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Authors

Guedikian, Annie A
Lee, Alexandria Y
Grogan, Tristan R
et al.

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Reproductive and metabolic determinants of granulosa cell dysfunction in normal-weight polycystic ovary syndrome women

Annie A. Guedikian, B.S.[a], **Alexandria Y. Lee**[a], **Tristan R. Grogan, M.S.**[b], **David H. Abbott, Ph.D.**[c], **Karla Largaespada, B.S.**[a], **Gregorio D. Chazenbalk, Ph.D.**[a], and **Daniel A. Dumesic, M.D.**[a]

[a]Department of Obstetrics and Gynecology, University of California, Los Angeles, 10833 Le Conte Ave. Los Angeles, California, United States, 90095

[b]Department of Medicine Statistics Core, University of California, Los Angeles, 911 Broxton Ave. Los Angeles, California, United States, 90024

[c]OB/GYN, Wisconsin National Primate Research Center, 1223 Capitol Court, University of WI, Madison, Madison, Wisconsin, United States, 53715

Abstract

Objective—To determine the degree to which estradiol (E2) hyperresponsiveness to follicle-stimulating hormone (FSH) and anti-mullerian hormone (AMH) overproduction in normal-weight women with polycystic ovary syndrome (PCOS) correlate with increased antral follicle number (AFN), hyperandrogenism and/or metabolic dysfunction.

Design—Prospective cohort study

Setting—Academic medical center

Patients—Seven normal-weight PCOS women (1990 NIH criteria) ages 20–34 years and 13 age- and body mass index (BMI, 18.5–25 kg/m^2)-matched normoandrogenic ovulatory women were studied.

Intervention(s)—All women underwent basal serum hormone and metabolic measurements, FSH stimulation testing with transvaginal ovarian sonography, frequently-sampled intravenous glucose tolerance testing and whole-body dual-energy x-ray absorptiometry.

Main Outcome Measure(s)—Serum hormone/metabolite levels, 24-hour serum E2 response to 150 IU recombinant human (rh) FSH infusion, AFN, insulin sensitivity and body mass measurements.

Results—Serum E2 responsiveness to rhFSH and AMH levels were greater in PCOS than BMI- and age-matched control women, as were serum androgen levels, AFN and abdominal fat mass. In

Corresponding author: Daniel A. Dumesic, M.D., Department Obstetrics and Gynecology, David Geffen School of Medicine at UCLA, 10833 Le Conte Avenue, Room 22-178 CHS, Los Angeles, California 90095, Telephone 310-794-5542; FAX 310-206-2057; ddumesic@mednet.ucla.edu.

all women combined, serum E2 responsiveness to rhFSH was associated with AFN. Serum AMH levels, however, positively correlated with AFN but remained positively correlated with serum luteinizing hormone and free testosterone levels and negatively correlated with total body fat and percent body fat, adjusting for AFN.

Conclusion—In normal-weight PCOS women, serum E2 hyperresponsiveness to rhFSH represents increased AFN, while elevated serum AMH levels reflect opposing effects of stimulatory reproductive (hyperandrogenism and increased AFN) versus inhibitory metabolic (body fat) factors. Given the small number of subjects reported, additional follow-up studies are required to confirm these data.

Keywords

anti-mullerian hormone; PCOS; hyperandrogenism; estradiol; adiposity

Human folliculogenesis is a complex process, whereby primordial follicles are recruited into a cohort of growing follicles, from which one antral follicle is selected to ovulate. Central to this process are granulosa cell-derived factors that coordinate primordial follicle recruitment (1, 2) with selection of the antral follicle as it becomes responsive to follicle-stimulating hormone (FSH) and eventually ovulates (2, 3). While many primordial follicles initiate growth, only a handful reach a selectable antral follicle size by the beginning of a normal menstrual cycle, and just one usually becomes dominant to proceed onwards to ovulation (4).

