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The Role of GnRH Receptor Autoantibodies in Polycystic Ovary Syndrome

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Objective: Is polycystic ovary syndrome (PCOS) associated with activating autoantibodies (AAb) to the second extracellular loop (ECL2) of gonadotropin-releasing hormone receptor (GnRHR)?

Design and Methods: We retrospectively screened sera from 40 patients with PCOS and 14 normal controls (NCs) with regular menses using enzyme-linked immunosorbent assay (ELISA) for the presence of GnRHR-ECL2-AAb. We obtained similar data from 40 non-PCOS ovulatory but infertile patients as a control group (OIC) of interest. We analyzed GnRHR-ECL2-AAb activity in purified immunoglobulin (Ig)G using a cell-based GnRHR bioassay.

Results: The mean ELISA value in the PCOS group was markedly higher than the NC ($P = .000036$) and the OIC ($P = .0028$) groups. IgG from a sample of 5 PCOS subjects, in contrast to a sample of 5 OIC subjects, demonstrated a dose-dependent increase in GnRHR-stimulating activity qualitatively similar to the acute action of the natural ligand GnRH and the synthetic agonist leuprolide. The GnRHR antagonist cetrorelix significantly suppressed ($P < .01$) the elevated GnRHR activity induced by IgG from 7 PCOS patients while the IgG activity level from 7 OIC subjects was unchanged. Five other OIC subjects had relatively high ELISA values at or above the 95% confidence limits. On further study, 3 had normal or low activity while 2 had elevated IgG-induced GnRHR activity. One suppressed with cetrorelix while the other did not. The copresence of PCOS IgG increased the responsiveness to GnRH and shifted the dosage response curve to the left ($P < .01$).

Conclusions: GnRHR-ECL2-AAb are significantly elevated in patients with PCOS compared with NCs. Their presence raises important etiological, diagnostic, and therapeutic implications.

Key Words: autoantibodies, GnRH receptor, polycystic ovary syndrome

Abbreviations: AAb, activating autoantibodies; AMH, anti-Müllerian hormone; BMI, body mass index; CV, coefficient of variation; ECL2, second extracellular loop; ELISA, enzyme-linked immunosorbent assay; FSH, follicle-stimulating hormone; GnRHR, gonadotropin-releasing hormone receptor; GnRHR, GnRH receptor; GWAS, genome-wide association study; GPCR, G protein–coupled receptor; Ig, immunoglobulin; LH, luteinizing hormone; NC, normal control; OD, optical density; OIC ovulatory infertile control; PCOS, polycystic ovary syndrome.

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Polycystic ovary syndrome (PCOS) is a poorly understood systemic disease characterized by ovulatory dysfunction, hyperandrogenism, and/or polycystic appearing ovaries on transvaginal ultrasound. Its prevalence in women of reproductive age ranges from 5% to 9% according to the 1990 National Institutes of Health criteria and is even higher using the broader 2003 Rotterdam criteria [1].

In ovulatory women, gonadotropin-releasing hormone (GnRH) is released in an episodic manner from the hypothalamus, resulting in pulsatile secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then communicate with the ovaries to result in ovulation [2]. In contrast, PCOS is characterized by a variable elevation of LH and testosterone of unknown pathophysiology [3, 4]. PCOS is frequently associated with significant metabolic sequelae leading to long-term healthcare issues, including obesity, hyperandrogenism, type 2 diabetes mellitus, hypercholesterolemia, endometrial hyperplasia, hypertension, and increased risk for cardiovascular disease and endometrial cancer [5-7]. There is no known etiology of this disorder and therefore no specific therapy [8-10]. Generally, physicians work with patients to manage the individual manifestations and the long-term health concerns. Several studies have suggested a weak polygenic background primarily from familial associations [11-14]. Several twin studies have supported a genetic predisposition for PCOS in monozygotic twins compared with dizygotic twins [15, 16]. Genome-wide association studies (GWASs) have been carried out in PCOS and support a potential oligogenetic component, but fail to identify a likely pathogenetic basis for the disease [17, 18]. However, a recent GWAS of deoxyribonucleic acid methylation found differential methylation on chromosome 6 within the major histocompatibility complex region, implicating expression of immune-related genes in the pathogenesis of PCOS [19]. An effect of increased testosterone in utero has been supported by certain animal studies, but the studies are insufficient to account for the large majority of subjects [20-23]. A recent report supports a possible role of anti-Müllerian hormone (AMH) interactions with hypothalamic GnRH neurons [24]. The role of hypothalamic AMH has not been clarified but its presence can mimic certain effects in PCOS and direct blockade of the central GnRH receptor (GnRHR) has been reported to ameliorate some of these issues [24].

