyses were performed by the University of Colorado Anschutz Research core laboratory or the Children's Hospital Colorado clinical laboratory, except as noted otherwise. Plasma glycerol (*R*-Biopharm, Marshall, MI), FFAs (Wako Chemicals, Inc., Richmond, VA), and triglycerides, total cholesterol, high-density lipoprotein cholesterol (Hitachi 917 auto-analyzer; Boehringer Mannheim Diagnostics, Indianapolis, IN) were analyzed enzymatically. The Friedewald equation was used to calculate the low-density lipoprotein cholesterol concentrations [39]. Diabetes Control and Complications Trial-calibrated high-performance ion-exchange liquid chromatography was used to determine the hemoglobin A1c level (Bio-Rad Laboratories, Hercules, CA). A radioimmunoassay was used to analyze serum adiponectin, leptin, and insulin (EMD Millipore, Billerica, MA). The immunoturbidimetric assay was used for highly sensitive C-reactive protein determination (Beckman Coulter, Brea, CA). The chemiluminescent immunoassay was used for C-peptide (DiaSorin, Stillwater, MN), VITROS 5600 was used to measure aspartate aminotransferase and alanine transferase (Ortho Clinical Diagnostics, Rochester, NY), the white blood cell and platelet counts were measured using a Sysmex analyzer (Sysmex, Lincolnshire, IL), a chemiluminescent

immunoassay was used for progesterone and estradiol (Beckman Coulter, Brea, CA), and an electrochemiluminescence immunoassay was used for dehydroepiandrosterone-sulfate, sex hormone-binding globulin (SHBG), and anti-Müllerian hormone (AMH) (Esoterix, Calbassas Hills, CA). Liquid chromatography–tandem mass spectrometry was used to measure total testosterone (Esoterix, Calbassas Hills, CA). The free androgen index (FAI) was calculated from the total testosterone and SHBG levels. Gas chromatography mass spectrometry was performed in the laboratory of Dr. Bergman for the analysis of $^2\text{H}_5$ glycerol and 6,6- $^2\text{H}_2$ glucose [21, 40].

I. Tracer Calculations

The glucose and glycerol rate of appearance (Ra) per phase and mean clearance rate (MCR) of glucose were calculated, as previously described [3]. The glycerol Ra at the 10-mU/m²/min phase was used to define adipose IS, and the glucose Ra at the 16-mU/m²/min phase was used to define hepatic IS. The percentage of suppression of the glycerol Ra was calculated as the percentage of change in the Ra from basal to the 10-mU/m²/min phase, and the glucose Ra was calculated as the percentage of change in the Ra from basal to the 16-mU/m²/min phase. Additionally, modeling to account for the entire curve was performed [41]. The 50% inhibitory concentration (IC₅₀) was calculated to explain the relationship between the Ra at different insulin concentrations across the phases [21]. Log Ra vs log insulin were plotted for each of the four phases of the clamp for each participant. The slope of the curve was used to determine the concentration of insulin at the point on the curve that was equal to 50% suppression of the basal Ra for each individual [21].

J. Statistical Analysis

The results are presented as the mean \pm standard deviation or median (25%, 75%). Group comparisons were made using the χ^2 test or Fisher's exact test for proportions and the *t* test or Kruskal-Wallis test for continuous variables. The *a priori* primary outcome was the GIR (mg/kg/min), and the major secondary outcomes were the glucose Ra at the 16-mU/m²/min phase, glycerol Ra at the 10-mU/m²/min phase, percentage of hepatic fat, and ADP time constant. Repeated measures mixed models were used to compare the data outcomes measured at multiple time points during the clamp test. Spearman's correlation coefficients were used to examine the relationships between GIR and variables known to be associated with IS in other populations, including body composition markers (*i.e.*, BMI, percentage of body fat, visceral fat, liver fat, and waist circumference), physical activity (*i.e.*, METs from 3DPAR, VO₂peak, and time sedentary from accelerometer), PCOS markers (*i.e.*, total testosterone and FAI), and tissue-specific IS. *P* < 0.05 was considered statistically significant, and a correction for multiple testing was not used owing to the limited number of defined primary and secondary outcomes. The remainder of comparisons were descriptive. All statistical analyses were performed with SAS software, version 9.4 (SAS Institute, Cary, NC) and SigmaStat, version 13.1 (Systat Software, Inc., San Jose, CA).

