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Distinct Early Phenotypes in Girls at Risk for PCOS

Distinctive Reproductive Phenotypes in Peripubertal Girls at Risk for Polycystic Ovary Syndrome

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Context: Increased testosterone (T) levels are a cardinal feature of PCOS. Female relatives of affected women, including postmenopausal mothers and premenarchal daughters (PCOS-d), have elevated T levels consistent with a genetic susceptibility to this phenotype. Obese girls (OB-g) also have increased T levels throughout puberty, which may indicate risk for PCOS.

Objective: We tested the hypothesis that premenarchal PCOS-d have distinctive phenotypic features compared to OB-g.

Design, Setting, and Participants: 48 PCOS-d, 30 OB-g and 22 normal weight (NW-g) premenarchal girls were studied. Mothers of OB-g and NW-g had no clinical evidence for PCOS.

Main Outcome Measures: Reproductive hormones were measured.

Results: BMI differed by design, highest in OB-g, followed by PCOS-d ($P > 0.001$ between all groups). PCOS-d and OB-g had similar increases in free T levels compared to NW-g (PCOS-d v NW-g, $P = 0.01$; OB-g v NW-g, $P = 0.0001$; PCOS-d v OB-g, $P = 0.19$). SHBG levels were lowest in OB-g, and lower in PCOS-d compared with NW-g (PCOS-d v NW-g, $P = 0.005$; OB-g v NW-g, $P < 0.0001$; PCOS-d v OB-g, $P < 0.0001$). AMH levels in PCOS-d were significantly increased

compared to OB-g, who tended to have lower AMH levels than NW-g (PCOS-d v OB-g, $P < 0.0001$; OB-g v NW-g, $P = 0.07$; PCOS-d v NW-g, $P = 0.10$).

Conclusions: Despite similarly elevated free T levels, PCOS-d had increased AMH levels compared to OB-g. This finding suggests that OB-g lack alterations in ovarian folliculogenesis, a key reproductive feature of PCOS. Causal mechanisms may differ in PCOS-d or OB-g, or elevated T in OB-g may not be an early marker for PCOS.

We found similar degrees of hyperandrogenemia in premenarchal PCOS daughters and obese girls but differing anti-Müllerian hormone levels, suggesting ovarian folliculogenesis differs in these groups.

Introduction

Hyperandrogenemia is a cardinal reproductive phenotype of PCOS and may play a causal role in disease pathogenesis (1). Approximately 40% of reproductive-age sisters of affected women have elevated total or bioavailable testosterone (T) levels (1). Further, male (2) as well as non-reproductive-age female (i.e. postmenopausal and premenarchal (3,4)) first-degree relatives have hyperandrogenemia, suggesting a genetic susceptibility to this phenotype. In animal models, including non-human primates (5-7), androgen exposure *in utero* (5,8), neonatally (9) or peripubertally (7) can result in phenocopies of PCOS. Accordingly, we have hypothesized that genetic variation resulting in hyperandrogenemia during key developmental windows programs the phenotypic features of PCOS (4,10). Consistent with this hypothesis, we have found that daughters of affected women (PCOS-d) have evidence for increased global 5 α -reductase activity in early childhood (10) and elevated T levels beginning in early puberty (4). In addition to these early differences in androgen production and metabolism, PCOS-d exhibit other reproductive and metabolic phenotypes, including elevations of AMH over the course of childhood, as well as early metabolic and β -cell dysfunction by the peripubertal years (4,11,12).

Obese girls (OB-g) also have increased total and free T levels during the pubertal transition (13). Because of the proposed central role of androgens in the pathogenesis of PCOS (14), it has been hypothesized that hyperandrogenemic OB-g will develop PCOS after puberty (15,16). Thus, elevated T levels may be an early biomarker for PCOS (16). However, no longitudinal studies have been performed to test this hypothesis.

An important distinguishing feature between PCOS-d and OB-g is their differing genetic risk for PCOS. All of the mothers of PCOS-d are genetically affected by definition. Accordingly, a substantial percentage of PCOS-d will have inherited one or more PCOS susceptibility variants (17-19) from their affected mothers (20). In contrast, the prevalence of PCOS susceptibility loci in OB-g and their mothers would be expected to reflect the background 7-15% population prevalence of PCOS (21). Therefore, the mechanisms for early hyperandrogenemia and the risk for future development of PCOS may differ in these distinct groups. We performed this study to test the hypothesis that the premenarchal reproductive phenotypes differ in OB-g compared to those in PCOS-d.

