

Clinical Research Article

# Serum Testosterone to Androstenedione Ratio Predicts Metabolic Health in Normal-Weight Polycystic Ovary Syndrome Women

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**Abbreviations:** A4, androstenedione; AIRg, acute response to glucose; AKR1C3, aldo-keto reductase 1C3; ASC, adipose stem cell; BMI, body mass index; CV, coefficient of variation; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; DI, disposition index; DMEM, Dulbecco's modified Eagle's medium; E1, estrone; E2, estradiol; FFA, free fatty acid; FSH, follicle-stimulating hormone; FSIVGTT, frequently sampled intravenous glucose tolerance testing; HDL-C, high-density lipoprotein cholesterol; HMW, high-molecular-weight; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; LH, luteinizing hormone; NIH, National Institutes of Health; PCOS, polycystic ovary syndrome; SC, subcutaneous; Sg, glucose effectiveness; SHBG, sex hormone-binding globulin; Si, insulin sensitivity; T, testosterone; TG, triglyceride; UCLA, University of California, Los Angeles.

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## Abstract

**Context:** Increased aldo-keto reductase 1C3 (AKR1C3)-mediated conversion of androstenedione (A4) to testosterone (T) promotes lipid storage in subcutaneous (SC) abdominal adipose in overweight/obese polycystic ovary syndrome (PCOS) women.

**Objective:** This work examines whether an elevated serum T/A4 ratio, as a marker of enhanced AKR1C3 activity in SC abdominal adipose, predicts metabolic function in normal-weight PCOS women.

**Methods:** This prospective cohort study took place in an academic center and comprised 19 normal-weight PCOS women and 21 age- and body mass index-matched controls. Interventions included circulating hormone/metabolic determinations, intravenous glucose tolerance testing, total body dual-energy x-ray absorptiometry, and SC abdominal fat biopsy. Serum T/A4 ratios, hormone/metabolic measures, and AKR1C3 expression of adipocytes matured in vitro were compared between female types; serum T/A4 ratios were correlated with serum lipids, adipose insulin resistance (adipose-IR), homeostatic model assessment of insulin resistance (HOMA-IR) and insulin sensitivity (Si).

**Results:** Increased serum T/A4 ratios ( $P = .040$ ) and log adipose-IR values ( $P = .002$ ) in PCOS women vs controls were accompanied by *AKR1C3* messenger RNA overexpression of PCOS adipocytes matured in vitro ( $P = .016$ ). Serum T/A4 ratios in PCOS women, but not controls, negatively correlated with log triglycerides (TGs:  $R = -0.65$ ,  $P = .002$ ) and the TG index ( $R = -0.57$ ,  $P = .011$ ). Adjusting for serum free T, serum T/A4 ratios in PCOS women remained negatively correlated with log TG ( $R = -0.57$ ,  $P = .013$ ) and TG index ( $R = -0.50$ ,  $P = .036$ ), respectively, without significant relationships with other metabolic measures.

**Conclusion:** An elevated serum T/A4 ratio, as a marker of enhanced *AKR1C3* activity in SC abdominal adipose, predicts healthy metabolic function in normal-weight PCOS women.

**Key Words:** adipose, polycystic ovary syndrome, adipocyte, *AKR1C3*, testosterone, androstenedione

As the most common reproductive-metabolic disorder of reproductive-aged women, polycystic ovary syndrome (PCOS) is characterized by ovarian hyperandrogenism from altered hypothalamic-pituitary-ovarian function in combination with insulin resistance (IR) [1]. Major reproductive-metabolic manifestations of PCOS include hirsutism and oligoanovulation linked with glucose intolerance, dyslipidemia, adipose insulin resistance (adipose-IR), and preferential abdominal fat deposition [2, 3]. Consequently, most women with PCOS have IR from perturbed insulin receptor signaling, altered adipokine secretion, and abnormal steroid metabolism [1]. Almost one-half of PCOS women in the United States also have metabolic syndrome, which is greater in prevalence than that of age-matched women without PCOS and worsened by obesity [4].

Adipose-specific aldo-keto reductase 1C [*AKR1C*] enzymes control body fat distribution in humans through local androgen metabolism [5]. As a major *AKR1C* isoform with significant  $17\beta$ -hydroxysteroid dehydrogenase activity, *AKR1C3* promotes androstenedione (A4) conversion to testosterone (T), with higher activity in subcutaneous (SC) than intra-abdominal fat [6, 7]. Linked with percentage of trunk fat mass in women [5], increased *AKR1C3*-mediated conversion of A4 to T in SC abdominal adipose of overweight/obese PCOS women increases lipogenesis (lipid formation) and decreases lipolysis (lipid breakdown) [8]. Such increased *AKR1C3*-mediated T production in PCOS could promote triglyceride (TG) storage in SC adipose, which normally protects against IR, with intra-abdominal fat deposition having the opposite effect [9].

Such a mechanism favoring SC abdominal fat storage in normal-weight PCOS women with IR [2, 3], however, could also predispose to lipotoxicity through ectopic lipid accumulation with weight gain [8, 10-12]. The present study, therefore, examines whether an elevated serum T/A4 ratio (ie, total T/A4 ratio), as a marker of enhanced *AKR1C3* activity in SC abdominal adipose, predicts metabolic function

in normal-weight PCOS women compared to age- and body mass index (BMI)-matched normoandrogenic ovulatory (control) women. The study also examines whether enhanced *AKR1C3* gene expression occurs in PCOS stem cells characterized by accelerated lipid accumulation during adipocyte maturation in vitro.

## Materials and Methods

### Study Participants

Nineteen normal-weight PCOS and 21 control women (aged 19-35 years; BMI = 19-25) who had previously participated in our National Institutes of Health (NIH)-funded study (P50 HD071836) examining adipose dysfunction in PCOS were studied [2, 3, 13, 14]. All participants in the present study were healthy women, as published previously [2, 3, 13, 14].