2. Results

The present study included 38 normal-weight participants, 18 with PCOS (age 15.9 \pm 1.8 years) and 20 normal-weight controls (NWCs; age 15.0 \pm 2.1 years). All the girls were at Tanner stage 5, and the mean interval from menarche to study day was 3.8 years for both groups. Because of scheduling difficulties, equipment failure, and patient preference, not all subjects completed all procedures. Of the 18 PCOS participants and 20 NWCs, 13 and 11 had data available for the primary outcome, the hyperinsulinemic euglycemic clamp test, respectively. For the mitochondrial assessment, 13 PCOS and 9 NWCs completed the testing. The demographic characteristics and physical attributes of the entire cohort are listed in [Table 1](#). The groups had similar demographic and physical characteristics, including age,

race/ethnicity, BMI percentile, age of menarche, pubertal stage, percentage of total body fat, waist/hip ratio, and percentage of visceral fat. The percentage of liver fat was significantly greater in the PCOS group ($P = 0.017$), although the median for both groups was well below the threshold of 5.5% used to define clinically relevant hepatic steatosis. The habitual dietary intake, including total daily kilocalories, fat grams, protein grams, carbohydrate grams, and fructose milligrams were similar (Table 1), as was the habitual daily activity, assessed using either the 3DPAR questionnaire or the accelerometer. In subgroup analyses of the girls who had undergone clamp testing, the between group trends were the same as those for the entire cohort, except that the NWC girls with clamp testing were slightly younger than the PCOS group with clamp testing (14.5 ± 2.4 vs 16.3 ± 1.4 years; $P = 0.04$) and had a smaller waist circumference (68 ± 5 vs 74 ± 8 cm; $P = 0.04$).

The fasting biochemical measurements are listed in Table 2. Total testosterone, FAI, AMH, and dehydroepiandrosterone-sulfate were significantly higher in the PCOS group, as expected. However, the PCOS and NWC groups had similar SHBG, estradiol, leptin, adiponectin, hemoglobin A1c, fasting glucose, and insulin levels. Inflammatory markers, including highly sensitive C-reactive protein, white blood cell count, and platelet count, and the serum lipid profile, including cholesterol, high-density lipoprotein, low-density lipoprotein, and triglycerides, were also similar. The results of the comparisons within the subgroups with clamp testing were similar to those observed in the entire cohort.

Figure 1 shows data from the hyperinsulinemic euglycemic clamp test. The serum glucose concentrations remained steady throughout the clamp, and the groups were not significantly different across the phases of the clamp [$P = 0.26$; Fig. 1(a)]. The serum insulin concentrations

Table 1. Participant Characteristics

Characteristic	Control Group	PCOS Group	P Value
Participants, n	20	18	NA
Age, y	15.0 ± 2.13	15.9 ± 1.83	0.15
BMI, kg/m ²	21.3 ± 2.9	22.7 ± 2.3	0.13
BMI percentile	60 ± 21	68 ± 18	0.20
Ethnicity, n (%)			NS
White	10 (50)	7 (39)	
Hispanic	2 (10)	7 (39)	
Black	6 (30)	4 (22)	
Other	2 (10)	0 (0)	
Menarche age, y	12.0 (11.8, 13.5)	12.5 (11.3, 13.0)	0.92
Time from menarche, y	3.8 ± 2.4	3.8 ± 1.3	1.00
Waist circumference, cm	69.6 ± 6.4	74.0 ± 8.0	0.09
Waist/hip ratio	0.79 ± 0.07	0.82 ± 0.07	0.15
Total body fat, %	29.4 (22.5, 33.6)	33.0 (26.9, 36.1)	0.21
Liver fat, %	0.00 (0.00, 0.72)	1.13 (0.64, 1.91)	0.017 ^a
Visceral fat, %	16.7 ± 4.31	15.0 ± 4.24	0.40
Food intake from FFQ			
Total daily, Kcal	1577 (1081, 2197)	1379 (1164, 1787)	0.69
Fat, g	65 ± 26	66 ± 28	0.85
Protein, g	57 (42, 71)	47 (36, 66)	0.44
Total carbohydrates, g	195 (161, 290)	175 (137, 213)	0.34
Fructose, mg	23 (17, 43)	21 (16, 31)	0.43
Daily METs from 3DPAR	60 (52, 72)	52 (47, 62)	0.18
Accelerometer			
Sedentary, %	0.63 ± 0.15	0.69 ± 0.13	0.44
Lifestyle/light, %	0.30 ± 0.12	0.27 ± 0.12	0.58
Moderate/vigorous/very vigorous, %	0.03 (0.02, 0.07)	0.05 (0.04, 0.06)	0.45
VO ₂ peak (mL/kg/min)	30.3 (25.2, 33.5)	29.1 (24.8, 31.0)	0.24