Methods

Premenarchal PCOS-d (n=48), OB-g (n=30) and normal weight control girls (NW-g, n=22) aged 8-12 years with breast Tanner stage I-III were studied. OB-g had the additional body mass index (BMI) inclusion criterion of BMI greater than or equal to the 97th percentile and NW-g had a BMI criterion of BMI less than the 85th percentile; PCOS-d of any BMI were included. All girls were in good health and not taking any medications known to alter reproductive hormone metabolism or glucose homeostasis for at least 1 month prior to study. PCOS-d had a mother

who fulfilled NIH criteria for PCOS (hyperandrogenism and oligomenorrhea with exclusion of other reproductive disorders (21)) as confirmed by us prior to the current study or by their personal physician. Mothers of OB-g and NW-g had regular menses every 27-35 days as well as no history of reproductive disorders and no signs or symptoms of androgen excess by validated questionnaire (1). Participants were recruited by contacting women who have previously participated in our studies of PCOS and control adult women, through recruitment letters sent to eligible patients at our medical centers, as well as by advertisements in local media and on-line.

All girls were in good health and not taking any medications known to alter reproductive hormone metabolism or glucose homeostasis for at least one month prior to study. PCOS-d, OB-g, and NW-g were studied at Ann & Robert H Lurie Children's Hospital, additional PCOS-d were studied at the Milton S Hershey Medical Center-Penn State College of Medicine. The Institutional Review Boards of the Feinberg School of Medicine, Northwestern University, Ann & Robert H Lurie Children's Hospital, and Penn State College of Medicine approved this study. Written informed consent was obtained from a parent of all girls, and written assent was obtained from each girl aged 12 years or older prior to participation. Clinical and biochemical data from some of the study subjects have been previously reported (4,22).

A physical exam including breast Tanner staging determined by visualization and palpation was performed by a single pediatric endocrinologist at Ann & Robert H Lurie Children's Hospital, and by a trained study coordinator at Hershey Medical Center-Penn State. A fasting early morning blood sample was collected for measurement of anti-Müllerian hormone (AMH), T, SHBG, ultra-sensitive estradiol, dehydroepiandrosterone sulfate (DHEAS), and androstenedione levels.

Assays

Androstenedione, ultra-sensitive estradiol, AMH, SHBG, and DHEAS were measured at the University of Virginia Ligand Core Lab, Charlottesville, VA. Androstenedione was measured by using the Siemens Diagnostics (Los Angeles, CA) RIA system (sensitivity 0.1 ng/mL, intra-assay CV 4.9%, interassay CV 7.0%), ultra-sensitive estradiol was measured using the Siemens Diagnostics (Los Angeles, CA) RIA system (sensitivity 10 pg/mL, intra-assay CV 6.3%, interassay CV 8.1%), and AMH was measured using the Beckman Coulter (Brea, CA) 2-site ELISA System (sensitivity 0.16 ng/mL, intra-assay CV 3.9%, interassay CV 6.2%) (23). SHBG and DHEAS were measured by the Siemens Diagnostics (Los Angeles, CA) chemiluminescence system (SHBG sensitivity 2 nmol/L, intra-assay CV 2.7%, interassay CV 5.2%; DHEAS sensitivity 150 ng/mL, intra-assay CV 5.4%, interassay CV 6.5%). T levels were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Brigham Research Assay Core, Boston, MA, sensitivity 2 ng/dL, intra-assay coefficient of variation (CV) 9.0% at 16 ng/dL, interassay CV 15.8% at 12 ng/dL) (12). Free T was calculated as reported (24).