PCOS was diagnosed by 1990 NIH criteria and biochemical hyperandrogenism, as previously defined by an elevated mean serum total or free T level from 2 separate blood samples more than 2 SD above the normal ranges of the age- and BMI-matched control group [2, 3, 13, 14]. Control women had normal menstrual cycles at 21- to 35-day intervals and a luteal-phase progesterone level without signs of androgen excess [1]. Exclusion criteria, including late-onset congenital adrenal hyperplasia, thyroid dysfunction, and hyperprolactinemia, have previously been reported [2, 3, 13, 14]. All studies were performed according to the Declaration of Helsinki after approval by the University of California, Los Angeles (UCLA) Institutional Review Board and signed informed consent by each participant.

### Body Fat Distribution

Waist and hip measurements were determined in all participants [2, 3, 13, 14]. Total body dual-energy x-ray

absorptiometry scan was measured with a Hologic QDR Discovery A densitometer (Hologic Inc) as previously reported [2, 3, 13, 14]. Android and gynoid fat regions were from the first lumbar vertebra to the top of pelvis and from the femoral head to the mid-thigh, respectively.

### Blood Sampling

All blood sampling was performed during the follicular phase (days 5-10 of the menstrual cycle) in control women and during documented oligoanovulation by a low serum follicular-phase progesterone level in PCOS women, as previously reported [2]. Fasting blood samples were collected immediately before frequently sampled intravenous glucose tolerance testing (FSIVGTT) for total and free T, A4, dihydrotestosterone (DHT), dehydroepiandrosterone sulfate (DHEAS), gonadotropins, estrone (E1), estradiol (E2), glucose, free fatty acid (FFA), insulin, sex hormone-binding globulin (SHBG), and lipid (total cholesterol, high-density [HDL], low-density lipoprotein [LDL], and triglyceride [TG]) measurements. Fasting blood values were used to calculate adipose-IR (product of fasting circulating FFA [mmol/L] and insulin levels [pmol/L]) and homeostatic model assessment of insulin resistance (HOMA-IR: [insulin ( $\mu$ U/mL)  $\times$  glucose (mmol)]/L/22.5), recognizing that adipose-IR positively correlates with hepatic fat content from ectopic fat [15, 16]. The triglyceride glucose index (TG index;  $\ln$ [TG (mg/dL)  $\times$  glucose (mg/dL)/2]) also was calculated as previously described [3].

The FSIVGTT was performed in all women using the modified minimal model of Bergman [17], except in one control who declined the study. Briefly, glucose in 50% concentration (0.3 g/kg) and regular human insulin (0.03 units/kg) were injected intravenously under fasting conditions at 0 and 20 minutes, respectively, and blood was collected at -20, -15, -5, 0, 2, 4, 8, 19, 22, 30, 40, 50, 70, 90, and 180 minutes for glucose and insulin determinations. Mathematical modeling of circulating glucose and insulin levels defined: insulin sensitivity ( $S_i$ , ie, insulin action to accelerate glucose uptake and suppress glucose production), glucose effectiveness ( $S_g$ , ie, combined effect of glucose to enhance glucose uptake and suppress endogenous glucose production at fasting insulin levels), acute response to glucose (AIR $_g$ , ie, pancreatic  $\beta$ -cell response to glucose infusion), and disposition index (DI, ie,  $\beta$ -cell compensation index [product of  $S_i$  and AIR $_g$ ]).

### Hormonal and Metabolic Assays

Serum levels of DHEAS, A4, total T, DHT, and E1 were measured by LC-MS/MS (Quest Diagnostics Nichols Institute). The intra-assay coefficient of variation (CVs) were DHEAS, 2.6%; A4, 3.9%; total T, 10.9%; DHT, 7.8%; and E1, 10.2%. The inter-assay CVs were DHEAS, 4.4%; A4, 3.5%; total T,

10.3%; DHT, 7.0%; and E1, 9.5%. Free T was calculated from the concentrations of total T, SHBG (Beckman Coulter catalog No. A48617, RRID:AB\_2893035; [http://antibodyregistry.org/AB\\_2893035](http://antibodyregistry.org/AB_2893035)) [18], and albumin. The intra-assay and interassay CVs for free T were 5.6% and 6.2%, respectively.

Serum measurements of insulin (Roche catalog No. 12017547, RRID:AB\_2756877; [http://antibodyregistry.org/AB\\_2756877](http://antibodyregistry.org/AB_2756877)) [19], luteinizing hormone (LH; Roche catalog No. 11732234, RRID:AB\_2800498; [http://antibodyregistry.org/AB\\_2800498](http://antibodyregistry.org/AB_2800498)) [20], follicle-stimulating hormone (FSH; Roche catalog No. 11775863, RRID:AB\_2800499; [http://antibodyregistry.org/AB\\_2800499](http://antibodyregistry.org/AB_2800499)) [21], and E2 (Roche, catalog No. 03000079, RRID:AB\_2893079; [http://antibodyregistry.org/AB\\_2893079](http://antibodyregistry.org/AB_2893079)) [22] by electrochemiluminescence; glucose by a hexokinase method; and fasting lipids by spectrophotometry were performed at the UCLA Center for Pathology Research Services. The laboratory intra-assay and interassay CVs also have previously been reported [2, 3, 13, 14] and were less than 7% and 11%, respectively.

Serum FFAs were measured by quantitative spectrophotometry (ARUP Laboratories). The intra-assay and interassay CVs for FFAs were 1.8% and 1.2%, respectively. Serum levels of high-molecular-weight (HMW) adiponectin by enzyme-linked immunosorbent assay (ALPCO Diagnostics catalog No. 80-ADPHU-E01, RRID:AB\_2892778; [http://antibodyregistry.org/AB\\_2892778](http://antibodyregistry.org/AB_2892778)) [23] and leptin by radioimmunoassay (Millipore catalog No. HL-81K, RRID:AB\_2756879; [http://antibodyregistry.org/AB\\_2756879](http://antibodyregistry.org/AB_2756879)) [24] were measured at the Endocrine Technologies Support Core Lab, Oregon National Primate Research Center. The intra-assay CVs for HMW adiponectin and leptin were 7.6% and 5.1%, respectively; the interassay CVs for HMW adiponectin and leptin were 8.0% and 10.1%, respectively.