Previous attempts to identify an autoimmune cause in PCOS have focused on both hypothalamic–pituitary and peripheral glands, but have been relatively uninformative [25-27]. In recent years, a new type of autoantibody has gained increasing attention. These autoantibodies bind to the G protein–coupled receptors (GPCRs), preferentially the second extracellular loop (ECL2), and can activate *or* inhibit specific GPCR signaling pathways, which contribute to the pathogenesis of various diseases including cardiovascular, nervous, and other systemic disorders [28-30]. These autoantibodies differ significantly from natural GPCR ligands in their impact on the GPCR. The natural ligand invariably leads rapidly to homologous desensitization of the receptor as is evident with therapies employing GnRH or its synthetic analogs such as leuprolide. By contrast, GPCR-directed autoantibodies are unable to induce such desensitization [31, 32]. These autoantibodies can by a direct action stimulate their target GPCR; but importantly they also possess the ability to either positively or negatively alter the GPCR–natural ligand interaction by their ability to bind allosterically to non–pocket-related receptor structures [30, 33]. With this background in mind, we hypothesized that activating autoantibodies (AAb) to the ECL2 of GnRHR, a GPCR, would be present in PCOS patients, contribute to its pathophysiology, and possess diagnostic value.

1. Materials and Methods

A. Study subjects

Sera from 40 matched pairs of patients (subjects) with PCOS and ovulatory infertile controls (OICs) seen at our academic infertility clinic from 2012 to 2016 were included as 2 groups in the study. Subjects with PCOS were diagnosed based on the presence of at least 2 of the 3

Rotterdam criteria, 1 of which was ovulatory dysfunction for all subjects; the other consisted of clinical or laboratory evidence of hyperandrogenism, and/or polycystic appearing ovaries on transvaginal ultrasound with at least 12 or more follicles measuring 2 to 9 mm. Other causes of ovulatory dysfunction (such as hypothyroidism, hyperprolactinemia, or forms of congenital adrenal hyperplasia) were excluded [34, 35], and all patients in the convenience sample had stored serum from clinical care that was available for testing. The OIC group consisted of ovulatory patients seen for infertility in the same clinic from 2012 to 2016 who had a stored serum sample drawn prior to beginning infertility treatments. These were matched 1 to 1 with the PCOS patients for race/ethnicity, age ± 3 years, and body mass index (BMI) ± 5. Normal controls (NCs) consisted of 14 women (age 18-35 years) with no history of autoimmune disease who were recruited for a concurrent study. These subjects were similar to the other 2 matched groups for race/ethnicity and BMI ± 5 but were slightly younger in age (–5 years on average). They had a history of regular periods and denied hirsutism or infertility. Patient charts were reviewed for clinical and biochemical measures including AMH were obtained at the discretion of the treating physician in accordance with clinically indicated care. Blood samples were generally obtained at the end of each patients' visit. Most (2/3) were seen in the morning and 1/3 in the afternoon. All of the NC samples were obtained in the morning. Serum was frozen and stored at –80°C. All serum samples were deidentified and sent frozen to the research laboratory. The identity of the sera from the PCOS patients and controls was blinded in all cases to the laboratory personnel performing the enzyme-linked immunosorbent assays (ELISAs). These data were sent to the referring reproductive endocrinology group for identification and subsequent independent analysis by the statistician.