Statistical Analysis

Data were log or square root transformed when necessary to achieve homogeneity of variance. Pearson correlation was performed to assess the association of the potential confounders of age and breast Tanner Stage with all endpoints. For variables that were significantly correlated with age and/or breast Tanner Stage, Analysis of Covariance (ANCOVA) adjusting for these parameters was applied. Otherwise, unadjusted ANOVA was used to assess differences in endpoint variables between the groups. Tukey post-hoc testing was used to determine which groups differed significantly. Categorical variables were compared by Fisher's exact test. We performed a subgroup analysis in obese PCOS-d and OB-g of comparable BMI to control for the independent impact of obesity on significant endpoints. In order to isolate a cohort of PCOS-d

and OB-g with comparable BMI, PCOS-d with the lowest BMI and OB-g with the highest BMI were excluded in a stepwise fashion until we achieved a cohort with comparable BMI (n=27, limited to participants with BMI z scores of 1.7 to 2.6). For this analysis, Student's *t*-tests were applied. Normative ranges for endpoint variables were defined by breast Tanner stage-specific thresholds of ± 2 SD in the L-g. Statistical analyses were performed with SAS 9.4 (SAS Institute, Inc., Cary, NC). Data are reported as the mean $\pm$ SD with the level of alpha set at 0.05.

Results

BMI differed between the groups by design, being highest in OB-g (Table 1). Both OB-g and PCOS-d were heavier than NW-g (Table 1). There were minimal differences in age between the groups ($P=0.05$), but with a trend toward younger age in PCOS-d compared to OB-g and NW-g (Table 1). Consistent with their younger age, there were a higher percentage of Tanner I and II stage breast ($P=0.01$) and pubic hair ($P=0.01$) PCOS-d compared to OB-g and NW-g (Table 1). The groups also varied in race and ethnicity, with a higher prevalence of Hispanic and Black girls in the OB-g compared with NW-g and PCOS-d ($P<0.0001$).

Total T levels did not differ between the groups ($P=0.53$, Figure 1). SHBG levels were lowest in OB-g compared to PCOS-d ($P<0.0001$) and to NW-g ($P<0.0001$), and were also lower in PCOS-d compared to NW-g ($P=0.005$) (Figure 1). Free T levels were similarly increased in PCOS-d ($P=0.01$) and in OB-g ($P=0.0001$) compared to NW-g (Figure 1). In total, 39% of PCOS-d and 38% of OB-g had elevated free T levels. DHEAS ($P=0.34$) and androstenedione levels ($P=0.52$, Table 1) did not differ between the groups. Estradiol levels were also similar between the groups ($P=0.09$, Table 1), and were strongly correlated with breast Tanner stage ($r=.51$, $P<0.0001$). AMH levels were significantly higher in PCOS-d compared to OB-g ($P<0.0001$); there was a trend toward higher AMH levels in PCOS-d compared to NW-g ($P=0.10$) (Figure 1). 31% of PCOS with elevated AMH also had elevated free T.

In the subgroup analysis in obese PCOS-d and OB-g, BMI z score ($P=0.08$), age ($P=0.10$) and breast Tanner stage ($P=0.29$) did not differ. AMH levels ($P=0.03$) remained increased in PCOS-d. SHBG levels remained lower in OB-g ($P=0.03$), while free T levels tended to be higher in OB-g ($P=0.09$).

Discussion

We have found that peripubertal PCOS-d and OB-g have distinct reproductive phenotypes. Despite similar elevations in free T compared to NW-g, AMH levels were significantly higher in PCOS-d compared to OB-g. It is possible that obesity contributed in the lower AMH levels in OB-g since a negative correlation between BMI and AMH levels has been reported in some (25,26), but not all (27), previous studies in adult women, including those with PCOS (28). However, the significant increases in AMH levels in PCOS-d compared to OB-g persisted in subgroups of comparable BMI. The lack of elevated AMH levels in OB-g suggested that they did not have the alterations in ovarian folliculogenesis characteristic of PCOS (29).

Small preantral and antral ovarian follicles up to 6 mm in size are the primary source of circulating AMH in women and girls (30,31). Polycystic ovaries have a 2- to 3-fold increase in small preantral follicles (32,33), and increased circulating AMH levels are a key feature of the PCOS reproductive phenotype (21,26,28,34). Even prior to menarche, circulating AMH levels correlate with the number of small and medium ovarian follicles (35). Previous studies in PCOS-d have found increased AMH levels during infancy, childhood (11), and puberty (36), suggesting differences in ovarian folliculogenesis may begin early in development in these girls.