Data presented as mean \pm standard deviation or median (25%, 75%).

Abbreviations: FFQ, food frequency questionnaire; METs, metabolic equivalents of task.

^aStatistically significant difference.

Table 2. Fasting Morning Laboratory Test Results

Laboratory Test	Control Group	PCOS Group	P Value
Total testosterone, ng/dL	38 (24, 45)	46 (33, 62)	0.047 ^a
SHBG, nmol/L	47 (31, 62)	38 (28, 70)	0.74
FAI	2.2 (1.3, 3.9)	4.1 (2.7, 5.7)	0.036 ^a
DHEA-S, μg/dL	118 ± 53	186 ± 58	0.019 ^a
AMH, ng/mL	1.8 ± 1.6	6.8 ± 4.5	< 0.01 ^a
Estradiol, pg/mL	48 (28, 72)	41 (22, 63)	0.74
Leptin, ng/mL	12 (7, 18)	12 (11, 16)	0.68
Adiponectin, ng/mL	9.1 ± 3.1	8.0 ± 3.1	0.33
Cholesterol, mg/dL	146 ± 24	147 ± 27	0.95
HDL, mg/dL	47 ± 9	49 ± 9	0.54
TG, mg/dL	75 ± 21	77 ± 34	0.86
LDL, mg/dL	84 ± 21	83 ± 27	0.82
AST, U/L	23 (15, 31)	28 (23, 36)	0.05
ALT, U/L	23 ± 10	29 ± 10	0.09
WBC count, 10 ⁹ cells/L	6.5 ± 1.5	6.8 ± 2.2	0.30
Platelet count, 10 ⁹ cells/L	261 ± 45	269 ± 67	0.70
hs-CRP, mg/dL	0.4 (0.1, 1.1)	0.4 (0.2, 1.0)	0.47

Data presented as mean ± standard deviation or median (25%, 75%).

Abbreviations: ALT, alanine transferase; AST, aspartate aminotransferase; DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein; hs-CRP, highly sensitive C-reactive protein; LDL, low-density lipoprotein; TG, triglycerides; WBC, white blood cell.

^aStatistically significant difference.

[Fig. 1(b)] increased with each phase of the clamp similarly ($P = 0.50$) between the two groups. Figure 1c shows the glucose Ra, which decreased with each phase of the clamp, with complete suppression of the glucose Ra occurring in both groups by the end of the clamp test. Figure 1d shows the MCR, which increased with increasing insulin. The MCR was lower for the PCOS group than for the NWC group ($P = 0.016$). The serum FFA concentrations decreased equally between the two groups ($P = 0.80$) with increasing insulin doses. Figure 1f shows the glycerol Ra, which was suppressed from the second phase of the clamp onward, without a difference between the two groups ($P = 0.96$).

The data from the hyperinsulinemic-euglycemic clamp test are listed in Table 3. Peripheral IS assessed with the GIR (mg/kg/min and mg/kg fat-free mass/min) during the highest dose of insulin was significantly different between the two groups. However, no substantial difference was found between the PCOS and NWC groups in hepatic IS, assessed using the endogenous glucose Ra compared at the 16-mU/m²/min hepatic phase, the IC₅₀ method, or the percentage of suppression of the basal glucose Ra (Table 3). Similarly, no substantial difference was found in the adipose IS measured using the Ra during the 10-mU/m²/min adipose phase, glycerol IC₅₀, or as percentage of suppression of the basal glycerol Ra. Furthermore, the FFA IC₅₀ and percentage of suppression of the basal FFA concentration was similar between the NWC and PCOS groups.