B. ELISA

Sera from the PCOS and the 2 control groups were screened using ELISA for detection of GnRHR autoantibodies. Briefly, a 28-mer peptide corresponding to the amino acid sequence of human GnRHR ECL2 (DSSGQTKVFSQCVTHCSFSQWWHQAFYN) (UniProtKB accession number P30968) was synthesized (LifeTein, Somerset, NJ) and demonstrated to be >95% pure with high-performance liquid chromatography. It was used to coat 384-well ELISA plates at a concentration of 10 μg/mL in coating buffer. Sera were diluted 1:50, and goat antihuman IgG conjugated with alkaline phosphatase and its substrate para-nitrophenyl-phosphate 104 were used to detect antibody binding. The optical density (OD) values were read at 405 nm at 10 minutes. The intra-assay coefficient of variation is 5.9% (n = 156) and the interassay coefficient of variation is 3.2% (n = 10). Sera from the PCOS, NC, and OIC subjects were simultaneously assayed in triplicate in a 384-well plate to eliminate interassay variability. The sera from the PCOS and OIC subjects were also assayed 16 months previously, and comparison of the individual values from these subjects showed a Spearman correlation of $\rho = 0.999883$ ($P = .0001$) demonstrating the sample OD values were consistent after storage for over a year.

C. IgG purification

Serum IgG was purified according to the manufacturer's recommendations using NAb Protein A/G Spin Kits (Thermo Fisher Scientific). Sera were selected for purification from 7 PCOS patients and 7 OIC controls; 3 each were randomly selected from the top tertile, 2 from the middle, and 2 from the lower tertile of the ELISA data. Five of these IgG samples were used for the GnRHR-AAb activity assays and all 7 in the cetrorelix assays.

D. Cell-based GnRHR assay

GnRHR-AAb activity in serum IgG was measured with a calcium flux assay using a Ready-to-Assay GnRHR-expressing Chem-1 cell line (Eurofins Bioanalytics, St Charles, MO),

according to the manufacturer's protocol. Briefly, Chem-1 cells were dispensed into a 96-well microplate and incubated for 24 hours. After the plate was washed with Hanks balanced salt solution supplemented with 20 mM HEPES and 2.5 mM probenecid at pH 7.4, Fluo-8 NW (AAT Bioquest, Sunnyvale, CA), Ca^{2+} dye-loading solution was added to each well and incubated for 1 hour. Serum IgG, GnRHR agonist GnRH, or leuprolide (Sigma-Aldrich) was then added in constant volumes. Calcium flux response was recorded every 20 seconds for 180 seconds on a Hidex Sense microplate reader (Hidex, Turku, Finland). All samples were tested in triplicate. Data are expressed as a percentage of buffer baseline fluorescence signal to normalize the individual values. A value of 100% of basal activity represents the activity associated with the presence of buffer alone. A value of 100% of basal activity represents the activity associated with the presence of buffer alone. The intra-assay coefficient of variation is 8.2% (N = 58) and the interassay coefficient of variation is 8.0% (N = 8). Serum IgG (10-150 μg/mL) was used to assess activity dosage responses. IgG was tested at 4 different concentrations (0, 50, 100, and 150 μg/mL) to determine an optimal concentration for study. In separate experiments, IgG (100 μg/mL) was added in the absence and presence of the GnRHR antagonist cetrorelix (10^{-7} M) (Thermo Fisher Scientific) to measure specific receptor activity for individual subjects. GnRH (10^{-9}-10^{-6} M) in the absence and presence of serum IgG (100 μg/mL) was also tested to examine a possible allosteric effect of the PCOS IgG on the established GnRHR orthosteric ligand response.