Recent studies have suggested that AMH could play a role in the development of PCOS. We have found that ~3% of women with PCOS have mutations in *AMH* that decrease the bioactivity of the encoded protein, which may contribute to PCOS by decreasing AMH-mediated suppression of testosterone production (37). It is also possible that AMH plays a role in the pathogenesis of PCOS through neuroendocrine mechanisms. The AMH receptor is expressed in the hypothalamus on GnRH neurons in mice and humans (38). AMH increases GnRH-dependent LH secretion (38). Prenatal administration of high doses of AMH in pregnant mice induced GnRH-mediated hyperandrogenism and aromatase blockade in the mothers, resulting in intrauterine androgen exposure and ultimately a PCOS reproductive phenotype in their female offspring (39).

There is considerable evidence that androgens play a central role in the development of PCOS. Androgen excess during critical developmental windows produces phenocopies of PCOS in animal models (5-7). In humans, androgens antagonize the ability of estradiol and progesterone to slow the GnRH pulses contributing to the disordered gonadotropin secretion characteristic of PCOS (14). Hyperandrogenemia is a consistent reproductive phenotype in male as well as female relatives of women with PCOS (1,2,4). In the daughters of affected women, there are T elevations beginning prior to menarche and evidence for global increases in 5 α -reductase in childhood, which could enhance the local conversion of T to its more potent metabolite, dihydrotestosterone (10). Therefore, it is biologically plausible that girls with elevated free T levels will develop PCOS (40). However, there have been no prospective studies to test this hypothesis.

Our findings suggest that there may be additional phenotypic features in girls who are at increased genetic risk for PCOS, PCOS-d, because they have inherited one or more PCOS susceptibility alleles from their affected mothers (21). However, a subset of PCOS-d will not have inherited maternal PCOS susceptibility alleles. Accordingly, the PCOS-d cohort represents a mix of genetically affected and unaffected individuals. Nevertheless, the increased mean AMH levels in the PCOS-d suggest that at least some of these girls have the changes in folliculogenesis characteristic of PCOS, in addition to elevated free T levels. Longitudinal studies will be needed to determine if these girls will develop PCOS.

Our study had several limitations. Since our primary objective was to compare early reproductive phenotypes in two distinct putative PCOS risk groups, premenarchal PCOS-d and OB-g, BMI differed between our study groups by design. Differences in AMH levels remained significant in our subgroup analysis of OB-g and obese PCOS-d of comparable BMI but we lacked adequate power to determine whether reproductive phenotypes differed in normal weight PCOS-d and NW-g of comparable BMI. However, previous investigators have reported a distinct reproductive phenotype of elevated AMH levels during infancy and childhood (11), and elevated total testosterone levels by late puberty in normal weight PCOS-d compared to control girls of similar weight (12). Further, we did not have adequate power to perform separate analyses by individual Tanner stage. Nevertheless, we did account for the independent impact of pubertal stage by including breast Tanner stage as a covariate in our analyses. In addition, our subjects were not stratified by race or ethnicity since there was no evidence for racial or ethnic differences in circulating reproductive hormones, including AMH (41,42) and androgen levels (43).

In summary, we have shown that despite similar increases in free T, premenarchal PCOS-d and OB-g have distinct reproductive phenotypes. In contrast to OB-g, PCOS-d have elevated AMH levels, a biomarker for altered follicular development. The distinct phenotype in PCOS-d

may reflect increased genetic risk for PCOS compared to OB-g. It remains possible that both groups are at increased risk for PCOS by different causal mechanisms.

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Disclosure Summary:

The authors report no potential conflicts of interest relevant to this article.

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Figure 1. Reproductive hormone levels in PCOS-d and OB-g. While total T did not differ between PCOS-d, OB-g, and NW-g (upper left panel, ANCOVA $P=0.53$), PCOS-d and OB-g did have similarly decreased SHBG levels compared with NW-g (upper right panel, ANCOVA $P<0.0001$, NW-g v OB-g $P<0.0001$, PCOS-d v OB-g $P<0.0001$, PCOS-d v NW-g $P=0.005$). Accordingly, PCOS-d and OB-g had significant increases in free T compared with NW-g (lower left panel, ANCOVA $P=0.002$, NW-g v OB-g $P=0.0001$, PCOS-d v OB-g $P=0.19$, PCOS-d v NW-g $P=0.01$). AMH levels also differed between the groups, with PCOS-d having significantly increased AMH levels compared with OB-g and a trend toward increased AMH levels compared with NW-g which did not reach statistical significance (lower right panel, ANCOVA $P<0.0001$, NW-g v OB-g $P=0.07$, PCOS-d v OB-g $P<0.0001$, PCOS-d v NW-g

P=0.10). Unadjusted means and SEM pictured, statistical analyses adjusted for differences in age and breast Tanner stage.