### Subcutaneous Abdominal Adipose Stem Cell Differentiation Into Adipocytes In Vitro

#### *AKR1C3* gene expression

Three PCOS and 3 age-BMI-matched control women were selected from the present cohort based on their previously determined SC abdominal stem cell characteristics of enhanced lipid accumulation and PPAR $\gamma$  as well as CEBP $\alpha$  overexpression during adipogenesis in vitro [13, 19]. Each participant underwent an SC abdominal fat biopsy as previously described [13, 25]. SC adipose was washed with Dulbecco's modified Eagle's medium (DMEM) and digested at 37 °C in DMEM (Corning) containing 0.075% collagenase (Sigma-Aldrich) for 45 to 60 minutes on a shaker. After centrifugation of the digested material, the resulting cell pellet was plated in 60-mm dishes containing

DMEM/10% fetal calf serum, 0.05 U/mL penicillin, 0.05 mg/mL streptomycin, 1.25 mg/mL fungizone, and cultured at 37 °C until confluency in 5% CO<sub>2</sub>. Adipose stem cells (ASCs) were then incubated in adipocyte differentiation medium (DMEM/Ham's F-12 (1:1, v/v), HEPES pH 7.4, fetal bovine serum, biotin, pantothenate, human insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), PPAR $\gamma$  agonist, penicillin, streptomycin, amphotericin B [Zen-Bio]) for 12 days to induce ASC differentiation into adipocytes. At days 0, 3, 7, and 12, RNA was extracted from the cells using RNeasy mini kit (Qiagen). RNA sequencing for AKR1C3 was determined as previously described [25].

### Lipid staining and immunofluorescence

At day 12, fixed cells were stained with Oil-Red-O (Sigma Aldrich) for 20 minutes at room temperature to visualize lipid droplets. Nuclei were identified by the nuclear staining marker 4',6-diamidino-2-phenylindole (DAPI; 1:3000 [Invitrogen]). After 4 washes with deionized water, lipid staining was quantified by immunofluorescence. Twenty representative images were taken of fluorescent cells with an EVOS FL Digital Inverted Fluorescence microscope (Westover Scientific Inc), and fluorescence units per cell number were quantified using ImageJ software (NIH).

### Statistical Analysis

An unpaired *t* test compared patient characteristics, clinical hormone/metabolic values between PCOS, and age- and BMI-pair-matched control individuals. Results were presented with mean  $\pm$  SD unless otherwise noted. Serum DHT levels in 2 controls and 3 PCOS individuals were undetectable and excluded from analysis. Pearson correlation coefficients examined associations by female type between serum total T/A4 ratios and clinical outcomes [26]. As a sensitivity analysis, partial correlations were examined with the same associations after adjusting for free T to determine whether free T was confounding the findings. For significant associations, we also fit linear regression models and the prediction lines were superimposed on the scatterplots. Owing to potential distributional assumption violations (eg, nonnormality), a log transform was applied to some measures before analysis (eg, LH, AIR, TG, adipose-IR). Statistical analyses were run using IBM SPSS v27, and *P* values less than .05 were considered statistically significant.

For RNA-sequencing analysis, sequencing reads were first mapped to the latest UCSC transcriptome set using Bowtie2 version 2.1.0 [27] and the gene expression level was estimated using RSEM v1.2.15 [28]. TMM (trimmed mean of M-values) was used to normalize gene expression. A 2-way analysis of variance with repeated measures (for time in

days) compared AKR1C3 gene expression and lipid content of cultured stem cells over time by female type, and *P* values less than .05 were considered statistically significant.

## Results

### Patient and Hormone Characteristics

Age, BMI, waist, and hip measurements were comparable between the PCOS and control women. Total and lean body mass as well as total and percentage of body fat also were comparable between PCOS and control women. The percentage of android fat relative to total body fat and the ratio of android to gynoid fat mass in PCOS women were within the high-normal ranges (Table 1). There were no female-type differences in serum FSH, estrogen, DHEAS, or fasting glucose, insulin, and cholesterol levels.

Serum log LH and total/free T as well as A4 levels were greater in PCOS than control women (LH, *P* = .004; androgens, *P* < .001), as were serum DHT levels (*P* = .007) (see Table 1). The serum total T to A4 ratio was also greater in PCOS than control women (*P* = .040). HOMA-IR and Si values in PCOS women were within the high-normal and low-normal ranges, respectively, without female-type differences in Sg, log AIRg, or DI values (Fig. 1). Log adipose-IR values were increased in PCOS vs control women (*P* = .002), as previously reported [3], due in part to high-normal fasting circulating FFA levels (*P* = .050). Log fasting serum TG concentrations, TG index, and serum HMW adiponectin as well as leptin levels were similar between female groups.

### Clinical Correlations

#### Polycystic ovary syndrome women

The serum total T/A4 ratio in PCOS women was not significantly correlated with log adipose-IR (*R* = -0.44, *P* = .061), despite serum levels of free T, A4, and DHEAS being positively correlated with log adipose-IR (free T: *R* = +0.51, *P* = .025; A4: *R* = +0.46, *P* = .049; DHEAS: *R* = +0.49, *P* = .032) (Fig. 2). In contrast, the serum total T/A4 ratio in PCOS women negatively correlated with log TG (*R* = -0.65, *P* = .002) and TG index (*R* = -0.57, *P* = .011) (Fig. 3), whereas serum A4 levels positively correlated with log TG (*R* = +0.52, *P* = .021) and TG index (A4: *R* = +0.51, *P* = .020), without other serum androgen interactions with log TG (total T: *R* = +0.24, *P* = .323; free T: *R* = +0.43, *P* = .066; DHEAS: *R* = +0.01, *P* = .970) or TG index (total T: *R* = +0.29, *P* = .230; free T: *R* = +0.35, *P* = .143; DHEAS: *R* = +0.02, *P* = .934). Serum DHT levels were unrelated to log adipose-IR (*R* = -0.23, *P* = .390), log TG (*R* = +0.27, *P* = .319), or TG index (*R* = -0.27, *P* = .303).