E. LH and total testosterone (T) assays

Sera from patients were used to determine circulating levels of LH and total Testosterone. The human LH ELISA assay kit (ENZKIT107-0001; RRID:AB_2848134 [36]) was obtained from Enzo Life Sciences (Farmingdale, NY). All assays were performed in duplicate and according to the manufacturers' instructions. The stated cross-reactivity for the LH assay was LH (100%), FSH (≤0.004%), human chorionic gonadotropin (≤0.004%), and thyrotropin (≤0.3%). Its range was from 1.2 to 280 mIU/mL. For LH the intra-assay coefficient of variation (CV) for the high and low standards (stds) values were 8.3% and 6.4%, respectively; the interassay CV for LH was 3.0%. Intra-assay CV for participant samples (n = 88) was 3.4%. The reliability coefficient for LH was 99.5%. The human total T ELISA assay kit (ADI-901-176; RRID:AB_2848133 [37]) was also obtained from Enzo Life Sciences. Its stated cross-reactivity is with testosterone 100%, androstenedione 16.4%, 19-hydroxytestosterone 7.6%, and dihydrotestosterone 2.7%, and all others were <1.0%. Its assay range was 0.04 to 34.7 nmol/L. For T the intra-assay CVs for the high and low std values were 4.3% and 7.7%, respectively; the interassay CV for total T was 8.0%. The intra-assay CV for participant samples (n = 88) was 3.1%. The reliability coefficient for total T was 99.8%.

F. Statistics

Data are expressed as mean ± standard error of the mean unless stated otherwise. Sampling distributions for the continuous variables did not show significant deviation from normality per the D'Agostino–Pearson omnibus normality test. Group comparisons used paired (PCOS vs OIC) or unpaired (NC vs either PCOS or OIC) Student t tests. K-means clustering analysis was used to determine subgroups within each group [38]. Statistical significance was set at $P < .05$. Calculations used R (version 3.6.1).

G. Study approval

All subjects had consented to their sera being used for assays relevant to their reproductive evaluation. All NC subjects had signed consent for use of their sera for metabolic studies relating to conditions with suspected autoimmune etiology. This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Board as conforming to the overlying ethics principles operative in the United States.

2. Results

Characteristics of the 40 matched PCOS–OIC pairs and 14 NC subjects are shown in Table 1. There was no significant difference in age ($P = .97$) or BMI ($P = .68$) between matched PCOS subjects and OIC. The NC group (24.6 ± 2.0 years) were younger than either the PCOS (29.9 ± 3.1 years) or OIC (29.9 ± 2.9 years) groups ($P = .0001$ for both), but were similar in BMI (PCOS vs NC $P = .084$; OIC vs NC $P = .094$). Serum LH was significantly higher in the PCOS group than either the OIC ($P = .0032$) or NC ($P = .0001$) groups. Serum testosterone was higher in the PCOS group than in either control group but only reached significance compared with the NC group ($P = .0079$), but not to the OIC group ($P = .084$). AMH mean levels were not significantly higher in the PCOS than OIC but the wide variance and relatively smaller number of assays available from the clinical subjects make this comparison of less value. No assays of AMH were available for the NC group. Sera were screened by ELISA for autoantibodies directed to the ECL2 of GnRHR (Fig. 1). The PCOS group had a marked and significantly elevated mean ELISA value compared with the NC controls (unpaired t: $P = .000036$). This ELISA was also performed on sera from a group of infertile women and had a history of at least 9 ovulatory cycles as determined by monthly temperature monitoring. These subjects were matched for sex, race, BMI, and age to the PCOS subjects. Their ELISA values also were significantly lower than the PCOS subjects ($P = .0028$). The mean ELISA value in the OIC subjects was elevated compared with the NC controls, but this did not reach significance (unpaired t: $P = .077$). The small sample size of each group suggests that the effect of potential outliers be considered. The PCOS group had 1 subject with an OD value over + 3 standard deviation (SD) above the group mean; this could be a statistical outlier, although there were no remarkable characteristics (eg, age, BMI, AMH) to consider her not a part of the PCOS group. The NC controls also had 1 participant whose OD value lay at the estimated 99th percentile (+2.6 SD above the group mean); this was considered a potential outlier. She had a history of being placed on oral contraceptive pills and at an early age for unknown reasons and later withdrawn. Omitting these 2 outliers and reanalyzing data changed the results minimally. The PCOS group remained significantly elevated compared with either the OIC (paired t: $P = .0054$) or the NC controls (unpaired t: $P = .000001$). The NC controls became significantly lower than the OIC subjects (unpaired t: $P = .017$). The OIC group had 5 values between the estimated 95th and 99th percentiles but no values stood out as outliers. A cluster analysis of the OIC controls suggested that the top 7 OD values formed a separate cluster from the remainder of this phenotypically heterogeneous group; however, other than the OD values no other characteristics were found that distinguished this cluster from the remainder. The dosage effects of the GnRHR agonists GnRH and the synthetic GnRH analog leuprolide on GnRHR activation in GnRHR-expressing Chem-1 cells are shown in Fig. 2. GnRH and leuprolide