As a granulosa cell-derived protein of the transforming growth factor-β (TGF- β) superfamily, anti-mullerian hormone (AMH) is produced by growing follicles (5, 6). Low AMH levels occur in primordial and primary follicles, which increase to maximal levels in large preantral and small antral stages, and then decline exponentially as follicles grow beyond 9 mm in size (6–9). Anti-mullerian hormone inhibits FSH-induced aromatase gene expression in human granulosa cells (10). Therefore, loss of AMH production coincides with rising granulosa cell cytochrome P450 aromatase expression that begins when follicles reach 6–8 mm in size (11, 12), allowing androgens produced by luteinizing hormone (LH)-stimulated theca cells to undergo FSH-stimulated aromatization to estradiol (E2). Accordingly, AMH produced by preantral and small antral follicles appears to decrease FSH sensitivity and limit the number of growing follicles, while a later decline of AMH in the selected follicle likely removes constraint on FSH-induced aromatase activity to promote E2 synthesis during further follicular growth (13, 14).

Serum AMH levels are elevated in normoandrogenic women with polycystic ovaries (PCO), and are further increased in hyperandrogenic women with PCO, independent of antral follicle number (AFN) (9). Consequently, serum AMH levels in women with polycystic ovary syndrome (PCOS) are elevated 2- to 3-fold and are positively correlated with AFN and serum androgen levels (15, 16), while AMH levels also are reduced in parallel with AFN by metformin administration and are diminished with obesity (17, 18). Paradoxically, exaggerated serum E2 responsiveness to recombinant human (rh) FSH also characterizes PCOS (19), being variably attributed to PCO (20), abundant aromatizable androgens (19) and enhanced *in vitro* granulosa cell sensitivity to FSH (21, 22).

Therefore, since obesity can alter granulosa cell function (19, 23), the present study examines the degree to which serum E2 hyperresponsiveness to FSH and AMH overproduction in normal-weight PCOS women correlate with increased AFN, hyperandrogenism and/or metabolic dysfunction.

MATERIALS AND METHODS

Study Participants

Approval by the UCLA Institutional Review Board was obtained for subjects to enroll in this study by signing informed consent before participation. Seven normal-weight PCOS women, ages 20–34 years, and 13 age- and BMI (18.5–25 kg/m^2)-matched normoandrogenic ovulatory control women were recruited. All study subjects were recruited from the general community via advertising on college campuses and in the local community, while one PCOS women had previously attended an outpatient clinic for androgen-related symptoms. Circulating hormone and metabolic characteristics of all subjects have previously been reported (24) and include 3 additional subjects (1 Control and 2 PCOS). All women were in good health and non-Hispanic Caucasian to avoid confounding differences by BMI, age and ethnicity (25). Subjects underwent: 1) blood sampling for steroid measurements by liquid chromatography-tandem mass spectrometry (LC-MS/MS), hormone levels and lipid analysis; 2) FSH stimulation testing following transvaginal ultrasound (TVUS) ovarian imaging; 3) frequently sampled intravenous glucose tolerance (FSIGT) testing; and 4) total body dual-energy x-ray absorptiometry (DXA).

All BMI- and age-matched control women had regular menstrual cycles at 21 to 35 day intervals, and a luteal phase progesterone (P4) level, without evidence of hirsutism, acne or alopecia (25). Subjects with PCOS were diagnosed by 1990 National Institutes of Health (NIH) criteria, excluding other endocrinopathies to avoid phenotypic variability of Rotterdam criteria (25). Hirsutism was defined by the modified Ferriman-Gallwey (mFG) method, with hirsutism defined as an mFG score ≥ 6 (26). Biochemical hyperandrogenism was defined as an elevated serum total or free testosterone (T) > 2 SD above the normal ranges of the BMI- and age-matched control group (24).

Exclusion criteria were thyroid dysfunction, hyperprolactinemia, late-onset congenital adrenal hyperplasia (screening serum 17-hydroxyprogesterone > 2 ng/mL), ovarian cyst(s) > 2 cm; present/past history of smoking (<1 year), cancer, alcohol abuse, drug addiction, depression; diabetes; uncontrolled hypertension (≥ 165/100); clinically significant hepatic or renal disease; or other major medical illness. Additional exclusion criteria were recent (<3 months) use of diabetes medications, antidepressants, beta-adrenergic blocking agents, weight loss medications, androgens, anabolic steroids or hormonal agents (i.e. oral contraceptives/insulin sensitizers).