**Table 1.** Patient characteristics and serum hormone and metabolic levels in normal-weight control vs polycystic ovary syndrome women<sup>a,b</sup>

Patient characteristics	NL (N = 21)	PCOS (N = 19)	P
Age, y	27.4 ± 4.8	25.2 ± 4.8	.161
BMI	21.6 ± 1.5	22.1 ± 1.9	.400
Waist, cm	75.0 ± 4.8	75.3 ± 4.9	.843
Hip, cm	88.5 ± 5.9	87.9 ± 5.2	.706
Total body mass, kg	60.7 ± 7.8	60.8 ± 6.7	.960
Lean body mass, kg	39.1 ± 5.1	39.4 ± 5.2	.843
Total body fat	19.3 ± 3.2	19.6 ± 2.8	.835
% body fat	31.8 ± 2.9	32.3 ± 3.9	.677
Android fat, kg	1.1 ± 0.3	1.2 ± 0.4	.213
% android fat	5.5 ± 0.7	6.1 ± 1.3	.111
Gynoid fat, kg	4.1 ± 0.8	4.0 ± 0.6	.692
% gynoid fat	21.4 ± 1.6	20.7 ± 1.5	.200
Android/gynoid fat mass ratio	0.26 ± 0.04	0.30 ± 0.07	.078
<b>Hormone/metabolic levels</b>			
Log LH, mIU/mL	0.89 ± 0.2	1.1 ± 0.2	.004
LH, mIU/mL	8.8 ± 4.9	14.1 ± 6.7	.007
FSH, mIU/mL	6.0 ± 2.3	5.4 ± 1.6	.390
E1, pg/mL	64.0 ± 32.0	71.8 ± 29.5	.429
E2, pg/mL	102.9 ± 104.6	77.3 ± 57.4	.339
Total T, ng/dL	30.1 ± 8.5	61.9 ± 18.6	<.001
Free T, pg/mL	2.3 ± 1.0	5.9 ± 1.8	<.001
A4, ng/dL	118.2 ± 37.6	202.7 ± 82.0	<.001
T/A4 ratio	0.27 ± 0.08	0.34 ± 0.13	.040
DHT, ng/dL	9.7 ± 2.7	15.9 ± 7.8	.007
DHEAS, µg/dL	178.4 ± 95.5	228.4 ± 68.2	.068
Fasting glucose, mg/dL	85.2 ± 6.0	85.7 ± 6.7	.819
Fasting insulin, µU/mL	4.6 ± 1.9	5.7 ± 2.1	.109
HOMA-IR	0.99 ± 0.5	1.2 ± 0.5	.161
Si, ×10 <sup>-4</sup> /min/µU/mL	5.9 ± 5.0	4.3 ± 1.9	.181
Log AIRg, µU/mL	2.5 ± 0.2	2.5 ± 0.2	.559
DI, Si × AIRg/100	20.0 ± 14.4	14.0 ± 7.6	.113
Sg, ×10/min	0.02 ± 0.01	0.02 ± 0.02	.882
SHBG, nmol/L	68.5 ± 32.4	52.5 ± 28.4	.107
HMW adiponectin, ng/mL	3212 ± 1653	2887 ± 2021	.579
Leptin, ng/mL	23.7 ± 13.2	20.9 ± 7.3	.415
Total free fatty acids, mmol/L	0.59 ± 0.2	0.75 ± 0.3	.050
Log adipose-IR	1.2 ± 0.2	1.4 ± 0.2	.002
Log TG, mg/dL	1.7 ± 0.2	1.8 ± 0.1	.195
HDL-C, mg/dL	64.1 ± 12.0	65.1 ± 10.6	.781
Non-HDL-C, mg/dL	91.8 ± 27.7	90 ± 26.0	.835
LDL-C, mg/dL	80.1 ± 24.2	77.3 ± 26.2	.721
Total C, mg/dL	156.7 ± 29.9	155.2 ± 29.8	.874
TG index	3.4 ± 0.2	3.4 ± 0.1	.246

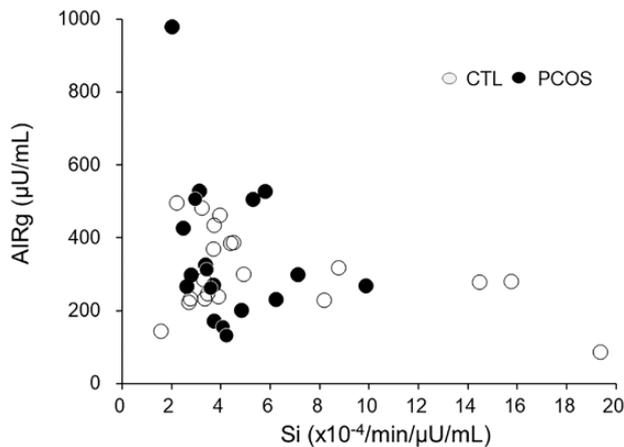
Mean ± SD. Conversion to SI units: T (× 0.0347 nmol/L), free T (× 3.47 pmol/L), A4 (× 0.0349 nmol/L), DHT (× 0.0344 nmol/L), DHEAS (× 0.0271 µmol/L), E1 (× 3.699 pmol/L), E2 (× 3.67 pmol/L), LH (× 1.0 IU/L), FSH (× 1.0 IU/L), glucose (× 0.0555 mmol/L), insulin (× 7.175 pmol/L), HMW adiponectin (× 0.001 µg/L), leptin (× 1.0 µg/L), total C (× 0.0259 mmol/L), HDL-C (× 0.0259 mmol/L), LDL-C (× 0.0259 mmol/L), non-HDL-C (× .0259 mmol/L), TG (× 0.0113 mmol/L).