Table 1. Clinical and biochemical characteristics of the study participants

		PCOS vs OIC				*P* value	
	PCOS	pairs	*P* value	OIC	NC	PCOS vs NC	OIC vs NC
Age (years)	29.9 (3.1) (40)	(40)	.97	29.9 (2.9) (40)	24.6 (2.0) (14)	.0001	.0001
BMI (kg/m±)	26.5 (5.7) (40)	(40)	.68	26.4 (5.3) (40)	24.0 (4.0) (14)	.0840	.094
LH (mIU/mL)	14.5 (5.7) (30)	(19)	.0032	8.3 (4.4) (26)	6.6 (2.4) (14)	.0001	.13
T (nmol/L)	302 (169) (30)	(19)	.084	193 (130) (26)	198.0 (77.4) (14)	.0079	.88
AMH (pmol/L)	40.7 (37) (16)	(9)	.68	22.8 (14) (25)	—	—	—

For PCOS, OIC, and NC entries are mean (SD) (N); for pair differences, the entries are mean (N); *P* values are from t-tests: paired for PCOS vs OIC, unpaired otherwise.
All data are mean (SD) (n).
Abbreviations: AMH, anti-Müllerian hormone; BMI, body mass index; LH, luteinizing hormone; NC, normal controls; OIC ovulatory infertile controls; T, testosterone.