Blood sampling

All blood sampling was performed during the follicular phase (days 5–10 of the menstrual cycle) in BMI- and age-matched control women and during a period of documented oligo-anovulation, as determined by serum P4 levels, in PCOS women. Fasting blood samples

were used to measure AMH, gonadotropins, total and free T, dihydrotestosterone (DHT), androstenedione (A4), dehydroepiandrosterone sulfate (DHEAS), estrone (E1), E2, sex hormone binding globulin (SHBG), and lipids (total cholesterol, high density lipoprotein [HDL], low density lipoprotein [LDL], triglycerides [TG]). Blood sampling for glucose and insulin was also performed under fasting conditions before FSIGT test, as previously described (24, 27).

Hormone and metabolite assays

Serum levels of DHEAS, A4, total T, DHT, and E1 were measured by LC-MS/MS (Quest Diagnostics Nichols Institute, San Juan Capistrano, CA). The intra-assay coefficient of variation (CVs) were: DHEAS, 5.4%; A4, 3.8%; total T, 10.7%; DHT, 15%; and E1, 3%. The inter-assay CVs were: DHEAS, 5.6%; A4, 6.5%; total T, 13.4%; DHT, 10%; and E1, 7%. Free T was calculated from the concentrations of total T, SHBG, and albumin. The intra- and inter-assay CVs for free T were 5.0% and 7.8%, respectively.

Serum determinations of LH, FSH, insulin, and E2 by electrochemiluminescence; glucose by a hexokinase method; and fasting lipids by spectrophotometry were performed at the UCLA Center for Pathology Research Services. The intra-assays CVs were: LH, 2.8%; FSH, 2.8%; insulin, 0.6%; E2, 7.0%; glucose, 1.1%; total cholesterol, 0.9%. LDL, 1.2%, HDL 1.1%, and TG, 1.0%. The inter-assays CVs were: LH, 2.6%; FSH, 2.6%; insulin, 2.1%; E2, 3.6%; glucose, 1.0%; total cholesterol, 0.9%; LDL, 1.3%; HDL, 1.1%; and TG, 1.0%.

Serum AMH levels were measured by ELISA (Ansh Labs, Webster, TX) in the Endocrine Technologies Support Core (ETSC) at the Oregon National Primate Research Center (ONPRC) following manufacturer's instructions. The assay range was 0.084 – 14.2 ng/ml with a limit of detection of 0.023 ng/ml. Ansh supplies two controls with the kit, Control I and Control II. Intra-assay CVs for Control I ranged from 2.1% to 8.8% and inter-assay CV was 8.2%. Intra-assay CVs for Control II ranged from 1.2 to 6.1% and inter-assay CV was 11.9%. Using an in-house nonhuman primate serum quality control pool with each assay, the intra-assay CVs ranged from 0.9% to 7.6%, and inter-assay CV for this pool was 11.6%.

FSH Stimulation Test

Transvaginal ultrasound ovarian imaging using a 4- to 8-mHz vaginal probe was performed in the follicular phase in BMI- and age-matched control women and during amenorrhea in PCOS women. Summed ovarian volume (both ovaries) were calculated by 2-dimensional imaging using a prolate ellipsoid formula (0.5237 X D1 X D2 X D3; D1, D2, and D3 being maximal longitudinal, anteroposterior, and transverse diameters). Total antral follicle number (2–9 mm in diameter) of both ovaries was determined by one investigator (D.A.D.); the intra-observer CV was 16.5%. Immediately after TVUS ovarian imaging, blood samples were drawn at 0.5-h intervals for 2 h before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 h after 150 IU rhFSH intravenous injection. This rhFSH dose has been shown to elicit an exaggerated E2 response to rhFSH in PCOS women (19). The serum E2 and FSH responses to rhFSH injection above basal values were measured by 24-hour (24-hr) area under the

curve [AUC] calculations using the trapezoidal method and the serum E2 response to rhFSH above basal was adjusted for total AFN.