Abbreviations: A4, androstenedione; AIRg, acute response to glucose; BMI, body mass index; C, cholesterol; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; DI, disposition index; E1, estrone; E2, estradiol; FSH, follicle-stimulating hormone; HDL-C, high-density lipoprotein cholesterol; HMW, high-molecular-weight; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; LH, luteinizing hormone; NL, normal-weight control; PCOS, polycystic ovary syndrome; Sg, glucose effectiveness; SHBG, sex hormone-binding globulin; Si, insulin sensitivity; T, testosterone; TG, triglycerides.

<sup>a</sup>Modified from references [2, 3, 13] and [14].

<sup>b</sup>Total body dual-energy x-ray absorptiometry studies (NL = 18, PCOS = 16); DHT values (NL = 19, PCOS = 16 because of undetectable levels in 2 controls and 3 PCOS individuals); frequently sampled intravenous glucose tolerance testing studies, log adipose-IR, and TG index studies (NL = 20, PCOS = 19).

Simultaneously, the serum total T/A4 ratio in PCOS women was not significantly correlated with fasting insulin ( $R = -0.38$ ,  $P = .112$ ) or HOMA-IR ( $R = -0.39$ ,  $P = .102$ ) (Fig. 4), although serum free T levels positively correlated with fasting insulin levels ( $R = +0.55$ ,  $P = .015$ ) and HOMA-IR values ( $R = +0.53$ ,  $P = .020$ ). Other serum androgen levels were unrelated to fasting insulin (total T:  $R = +0.11$ ,  $P = .651$ ; A4:  $R = +0.24$ ,  $P = .313$ ; DHT:  $R = -0.13$ ,  $P = .638$ ; DHEAS:  $R = +0.24$ ,  $P = .319$ ) or HOMA-IR (total T:  $R = +0.16$ ,  $P = .520$ ; A4:  $R = +0.29$ ,  $P = .224$ ; DHT:  $R = -0.16$ ,  $P = .542$ ; DHEAS:  $R = +0.26$ ,  $P = .289$ ).



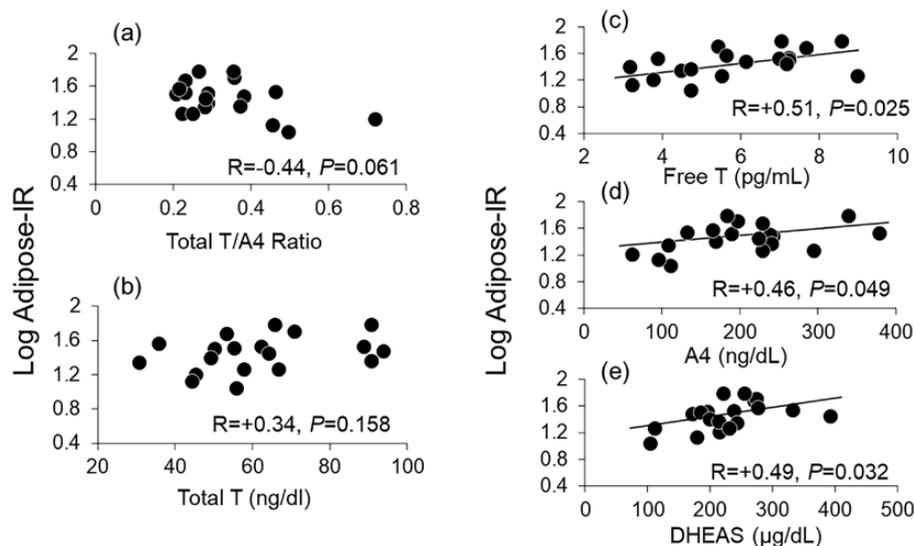
**Figure 1.** Relationships between Si and AIRg in 19 normal-weight polycystic ovary syndrome (PCOS) women and 20 age- and body mass index-matched normoandrogenic women (controls [CTL]). AIRg, acute insulin response to glucose (pancreatic  $\beta$ -cell response to glucose infusion); Si, insulin sensitivity (insulin action to accelerate glucose uptake and suppress glucose production). Open circles, controls; filled circles, PCOS women.

Similarly, the serum total T/A4 ratio in PCOS women was not significantly correlated with the android to gynoid fat mass ratio ( $R = -0.43$ ,  $P = .098$ ), whereas serum free T levels positively correlated to this ratio (free T:  $R = +0.53$ ,  $P = .036$ ; A4:  $R = +0.45$ ,  $P = .080$ ; DHT:  $R = -0.15$ ,  $P = .600$ ; DHEAS:  $R = +0.01$ ,  $P = .991$ ).