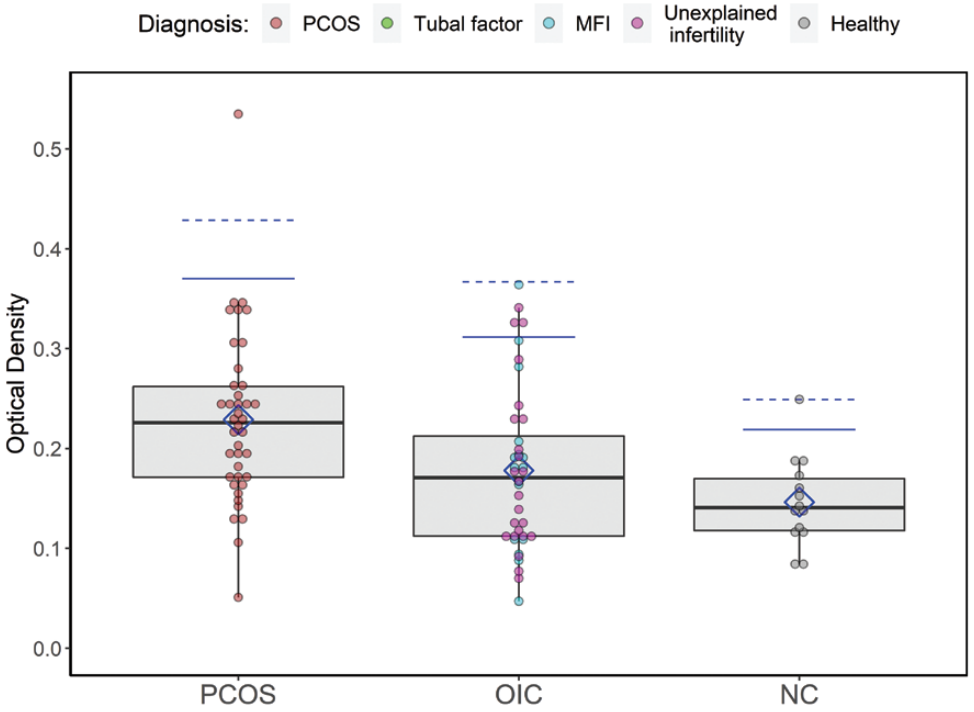

Figure 1. ELISA detection of GnRHR autoantibodies in patients with PCOS, ovulatory infertile controls (OICs), and normal controls (NC). The box plots indicate the median optical density (OD) value (middle black line) and the 75th and 25th percentiles (upper and lower edges, respectively). Mean OD value is indicated by the blue diamonds and the estimated 95th (solid) and 99th (dashed) percentiles are indicated by the blue lines. PCOS vs NC: $P = .000036$, unpaired t-test; PCOS vs OIC: $P = .0028$, paired t-test; OIC vs. NC: $P = .077$, unpaired t-test. MFI, male factor infertility.

reached their maximal effects at approximately 10^{-6} M and 10^{-7} M with an EC50 of 1 nM and 6 nM, respectively. Serum IgG purified from 5 PCOS subjects and 5 OIC subjects were likewise examined for their activation potential in GnRHR-expressing cells. IgG was tested at different concentrations to determine an optimal concentration for study. There was a significant dose effect of PCOS IgG on GnRHR activation ($P < .05$ vs OIC IgG) and the maximal effect occurred at 100 to 150 μg/mL of IgG (Fig. 3). No significant dose effect was observed for the OIC IgG.

The effect of the selective GnRHR blocker cetrorelix on GnRHR-AAb activity in vitro was tested in IgG purified from 7 PCOS patients and 7 OIC subjects. Cetrorelix markedly suppressed GnRHR-AAb activity in IgG from the PCOS group ($P < .001$) (Fig. 4). No significant effect of cetrorelix was observed in IgG from the OIC group. The effect of specific GnRHR blockade with cetrorelix was also tested for IgG from each of the 5 highest ELISA values in the OIC group (Fig. 5). Three of the 5 controls from this top-mode OIC group demonstrated relatively low specific GnRHR-AAb activity. One had a modest drop in activity following cetrorelix blockade. Of the other 2 with higher activity values, 1 had a significant decrease in GnRHR-AAb activity similar to that seen in the PCOS subjects while the second had a negligible change.

We examined the effect of GnRHR-AAb on orthosteric ligand activation of GnRHR using dosage responses for GnRH (10^{-9}-10^{-6} M) with and without serum IgG (100 μg/mL) (Fig. 6). IgG samples from the 7 PCOS patients and 7 OIC subjects were used for comparisons. Thus, 3 GnRHR dosage–response curves were generated in the presence of a constant concentration of PCOS IgG, for OIC IgG, and for the GnRHR alone. The PCOS IgG + GnRH activity dosage response was markedly increased over that of the OIC IgG + GnRH and for GnRHR