Total body DXA

Total body DXA was used to estimate body composition and distribution in our subjects because it was rapid and simple to perform and previously has confirmed fat mass as a predictor of mortality in United States adults participating in the National Health and Nutrition Examination Survey (NHANES) (28). For accuracy, all subjects were studied on a single DXA machine that was operated by one individual (29). Whole-body scans were measured with a Hologic QDR Discovery A densitometer (Hologic, Inc., Bedford, MA), using fast-array modem standard regions of interest, and Apex software versions 4.0.2 and 4.5.3.1. After standard regions of interest for the whole-body scan were set, Apex automatically defined android and gynoid regions according to set parameters: the android region extended between the superior aspect of the pelvis and the first lumbar vertebra (i.e., abdominal region), the gynoid region spanned from the head of the femur to mid-thigh. Intra-subject variabilities in whole body, android and gynoid fat masses by total body DXA assessment were 0.3%, 3.7% and 1.3%, respectively.

Statistical analysis

Comparisons of outcome variables between PCOS and BMI- and age-matched control women were analyzed using the Student's t-test. Measures with skewed distributions and outlying values (i.e., serum TG levels, AFN) were log transformed prior to analysis (30). Associations between reproductive and metabolic factors in all women combined were assessed using Pearson and partial correlation coefficients (adjusting for log AFN). Results/plots were summarized using mean ± SEM. Statistical analyses were performed using IBM SPSS V24 (Armonk, NY). P values <0.05 were considered statistically significant.

RESULTS

Patient characteristics

Age, BMI, waist and hip measurements were similar between the PCOS and BMI- and age-matched control women, while basal serum LH, total and free T, as well as A4 concentrations were greater in the PCOS than the control women (Table 1 Supplement). Basal serum FSH, E1, E2, DHT, and DHEAS levels were comparable between the two female types. Log AFN was greater in PCOS than BMI- and age-matched control women as was total ovarian volume.

Fasting plasma insulin levels were slightly higher in PCOS than BMI- and age-matched control women in the presence of normal serum glucose levels (Table 1 Supplement). Insulin sensitivities in PCOS and BMI- and age-matched control women were comparable (Table 2 supplement). Serum log TG and non-HDL levels tended to be higher in PCOS than these control women, while total cholesterol, LDL-C, HDL-C and SHBG concentrations were similar between the two female groups. Dual-energy x-ray absorptiometry showed similar amounts of total, lean and fat body mass as well as percent total body fat between PCOS and BMI- and age-matched control women. Android fat mass and percent android fat relative to

total body fat, however, were greater in PCOS than BMI- and age-matched control women. Gynoid fat mass and percent gynoid fat relative to total body fat were similar between PCOS and BMI- and age-matched control women.

FSH stimulation test

The overall serum E2 response to 150 IU rhFSH was greater in PCOS than BMI- and age-matched control women (P=0.009) (Fig. 1A) in the presence of similar serum FSH responses in both female groups (P=0.7) (Fig. 1B). The temporal pattern of serum E2 response to rhFSH over 24 hours also differed between PCOS and BMI- and age-matched control women (Fig. 1C & D). Specifically, the initial rise of serum E2 in all women occurred 4–6 hours after rhFSH injection, indicating the time interval required to induce granulosa cell aromatase activity *in vivo*. The subsequent rise in serum E2 to 12 hours after rhFSH injection, however, was exaggerated in PCOS women, leading to maximal serum E2 levels at 16 hours after rhFSH injection that were greater in PCOS than BMI- and age-matched control women. Thereafter, serum E2 levels remained elevated in PCOS compared to BMI- and age-matched control women and were followed by a slight decline of levels in PCOS, but not the control, women by 24 hours after rhFSH injection.