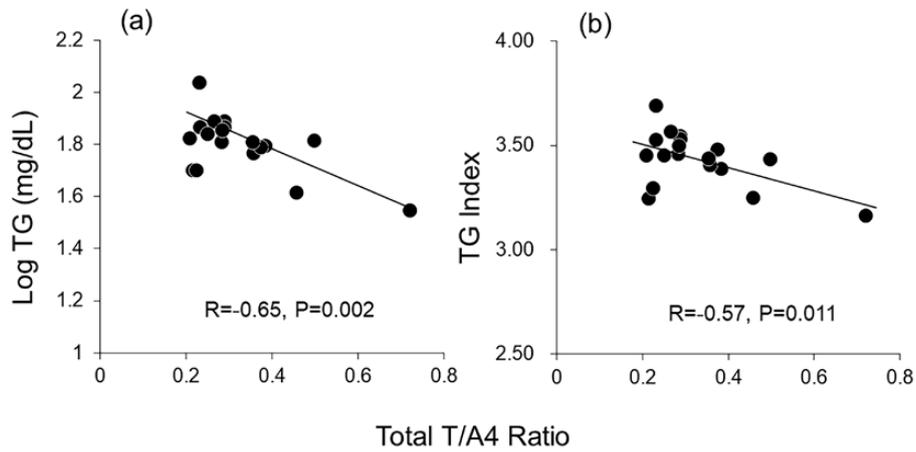
Partial correlations were examined after adjusting for serum free T to determine whether hyperandrogenism per se was confounding the findings. Adjusting for serum free T, the serum total T/A4 ratio in PCOS women remained negatively correlated with log TG ( $R = -0.57$ ,  $P = .013$ ) and TG index ( $R = -0.50$ ,  $P = .036$ ), respectively. The relationships between serum total T/A4 ratio and basal insulin ( $R = -0.18$ ,  $P = .476$ ), HOMA-IR ( $R = -0.20$ ,  $P = .420$ ), log adipose IR ( $R = -0.28$ ,  $P = .270$ ), and android to gynoid fat mass ratio ( $R = -0.26$ ,  $P = .354$ ) were not significant after adjusting for serum free T.

#### Control women

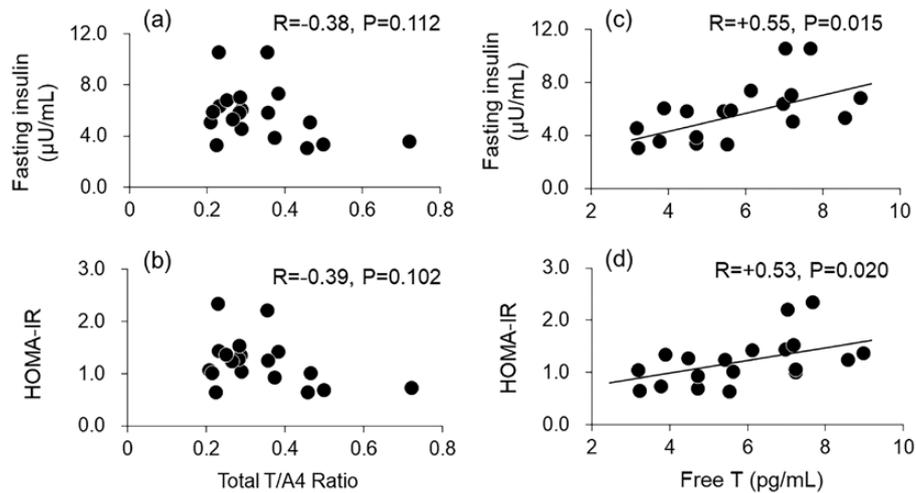
The serum total T/A4 ratio in control women was unrelated to log adipose-IR ( $R = -0.32$ ,  $P = .164$ ), log TG ( $R = -0.28$ ,  $P = .226$ ), TG index ( $R = -0.32$ ,  $P = .165$ ), HOMA-IR ( $R = -0.24$ ,  $P = .314$ ), fasting insulin levels ( $R = -0.27$ ,  $P = .241$ ) or android to gynoid fat mass ratio ( $R = +0.18$ ,  $P = .473$ ). Instead, it positively correlated with serum HMW adiponectin levels ( $R = +0.49$ ,  $P = .026$ ) (Fig. 5) without other significant serum androgen interactions (total T:  $R = +0.15$ ,  $P = .504$ ; free T:  $R = -0.20$ ,  $P = .386$ ; A4:  $R = -0.27$ ,  $P = .242$ ; DHT:  $R = -0.06$ ,  $P = .792$ ; DHEAS:  $R = -0.05$ ,  $P = .831$ ). The serum total T/A4 ratio in control women also negatively correlated with log AIRg ( $R = -0.52$ ,  $P = .019$ ) and positively correlated with SHBG ( $R = +0.52$ ,



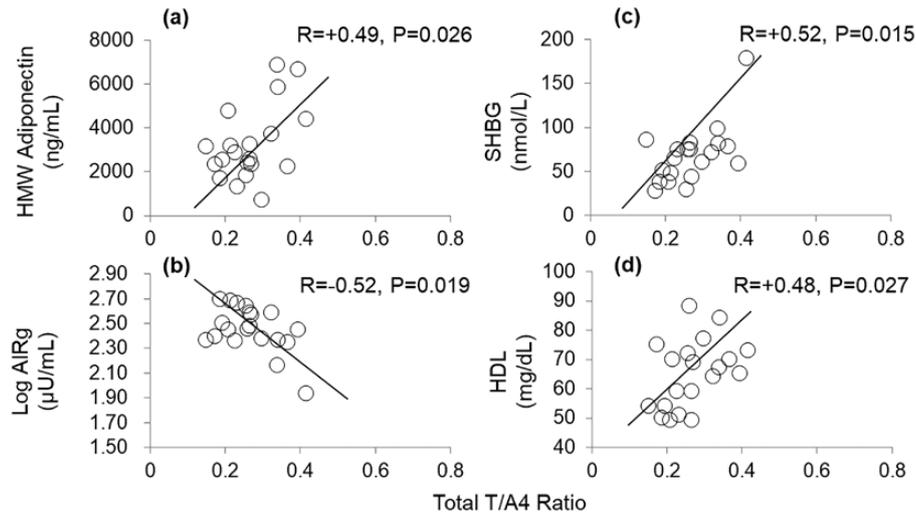
**Figure 2.** Correlations of log adipose-IR with serum A, total T/A4 ratio; B, total T; C, free T; D, A4; and E, DHEAS levels in normal-weight polycystic ovary syndrome (PCOS) women. Filled circles, PCOS women. All x-axes for log adipose-IR are identical in scale. A4, androstenedione; DHEAS, dehydroepiandrosterone sulfate; log adipose-IR, log adipose insulin resistance; T, testosterone.