The data from the OGTT are also listed in Table 3. Fasting insulin, glucose, C-peptide, and homeostatic model assessment (HOMA) for insulin resistance did not differ significantly between the two groups. However, the Matsuda score was lower in the PCOS group, and the insulin and glucose concentrations 2 hours after oral glucose were markedly greater in the PCOS participants ($P < 0.001$ for insulin; $P = 0.01$ for glucose).

The measurements of muscle mitochondrial function are listed in Table 4. Girls with PCOS generated less force per muscle area. The ADP ($P < 0.01$) and PCr ($P = 0.018$) time constants were significantly longer in those with PCOS, despite performing less work. Also, the rate of anaerobic glycolysis was significantly lower in those with PCOS ($P = 0.004$). In contrast, no statistically significant differences with respect to the rates of creatine kinase reaction ($P = 0.975$) or oxidative phosphorylation ($P = 0.089$) were found.

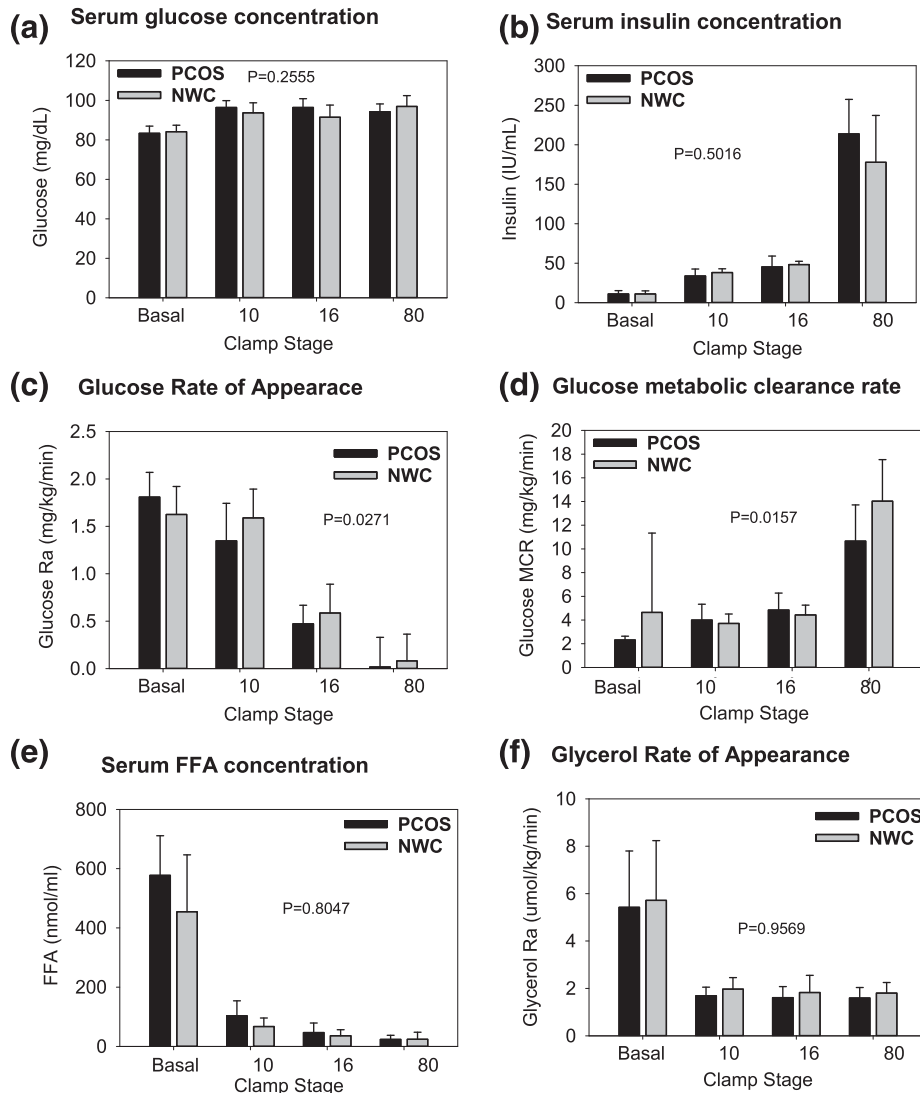


Figure 1. (a) Glucose serum concentration; (b) serum insulin concentration; (c) glucose Ra; (d) glucose metabolic clearance rate; (e) FFA concentration; and (f) glycerol Ra of four-phase hyperinsulinemic-euglycemic clamp test. Data shown for each of the four phases of the clamp identified per insulin dose for both NWC and PCOS adolescent groups. P values for the relationship between groups across all four time points.