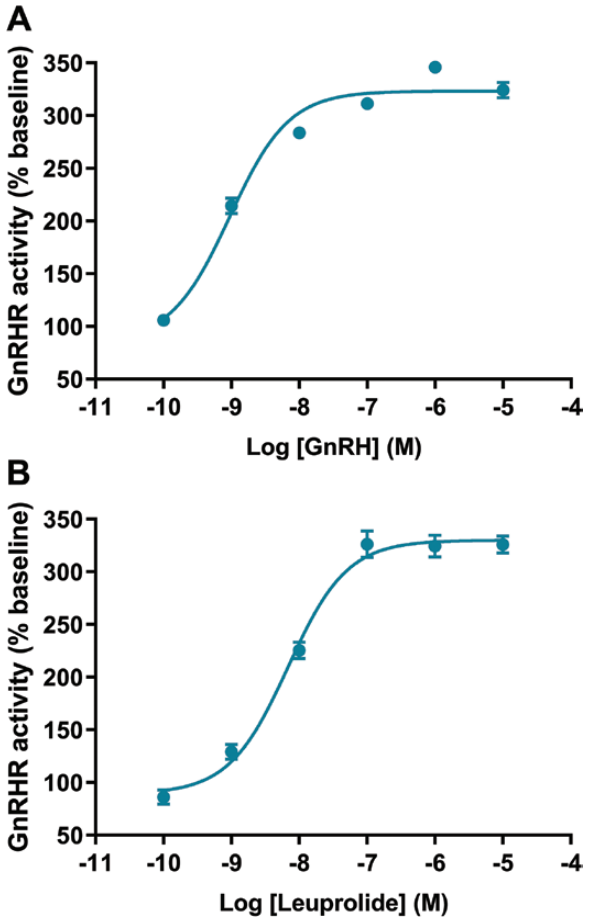

Figure 2. Dose–response curves of GnRH and leuprolide in the calcium flux assay. Both agonists were tested at concentrations from 10^{-10} to 10^{-6} M. The data are the mean ± standard error of the mean for 3 assays run in triplicate. The 10^{-10} M concentrations were not significantly different from the buffer baseline value, which was arbitrarily assigned a value of 100% for both agonists. The 2 response curves were performed in the same assay. There was a significant dosage response for each agonist.

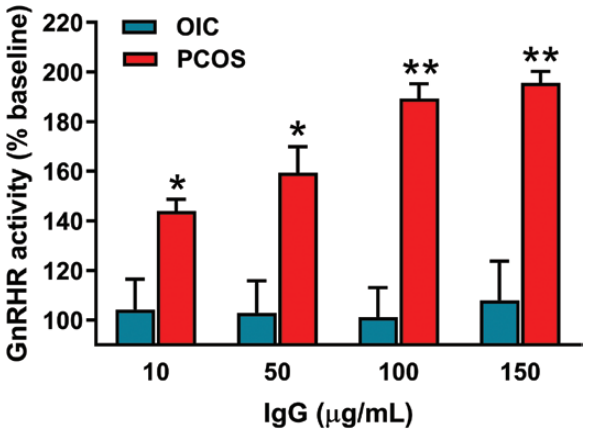

Figure 3. The dosage effects of serum IgG from PCOS and OIC subjects on GnRHR activation in the calcium flux assay. There was a significant dosage-dependent increase in PCOS IgG-induced GnRHR activation with a maximal effect at 100 to 150 μg/mL. The OIC IgG activity was not significantly different from the buffer baseline activity and no dosage effect was noted. $P < .05$, $^{**}P < .01$ vs OIC, n = 5.

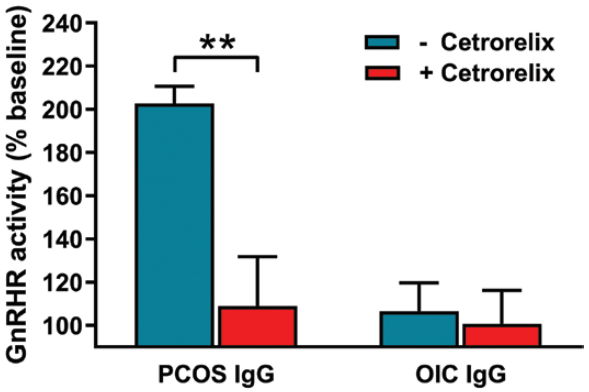

Figure 4. The effect of GnRHR blockade on serum IgG-induced GnRHR activation in the calcium flux assay. The selective GnRHR blocker cetrorelix (10^{-7} M) effectively suppressed the mean elevated GnRHR activity from the PCOS IgG (100 μg/mL, n = 7) to levels not significantly different for the baseline buffer alone. Cetrorelix produced no significant change in the already low mean GnRHR activity in the OIC IgG (100 μg/mL, n = 7). $^{**}P < 0.01$.