In all women combined, serum E2 response to rhFSH positively correlated with percent android fat (r=0.57, P=0.009), serum total T (r=0.49, P=0.03) level and log AFN (r=0.55, P=0.01) (Figure 2). Adjusting for log AFN confirmed that the serum E2 response to rhFSH was related to AFN, being unrelated to percent android fat or serum total T level independent of AFN (Table 1). In support of this, the serum E2 response to rhFSH per log AFN was similar in PCOS (2477.7±388.1) and BMI- and age-matched control women (1831.8±282.0 pg/mL/24 hrs/log AFN, P=0.19).

Serum AMH levels

Serum AMH values from one PCOS woman were omitted from analysis due to undetectable levels from an AMH mutation (31). Of the remaining 6 PCOS women, serum AMH levels were higher in PCOS (14.8±2.6 ng/mL) than BMI- and age-matched control women (4.1±0.7 ng/mL, P=0.007).

Serum AMH levels in all women combined positively correlated with percent android fat (r=0.68, P=0.001); serum levels of total T (r=0.64, P=0.003), free T (r=0.75, P<0.001), A4 (r=0.63, P=0.004) and LH (r=0.64, P=0.003); as well as log AFN (r=0.77, P<0.001) (Figure 3). Log AFN, however, only partially explained serum AMH levels. Adjusting for log AFN, serum AMH levels remained positively correlated with serum LH (r=0.55, P=0.02) and fT (r=0.49, P=0.04) levels and became negatively correlated with total body fat (r=−0.51, P=0.03) and % body fat (r=−0.53, P=0.03) (Table 1).

DISCUSSION

The present study confirms clinical observations that exaggerated serum E2 responsiveness to 150 IU rhFSH injection occurs in normal-weight PCOS women and resembles that of PCOS women who are obese (19). Furthermore, the exaggerated serum E2 response to rhFSH in our normal-weight PCOS women represented an increased number of small antral

follicles as an explanation for the previous clinical impression of enhanced granulosa cell sensitivity to FSH in PCOS (19). These findings are supported by evidence in PCOS versus normal women of a larger cohort of small antral follicles that are otherwise normally responsive to FSH (20, 32). Our clinical findings, however, differ from *in vitro* studies of PCOS granulosa cells showing heightened E2 sensitivity to FSH due to FSH receptor upregulation from hyperandrogenism (21, 33–35), perhaps because of a local aromatase inhibitor in small PCOS antral follicles with sufficient FSH availability (21, 22).

Although the initial rise of serum E2 at 4–6 hours after rhFSH injection in our normal-weight PCOS resembled that of previously-reported obese PCOS women, an exaggerated serum E2 rise thereafter in normal-weight PCOS subjects led to maximal serum E2 levels at 16 hours, which occurred later than that of obese PCOS women (19). Temporal differences in PCOS-related E2 hyperresponsiveness to rhFSH from increased adiposity in obese PCOS women may represent increased availability of aromatizable androgens (36) and inhibin (as a paracrine mediator of androgen synthesis) (37) and/or the ability of hyperinsulinemia to amplify LH- and insulin-like growth factor (IGF)-induced steroidogenesis (22, 38–40) by increasing serum IGF-I bioactivity and suppressing IGF-binding protein (41).

Serum AMH production positively correlated with log AFN, with both parameters being increased in normal-weight PCOS women. Adjusting for log AFN, however, serum AMH remained positively correlated with serum free T, consistent with findings in women with polycystic ovarian morphology that serum AMH levels are higher in women with hyperandrogenism than those without it (9). Since AMH levels reach maximal levels in large preantral and small antral stages before declining as follicles grow beyond 9 mm in size (6–9), the positive relationship between serum AMH and androgen levels independent of small AFN may represent the larger cohort of preantral follicles present in PCOS women (42), agreeing with T-induced growth of preantral and small antral follicles in nonhuman female primates (43, 44). It also may represent inherent granulosa cell dysfunction in PCOS, as evidenced by AMH overproduction by cultured granulosa cells of anovulatory PCOS patients (45).