**Figure 3.** Correlations of A, serum logTG level, and B, TG index value with serum total T/A4 ratio in normal-weight polycystic ovary syndrome (PCOS) women. Filled circles, PCOS women. A4, androstenedione; Log TG, log triglyceride; testosterone, T; TG index, triglyceride glucose index.



**Figure 4.** Correlations of A and C, fasting serum insulin level, and B and D, HOMA-IR value with A and B, serum total T/A4 ratio, and C and D, serum free T level in normal-weight polycystic ovary syndrome (PCOS) women. Filled circles, PCOS women. A4, androstenedione; HOMA-IR, homeostatic model assessment of insulin resistance; T, testosterone.



**Figure 5.** Correlations of serum A, HMW adiponectin (N = 21); B) log AIRg (N = 20); C, SHBG (N = 21); and D, HDL (N = 21) values with serum total T/A4 ratios in normal-weight controls. One control declined frequently sampled intravenous glucose tolerance testing. AIRg, acute insulin response to glucose; HDL, high-density lipoprotein; HMW, high-molecular-weight; SHBG, sex hormone-binding globulin.

$P = .015$ ) values, with opposite relationships of individual serum androgen levels with log AIRg (free T:  $R = +0.40$ ,  $P = .077$ ; A4:  $R = +0.44$ ,  $P = .053$ ) and SHBG (free T:  $R = -0.55$ ,  $P = .009$ ) values. The serum total T/A4 ratio in control women also positively correlated with serum HDL levels ( $R = +0.48$ ,  $P = .027$ ), with serum HDL and DHEAS levels positively interrelated ( $R = +0.51$ ,  $P = .017$ ).

The serum total T/A4 ratio in control women remained related to serum HMW adiponectin levels ( $R = +0.48$ ,  $P = .034$ ), log AIRg ( $R = -0.51$ ,  $P = .026$ ), SHBG ( $R = +0.56$ ,  $P = .009$ ), and serum HDL levels ( $R = +0.55$ ,  $P = .012$ ), after adjusting for serum free T.

### Subcutaneous abdominal stem cell characteristics

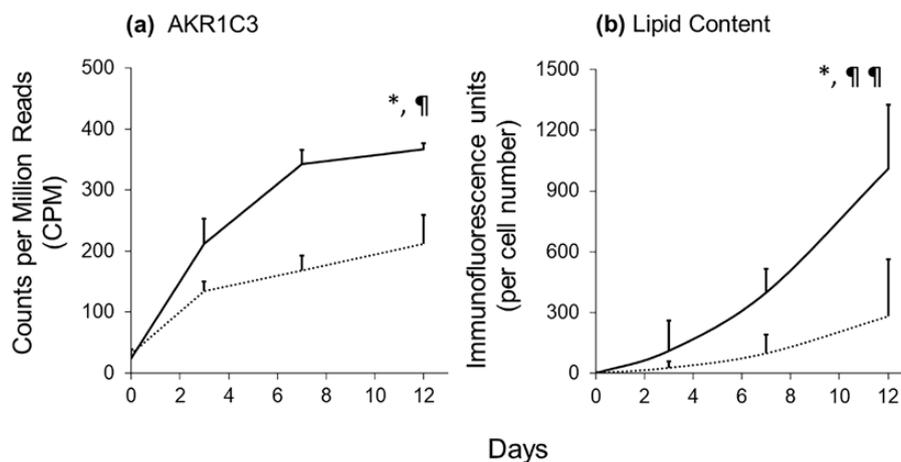
Significant female-type differences in *AKR1C3* gene expression occurred over time during SC abdominal stem cell development to adipocytes in vitro. In all 3 pair-matched cell samples, *AKR1C3* gene expression rose from day 3 to day 7 to reach maximal levels on day 12 (day effect,  $P = .002$ ), with greater *AKR1C3* messenger RNA expression in PCOS than control stem cells (PCOS effect,  $P = .016$ ; interaction,  $P = .137$ ) (Fig. 6). Lipid accumulation also rose from day 3 to day 12 (day effect,  $P = .003$ ), with greater lipid content in PCOS than control stem cells (PCOS effect,  $P = .009$ ; interaction,  $P = .068$ ) (Fig. 6). In PCOS women, the lowest and highest preadipocyte *AKR1C3* gene expressions corresponded with the lowest and highest serum T/A4 ratios, respectively ( $R = 0.98$ ,  $N = 3$ ); in controls, this relationship between preadipocyte *AKR1C3* gene expression and serum T/A4 ratio was not evident ( $R = 0.46$ ,  $N = 3$ ). Statistical testing ( $P$  values) were not performed because of the small sample size, but these results are suggestive.

## Discussion

Adipose-specific *AKR1C* enzymes control body fat distribution in humans, with *AKR1C3* expression being greater in SC than intra-abdominal adipose [6, 7]. In overweight/obese women with PCOS, increased *AKR1C3*-mediated T generation in SC abdominal adipose promotes lipid storage through increased lipogenesis and decreased lipolysis, implicating increased intra-adipose *AKR1C3* activity with metabolic function in PCOS [8]. Our present findings reaffirm these prior results by showing that an elevated serum total T to A4 ratio, as a marker of SC-enhanced abdominal adipose *AKR1C3* activity, in normal-weight women with PCOS accompanies *AKR1C3* overexpression in their SC abdominal stem cells during adipocyte maturation in vitro.

An elevated serum total T to A4 ratio, as a marker of enhanced *AKR1C3* activity in SC abdominal adipose in our PCOS women, offers a link between androgen inhibition of catecholamine-stimulated lipolysis in SC abdominal adipose [29] and catecholamine lipolytic resistance as reported in other PCOS women [30-32]. Such a mechanism in PCOS to promote SC lipid storage could lower circulating FFA levels via lipolytic suppression to diminish hepatic glucose production, according to the “single gateway hypothesis” [33], and thereby counterbalance increased adipose-IR to reduce hepatic fat content through decreased ectopic fat deposition [15, 16]. At a cellular level, therefore, SC abdominal adipocytes of PCOS women could simultaneously maintain lipid storage through acetyl-CoA carboxylase (as a crucial enzyme governing lipid metabolism) despite diminished glucose uptake from androgen inhibition of PKC zeta phosphorylation [34] and/or reduced GLUT-4 expression [35].