Within the participants with complete clamp data available, the correlates of GIR were assessed. The GIR was related to the fasting measures of IS, HOMA ($R = -0.526$; $P = 0.008$), and Matsuda score ($R = 0.731$; $P = 0.004$). Body composition markers also correlated with the GIR (mg/kg/min), including the percentage of body fat ($R = -0.57$; $P < 0.005$), absolute visceral fat ($R = -0.53$; $P = 0.0291$), and waist circumference ($R = -0.52$; $P < 0.01$). In addition, the GIR (mg/kg/min) was related to defects in muscle mitochondrial function, including the ADP time constant ($R = -0.577$; $P = 0.012$) and PCr time constant ($R = -0.602$; $P = 0.008$). However, the hormonal markers of PCOS (total testosterone, $R = -0.0775$, $P = 0.73$; FAI, $R = -0.21$, $P = 0.36$; and AMH, $R = 0.087$, $P = 0.743$) did not correlate with the GIR, diet, physical activity, or VO_{2peak} (data not shown).

3. Discussion

Although it is well established that obese women and adolescents have decreased IS compared with obese women with regular menses, less was known about normal-weight girls with

Table 3. Clamp Insulin Sensitivity and OGTT Measures

Variable	Control Group	PCOS Group	P Value
Peripheral IS			
GIR, mg/kg/min	13.5 ± 2.4	10.9 ± 2.7	0.02 ^a
GIR, mg/kg fat-free mass/min	18.8 ± 2.4	16.3 ± 3.1	0.04 ^a
Hepatic IS			
Glucose IC ₅₀ , IU/mL	52 ± 9	45 ± 15	0.30
Suppression glucose (basal to 16-mU/m ² /min phase), %	110 ± 14	101 ± 18	0.38
Adipose IS			
Glycerol IC ₅₀ , IU/mL	52 (42, 66)	45(42, 51)	0.25
Suppression glycerol (basal to 10-mU/m ² /min phase), %	61 ± 12	65 ± 11	0.52
FFA IC ₅₀ , IU/mL	34 (31, 34)	32 (26, 34)	0.15
Suppression FFA (basal to 10-mU/m ² /min phase), %	87 (85, 89)	85 (75, 91)	0.53
OGTT results			
Fasting glucose, mg/dL	78 (73, 83)	83 (81, 91)	0.08
Fasting insulin, μIU/mL	11 ± 4	11 ± 4	1.00
Fasting C-peptide, ng/mL	1.8 ± 0.4	1.6 ± 0.4	0.35
HbA1c, %	5.2 ± 0.3	5.2 ± 0.3	0.47
2-h Insulin, μIU/mL	41 ± 25	114 ± 26	<0.001 ^a
2-h Glucose, mg/dL	85 ± 23	119 ± 22	0.01 ^a
Matsuda score	21.6 (14.5, 36.3)	10.5 (8.4, 14.1)	<0.05 ^a
HOMA-IR	2.1 ± 0.4	2.6 ± 1.1	0.32

Data presented as mean ± standard deviation or median (25%, 75%).

Abbreviations: HbA1c, hemoglobin A1c; HOMA-IR, HOMA for insulin resistance.

PCOS. Overall, we found that normal-weight girls with PCOS have evidence of decreased peripheral IS. We also found that normal-weight adolescents with PCOS had evidence of postexercise muscle mitochondrial dysfunction and evidence of reduced glucose disposal postprandially. In contrast, despite the increased liver fat relative to normal-weight girls with regular menses, the hepatic and adipose IS were not different between the two groups. These findings indicate that certain aspects of metabolic alterations in PCOS, in particular, in the muscle, occur regardless of adiposity, and thus are likely related to PCOS status alone.