Serum AMH production also positively correlated with LH, independent of AFN. Although this relationship could be mediated through LH-stimulated theca cell androgen production (46), cultured granulosa cells from PCOS, but not BMI- and age-matched control, women produce AMH in response to LH treatment (45, 47), in part through premature LH receptor upregulation in PCOS women (48, 49). Furthermore, a recent multivariate analysis of serum AMH determinants showed serum LH as a positive predictor of serum AMH, adjusting for AFN and serum T, with serum LH levels contributing 18% of the variance of serum AMH levels, and AFN and serum T levels contributing an additional 5.3% and 9.5%, respectively (50).

To our knowledge, our study is the first to show that serum AMH levels in normal-weight women negatively correlate with total and percent body fat. This finding confirms some (50–52) but not all (53, 54), studies in women of an inverse relationship between serum AMH levels and BMI (51), independent of PCOS (52), AFN and circulating T and LH levels (50). Although the mechanism by which body fat affects AMH production remains unclear,

exposure of cultured human luteinized granulosa cells to leptin suppresses AMH mRNA expression through the JAK2/STAT3 pathway (23), implicating adipokines in the regulation of AMH.

Strengths of this study include the recruitment of young, normal-weight PCOS women by NIH criteria who were age- and BMI-matched to control women to eliminate multiple confounders affecting insulin sensitivity, while simultaneously studying a less severe PCOS phenotype within the general community rather than from a referral setting (55). Use of non-Hispanic Caucasian subjects also eliminated ethnic variations of body composition and fat distribution and the effect of BMI on AMH production (25, 52). Sophisticated techniques for radiographic and hormonal analysis also permitted stringent assessment of body fat, hyperandrogenism, and LH hypersecretion as additional correlates of AMH production, while performance of TVUS by a single investigator (D.A.D.) eliminated inter-observer variability of antral follicle counts.

On the other hand, limitations of our study include the inability to detect preantral follicles by TVUS, and use of 2D rather than 3D sonography, which may have altered accuracy of antral follicle determination (56). The findings from our normal-weight non-Hispanic Caucasian women with PCOS also may differ from other PCOS women of varying ethnicity and adiposity (19, 52). Importantly, the small number of subjects is a limitation of this paper, which is exploratory in nature.

In conclusion, in normal-weight PCOS women, serum E2 hyperresponsiveness to rhFSH represents increased AFN, while elevated serum AMH levels reflect opposing effects of stimulatory reproductive (hyperandrogenism and increased AFN) versus inhibitory metabolic (body fat) factors. Given the small number of subjects reported, additional follow-up studies are required to confirm these data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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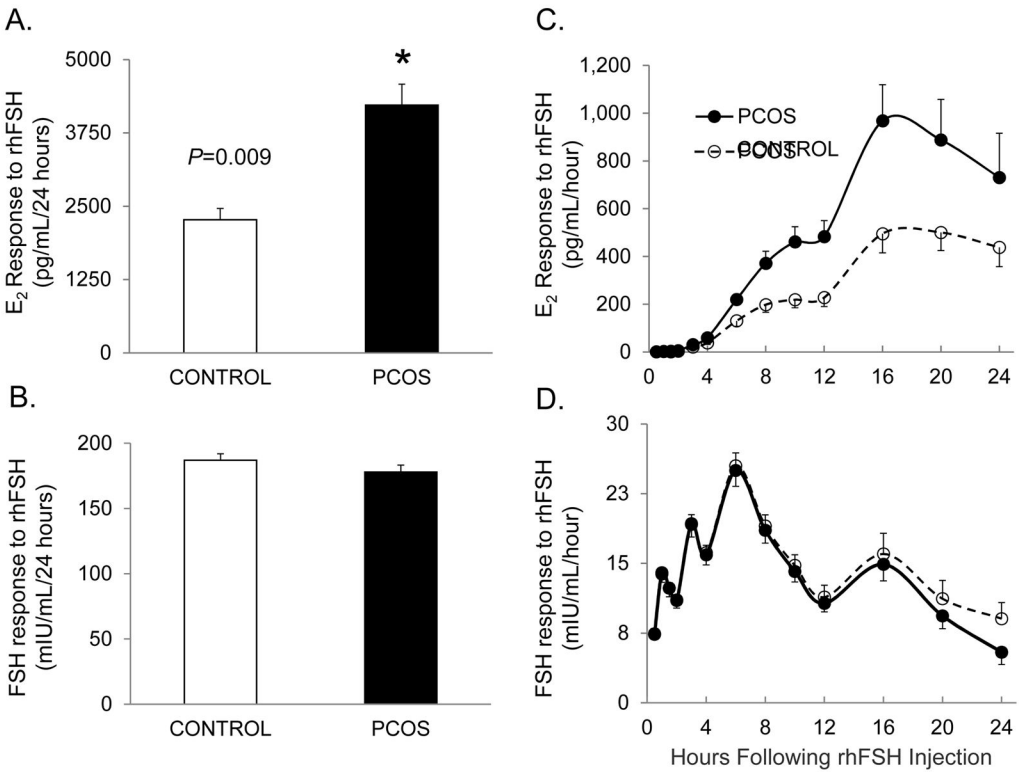