In support of this concept, the elevated serum total T/A4 ratio in normal-weight PCOS women negatively



**Figure 6.** A, *AKR1C3* messenger RNA levels, and B, lipid accumulation in polycystic ovary syndrome (PCOS) ( $N = 3$ ) vs control ( $N = 3$ ) subcutaneous (SC) abdominal stem cells during adipocyte maturation in vitro. These 3 PCOS and 3 age-body mass index-matched control women were selected from the present cohort based on their previously determined SC abdominal stem cell characteristics of enhanced lipid accumulation and *PPAR $\gamma$*  as well as *CEBP $\alpha$*  overexpression during adipogenesis in vitro [13, 25]. Mean  $\pm$  SD (day effect:  $*P < .005$ ; PCOS effect: ¶ $P < .025$ ; ¶¶ $P < .01$ ).

correlated with log serum TG levels and TG index values, and remained so after adjusting for serum free T. These correlations coincide with our previous findings in normal-weight PCOS women of enhanced lipid accumulation in SC abdominal ASCs during adipocyte maturation in vitro that both positively correlates with hyperandrogenemia and predicts reduced serum FFA levels and improves systemic insulin sensitivity in vivo [13, 14]. That such PCOS ASC behavior accompanies *AKR1C3* overexpression during its normal rise during adipocyte maturation in vitro [36] suggests that reduced SC abdominal TG turnover in normal-weight PCOS women favors Si, given that increased TG turnover in this adipose depot accompanies IR, preferential intra-abdominal fat accumulation, and fatty liver in obese girls [11, 12].

Importantly, *AKR1C3* gene expression and activity are greater in preadipocytes and adipose of gluteal compared to omental fat, with gluteal fat favoring androgen activation, and omental cells favoring androgen inactivation [7]. These differential actions of *AKR1C3*-mediated androgen activation by fat depot, combined with a positive association of hyperandrogenemia with intra-abdominal fat accumulation in normal-weight PCOS women [2], likely obscured any relationship of serum total T/A4 ratio with android to gynoid fat mass ratio in PCOS individuals.

Unlike the findings in women with PCOS, the serum total T/A4 ratio in controls positively correlated with serum adiponectin levels. As an insulin-sensitizing adipokine with higher expression in SC abdominal than omental fat [37], circulating adiponectin levels in humans suppress hepatic glucose production independent of glucose tolerance [38], presumably through inhibition of gluconeogenic enzyme expression as seen in mice [39, 40]. Adiponectin actions on hepatic function in controls may also underlie the other positive correlations of the serum total T to A4 ratio with SHBG and HDL levels [41]. In humans, serum adiponectin levels positively correlate with plasma HDL cholesterol levels [41] by inducing apo-AI and adenosine 5'-triphosphate-binding cassette transporter A1 during HDL assembly [42]; they also positively correlate with serum SHBG levels [43], likely through adiponectin-induced SHBG production, as seen in HepG2 cells [44]. Moreover, HDL induces DHEAS production in adrenocorticotropin-stimulated adult human adrenocortical cells in vitro [45], underlying the positive relationship of serum total T to A4 ratio with serum DHEAS level.

An important strength of this study was the use of healthy, normal-weight PCOS women according to NIH criteria who had a mild PCOS phenotype [46, 47] and were age- and BMI-matched to controls to eliminate the confounding effects of age, obesity, and referral bias on metabolic outcomes [15, 46-48], including Si [49, 50]. Our

experimental design also allowed us to study the relationship of the serum total T to A4 ratio, as a marker of SC abdominal *AKR1C3* activity, with metabolic outcomes in PCOS women with both high-normal HOMA-IR levels and low-normal Si values, adjusting for the confounding effects of circulating free T levels on glucose and lipid metabolism.

An important limitation of our study, however, was the use of serum, rather than adipose, to measure the total T to A4 ratio as a marker of SC abdominal *AKR1C3* activity, which may have led to underestimations in our findings, given that the elevation of this ratio is greater in the adipose than blood of PCOS women [8]. The small number of PCOS patients also diminished the statistical power to examine subtle interactions between various adipokines and clinical outcomes in vivo, and limited the applicability of our data to women of different PCOS phenotypes, ethnicity, adiposity, or age. Moreover, other *AKR1C* enzymes and hepatic insulin actions as well as their interactions with other steroids were not assessed and could have influenced the interactions examined. Also, we did not assess the role of TG catabolism, the effect of *AKR1C3* on 11-oxygenated androgen synthesis of adrenal origin [51], nor the circulating levels of 11-oxygenated androgen, which are elevated in overweight/obese women with PCOS [52]. Finally, *AKR1C3* overexpression in SC abdominal adipose PCOS stem cells was based on their enhanced cellular lipid accumulation and *PPAR $\gamma$*  as well as *CEBP $\alpha$*  overexpression during adipocyte maturation in vitro [13, 25], which may not apply to similar stem cells from other women with PCOS.

Our findings nevertheless suggest that, in healthy, normal-weight PCOS women, an elevated serum total T/A4 ratio as a marker of enhanced *AKR1C3* activity in SC abdominal adipose favors lipid storage. Such a metabolic adaptation in normal-weight PCOS women with low-normal Si may simultaneously enhance SC fat storage and increase circulating glucose availability as energy substrate for crucial target tissues, including brain and muscle.

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**Disclosures:** D.A.D. has consulted for Spruce Biosciences, Inc. A.T., M.M., R.N., G.L., G.D.C., T.R.G., and D.H.A have nothing to disclose.

**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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