To the best of our knowledge, we are the first to use the reference standard euglycemic hyperinsulinemic clamp and tracer method in normal-weight girls with PCOS. We demonstrated that these girls have peripheral insulin resistance, even when accounting for the possible contribution of the endogenous glucose Ra. In support of our findings, peripheral IS evaluated with the euglycemic hyperinsulinemic clamp test showed that adults with PCOS

Table 4. Mitochondrial Measures

Mitochondrial Measure	Control	PCOS	P Value
Exercise force/ calf area (kg/cm ²)	0.009 ± 0.002	0.007 ± 0.001	0.03 ^a
PCr time constant (s)	28.1 (24.9, 31.0)	36.0 (28.7, 41.0)	0.018 ^a
ADP time constant (s)	17.7 ± 3.3	22.8 ± 4.7	0.009 ^a
Oxidative phosphorylation (mmol/L/s)	0.21 ± 0.10	0.16 ± 0.05	0.09
Qmax (mmol/s)	0.41 (0.19, 0.57)	0.45 (0.38, 0.54)	0.72
Anaerobic glycolysis (mmol/L/s)	0.41 (0.19, 0.57)	0.15 (0.11, 0.20)	0.004 ^a
Creatine kinase ATP production (mmol/L/s)	0.05 (0.03, 0.09)	0.04 (0.01, 0.10)	0.98
Mitochondrial efficiency	0.15 (0.11, 0.20)	0.13 (0.11, 0.15)	0.64

Data presented as mean ± standard deviation or median (25%, 75%).

Abbreviations: ATP, adenosine triphosphate; Qmax, maximal mitochondrial capacity.

^aStatistically significant difference.

diagnosed via the more stringent NIH criteria and varied BMI were insulin resistant compared with non-PCOS adults with a similar BMI [8]. Furthermore, women with obesity and PCOS had the lowest IS, indicating an additive effect of obesity and PCOS status in adults with long-standing disease. Ketel *et al.* [7] found similar results using the hyperinsulinemic euglycemic clamp in a study of women with varied BMIs and with and without PCOS, as defined by the Rotterdam criteria. In contrast, when IS was assessed via 1/fasting insulin or HOMA in 78 women with untreated PCOS, no evidence was found of insulin resistance in the normal-weight women, with only a 29% prevalence of insulin resistance in the obese women [14]. Similarly, another study using a hyperinsulinemic euglycemic clamp reported that the prevalence of insulin resistance was similar in normal-weight women with PCOS diagnosed using the European Society of Human Reproduction and Embryology/American Society of Reproductive Medicine criteria compared with normal-weight women without PCOS [7]. Potential explanations for the variability in results include worse IS in females who present with PCOS as young as adolescence or differences in methods, including the definition of PCOS using the Rotterdam criteria (which does not identify women at risk of metabolic syndrome as well) vs NIH criteria, assessing IS with less-controlled surrogate measures, and the failure of some studies to control for important variables, including diet, acute physical activity, and habitual physical activity, which were strengths of our study.

Our findings for normal-weight girls with PCOS are similar to, although not as severe as, the alterations found in obese girls with PCOS. We, and others, have previously demonstrated that obese girls with PCOS have peripheral insulin resistance using the reference standard clamp methods [3, 5]. Using a 3-hour, 2-phase clamp test, Arslanian *et al.* [5] compared 12 obese PCOS adolescents and 10 non-PCOS obese adolescents and found that 50% of the PCOS girls had reduced peripheral tissue IS. Similarly, we found that in 41 obese PCOS and 30 obese non-PCOS adolescents, those with PCOS had a nearly 40% reduction in peripheral IS. Multiple studies assessing IS with surrogate measures have also demonstrated decreased IS in obese PCOS youth [5, 6, 17]. Thus, insulin resistance is clearly present in PCOS adolescents, regardless of their BMI.

We found the percentage of liver fat to be significantly greater in normal-weight PCOS adolescents, although the degree of hepatic fat remained clinically normal. Increased hepatic fat appears to become more remarkable with increasing BMI in the presence of PCOS, with 50% of obese PCOS adolescents having HS (hepatic fat >5%) in our previous study [3], with similar findings in obese adults with PCOS [13]. Typically, HS is associated with increased visceral fat, such as was seen in our obese PCOS adolescents [3]. The percentage of visceral fat was not greater and the waist circumference and waist/hip ratio only showed a trend toward being higher in our normal-weight PCOS adolescents [3]. Thus, the sample size might have been a limiting factor, although we did find that GIR was related to waist circumference. In both our previous study [3] and the study by Arslanian *et al.* [5], overweight/obese PCOS girls also showed evidence of hepatic insulin resistance with hyperinsulinemic euglycemic clamp testing, findings not present in our normal-weight PCOS adolescents. In our previous study, we also demonstrated adipose insulin resistance using hyperinsulinemic euglycemic clamp testing in obese PCOS adolescents, which, again, was not apparent in normal-weight PCOS girls [42].