Figure 1.

Twenty-four-hour (24-hr) area under the curve (AUC) determinations for serum E2 and FSH responses to 150 IU rhFSH intravenous infusion. The serum E2 response to rhFSH was significantly greater in PCOS (N=7) than BMI- and age-matched control (N=13) women (A) despite similar serum FSH responses to rhFSH injection in both female groups (B). The initial rise of serum E2 in all women occurred 4–6 hours after rhFSH injection, with the subsequent rise thereafter to maximal serum E2 levels at 16 hours being greater in PCOS than the control women (C). Serum FSH levels rose to maximal levels approximately 6 hours after 150 IU rhFSH intravenous infusion and then declined from these levels thereafter (D). *, P=0.009 *vs.* BMI- and age-matched control (mean ± SEM).

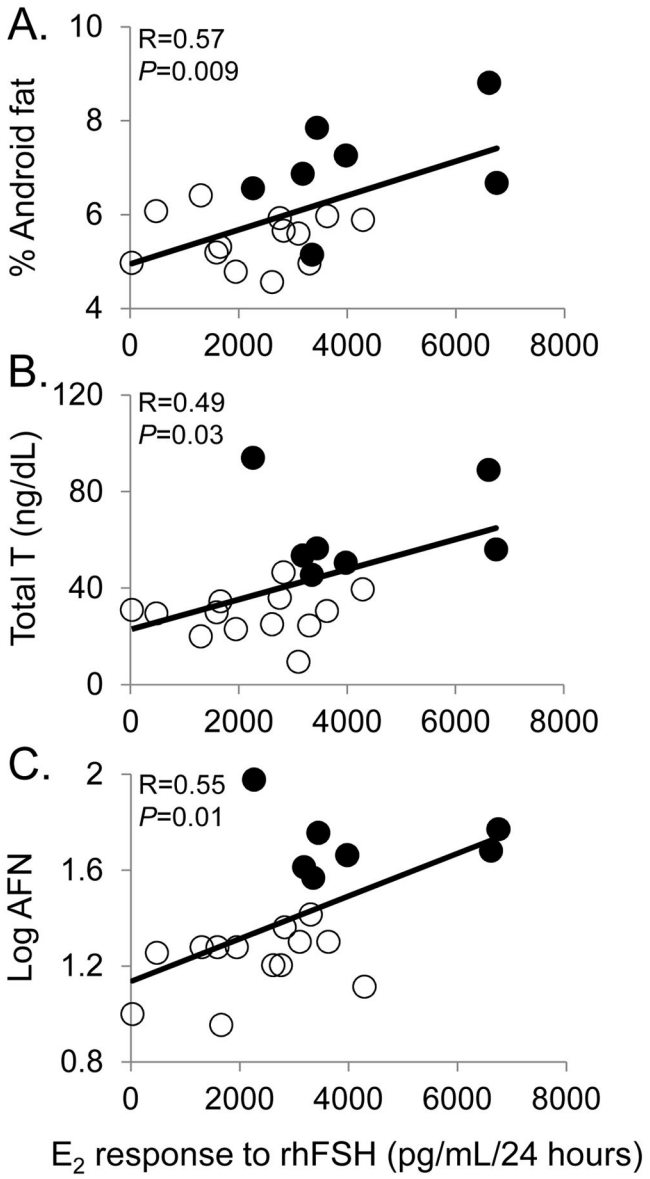

Figure 2.