Table 1: Baseline Clinical Characteristics and Reproductive Hormones

	PCOS-d n=48	OB-g n=30	NW-g n=22	P	PCOS-d v OB-g	PCOS-d v NW-g	OB-g v NW-g
Age (years)	9.7 ± 1.3* (8.0-12.3)	10.3 ± 1.1 (8.3-12.3)	10.3 ± 1.1 (8.3-12.7)	0.05	0.09	0.14	0.99
BMI percentile	80 ± 23 (16-99)	99 ± 1 (97-99)	50 ± 19 (19-84)	NA	NA	NA	NA
BMI z-score	1.1 ± 0.9 (-1.0-2.5)	2.4 ± 0.2 (1.9-2.9)	0.0 ± 0.5 (-0.9-1.0)	<0.0001	<0.0001	<0.0001	<0.0001
Breast Tanner Stage	44% I**	33% I	32% I	0.01 [†]	NA	NA	NA
	40% II	13% II	27% II				
	16% III	54% III	41% III				
Pubic Hair Tanner Stage	67% I	33% I	32% I	0.01 [†]	NA	NA	NA
	29% II	33% II	45% II				
	2% III	33% III	18% III				
	2% IV		5% IV				
Total T [‡] (ng/dL)	8 ± 6 (1-30)	9 ± 6 (1-22)	9 ± 8 (2-33)	0.53	NA	NA	NA
SHBG [§] (nmol/L)	54 ± 31 (9-180)	22 ± 10 (7-54)	71 ± 22 (38-121)	<0.0001	<0.0001	0.005	<0.0001
Free T [¶] (ng/dL)	0.13 ± 0.12 (0.01-0.58)	0.22 ± 0.15 (0.03-0.58)	0.10 ± 0.10 (0.02-0.40)	0.0002	0.19	0.01	0.0001
AMH [∇] (ng/mL)	3.28 ± 2.34 (0.13-13.00)	1.22 ± 0.72 (0.20-3.03)	2.08 ± 0.94 (0.79-4.52)	<.0001	<.0001	0.10	0.07
DHEAS ^Δ (ng/mL)	493 ± 358 (91-1730)	635 ± 349 (179-1555)	549 ± 362 (145-1770)	0.34	NA	NA	NA
Androstenedione [∞] (ng/mL)	0.47 ± 0.37 (0.13-1.27)	0.57 ± 0.37 (0.11-1.77)	0.50 ± 0.41 (0.11-1.78)	0.52	NA	NA	NA
Estradiol ^Σ (pg/mL)	13 ± 11 (4-59)	20 ± 14 (7-76)	14 ± 10 (5-43)	0.09	NA	NA	NA

* mean ± SD (range) **percentage of subjects included in each group are noted. P values listed from ANCOVA correcting for age and breast Tanner stage unless noted otherwise. BMI percentiles reported for descriptive purposes only. Statistical assessment for differences between the groups performed only on BMI z score. [†]Categorical variables breast and pubic hair Tanner Stage analyzed by Fisher's exact test. [‡]Total T levels missing in 3 PCOS-d. [§]SHBG levels missing in 2 PCOS-d. [¶]Free T levels missing in 4 PCOS-d. [∇]AMH levels missing in 9 PCOS-d, 7 OB-g and 4 NW-g. ^ΔDHEAS levels missing in 6 PCOS-d and 2 NW-g. [∞]Androstenedione levels missing in 28 PCOS-d.

^ΣEstradiol levels missing in 12 PCOS-d, 6 OB-g, and 4 NW-g. To convert DHEAS from ng/mL to nmol/L, multiply by 2.714; androstenedione from ng/dL to nmol/L, multiply by 0.0349; estradiol from pg/mL to pmol/L, multiply by 3.671.

ADVANCE ARTICLE