We found that normal-weight girls with PCOS also had abnormalities in their 2-hour postprandial glucose disposal, although the glucose concentrations were still within the normal glucose tolerance range. These findings are similar to those found in the published data, although the rates of IGT vary in PCOS. A recent large retrospective study of 163 adolescent PCOS girls with different BMIs documented IGT rates of 17% in obese girls but no IGT in normal-weight girls [43]. In contrast, Flannery *et al.* [17] found that 15% of 66 PCOS girls had IGT, with the same frequency in both obese and normal-weight groups. Palmert *et al.* [44] found that 33% of 27 girls with different BMIs had IGT, with just one of the normal-weight girls having IGT. Fulghesu *et al.* [45] found that the postglucose load insulin response was elevated in 48% of 79 girls with PCOS. Ibanez *et al.* [46] also documented hyperinsulinemia after an OGTT in girls with PCOS with different BMIs. Thus, girls with PCOS have evidence of abnormal postprandial glucose disposal, which could

implicate reduced glucose transport into the peripheral (muscle, brain, kidney, and/or adipose) or splanchnic tissues and/or reduced blood flow/glucose delivery.

We also found that normal-weight girls with PCOS had muscle mitochondrial dysfunction during exercise. The ADP time constant and PCr time constant, representative of the rates of recovery of PCr and ADP depletion after exercise, were slower in girls with PCOS, although they did not work as hard during the exercise session. Moreover, we also found decreased muscle glycolytic activity on MRI in our normal-weight PCOS adolescents. The combination of muscle mitochondrial dysfunction, reduced muscle glycolytic activity, peripheral insulin resistance, and reduced postprandial glucose disposal all support a defect in muscle insulin signaling and/or substrate delivery or metabolism in PCOS girls, especially because the GIR correlated tightly with both muscle ADP time constant and PCr time constant. Abnormalities in skeletal muscle insulin signaling have been reported in adults with PCOS [47]. Moreover, a relationship between muscle mitochondrial dysfunction and insulin resistance has been consistently supported by our previous studies of adolescents with type 1 diabetes, type 2 diabetes, and obese PCOS [4, 33, 37, 48].

We found that peripheral insulin resistance is associated with PCOS status in normal-weight girls but not to the absolute testosterone concentration. Similar findings have been demonstrated in adults [3, 6, 14]. This lack of association between insulin resistance and testosterone suggests that medical interventions targeting only testosterone lowering (*i.e.*, oral contraceptive pills) might not be adequate to address the insulin resistance present in normal-weight patients with PCOS. Thus, more research is needed to evaluate the differential responses to PCOS therapies in adolescents and the ideal approach to treating normal-weight PCOS.

Our study had several strengths and weaknesses. The strengths included similar demographic and physical characteristics between the two groups, including age, age of menarche, pubertal stage, dietary macronutrient patterns, BMI, and habitual physical activity, assessed using the 3DPAR and 7 days of accelerometer use. We used reference standard methods, including the hyperinsulinemic euglycemic clamp test, with stable isotope tracers to assess tissue-specific IS, *in vivo* mitochondrial function via ³¹P MRS, hepatic and visceral fat by MRI, cardiopulmonary fitness by VO₂peak, and physical activity using objective testing. We also standardized the acute physical activity, dietary intake, and stage of menstrual cycle before performing the outcomes assessments. However, owing to the deep phenotyping of each participant, we had a relatively small sample size. Also, because of the age range of 12 to 19 years, it is possible that differential effects of the growth hormone axis were present on the results. We were unable to evaluate for this; however, both groups were approximately 4 years after menarche.

In conclusion, adolescents with PCOS who are of normal weight have peripheral insulin resistance, which relates to muscle mitochondrial defects. They also have changes in postprandial glucose disposal and hyperinsulinemia and an increase in liver fat, although technically still within the “normal” range. Clinicians must be aware that insulin resistance in this patient population is subtle, but clearly present, arguing for PCOS treatment that address insulin resistance, even for those with a normal BMI.

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