Serum E2 response to rhFSH positively correlated with (A) percent android fat, (B) serum total T level and (C) log AFN in all women combined (13 BMI- and age-matched controls [open circles], 7 PCOS [dark circles]: total, 20 women).

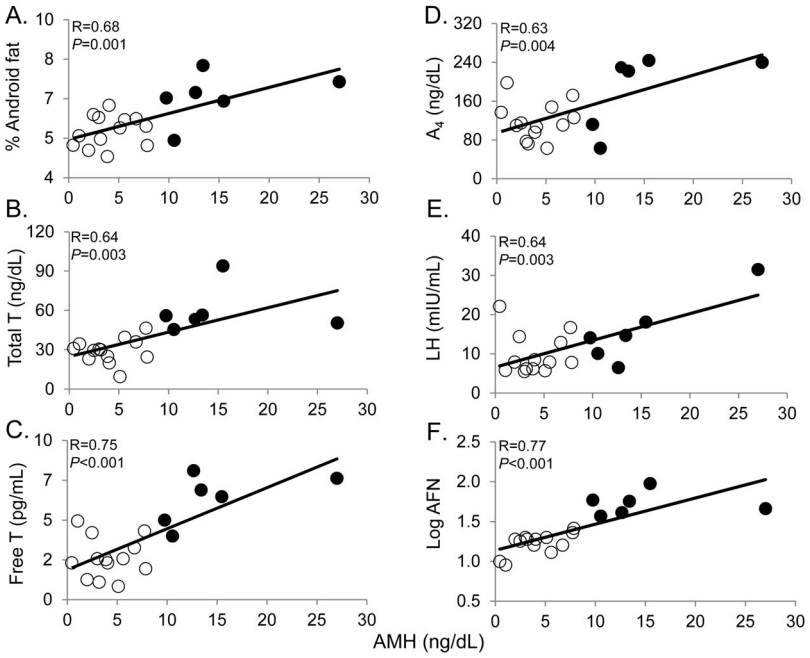

Figure 3.

Serum AMH levels positively correlated with (A) percent android fat; serum levels of (B) total T, (C) free T, (D) A4 and (E) LH; as well as (F) log AFN in all women combined (13 BMI- and age-matched controls [open circles], 6 PCOS [dark circles]: total, 19 women).

Table 1

Correlations of serum E2 response to rhFSH and AMH levels with reproductive and metabolic hormones.

	Pearson Correlation (unadjusted for Log AFN)	*P* value	Partial Correlation (adjusted for Log AFN)	*P* value
Serum E2 response to FSH (pg/mL/24 hours)				
Log AFN	0.55	**0.01**	----	----
Percent android fat (%)	0.57	**0.009**	0.33	0.16
Serum total T (ng/dL)	0.49	**0.03**	0.14	0.57
Serum AMH (ng/mL)				
Log AFN	0.77	**<0.001**	----	----
Percent android fat (%)	0.68	**0.001**	0.36	0.14
Serum total T (ng/dL)	0.64	**0.003**	0.13	0.6
Serum fT (pg/mL)	0.75	**<0.001**	0.49	**0.04**
Serum A4 (ng/dL)	0.63	**0.004**	0.46	0.05
Serum LH (mIU/mL)	0.64	**0.003**	0.55	**0.02**
Total body fat (kg)	−0.03	0.9	−0.51	**0.03**
Percent body fat (%)	−0.2	0.9	−0.53	**0.03**