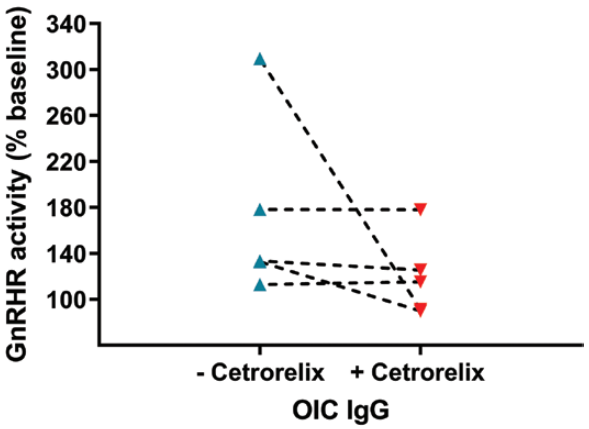

Figure 5. The effect of cetrorelix on GnRHR activation induced by serum IgG from the five OIC subjects with the highest ELISA OD values. Two of these OIC subjects had an elevated GnRHR activity. One suppressed with cetrorelix (10^{-7} M) to levels not significantly different from the buffer baseline while the other did not change. The other 3 of these 5 OIC subjects had relatively low baseline GnRHR activity near that for the baseline buffer values. Two were unchanged by cetrorelix blockade while the third had a small decrease clearly into a level not different from the buffer alone. The absence of a significant decrease in 4 of these 5 subjects following cetrorelix is quite similar to that observed in the control subjects in Fig. 4.

alone ($P < .01$). This increased GnRHR activity shifted the dosage–response curve toward the left. The incremental increase in GnRHR activity could not be accounted for by the constant concentration of PCOS-IgG in each well. The OIC-IgG showed minimal modulating effects compared with the PCOS-IgG.

3. Discussion

Our laboratory previously has identified several other syndromes that have proven to have a significant autoimmune pathophysiology because of GPCR-AAb [39-41]. There are preexisting circumstances in PCOS that are compatible with an autoimmune component including a young age of onset and copresence of other autoimmune diseases [42, 43]. We therefore hypothesized that AAb similarly targeting the GnRHR ECL2 could play a role in the pathophysiology of PCOS. Our study is the first to examine and demonstrate significantly

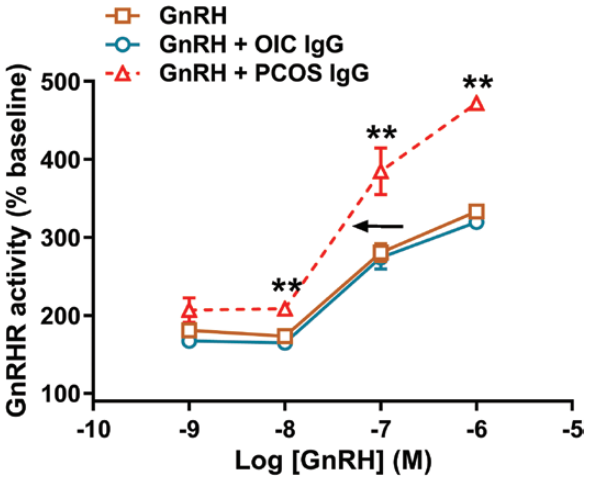

Figure 6. The effects of PCOS and OIC IgG on GnRH-induced GnRHR activation in the calcium flux assay. *The addition of a constant concentration* of PCOS IgG (100 μg/mL, n = 7) significantly increased the GnRHR-induced activity dosage response of GnRH alone (10^{-9} to 10^{-6} M). Addition of a constant concentration of OIC IgG (100 μg/mL, n = 7) had no significant effect on the GnRH dosage response curve. There was no significant rise in the 10^{-9} M values compared with the 10^{-8} M values in this particular group of transfected cells, although there was a small difference in activity the presence of the PCOS IgG. This leftward shift of the GnRH curve in the presence of a constant concentration of PCOS IgG compared with the GnRH and GnRH + OIC IgG is significantly more than additive. $^{**}P < 0.01$ vs GnRH or GnRH + OIC IgG alone.

elevated GnRHR-ECL2-AAb levels in PCOS subjects compared with OIC subjects and to a group of NC women with no previously identified autoimmune disease.

The activation potential of this circulating GnRHR-AAb was examined and confirmed in sera from a subgroup of PCOS subjects using purified serum IgG in a cell-based GnRHR bioassay. We have demonstrated a significant dose–response curve for GnRHR activation in this assay using the normal ligand GnRH as well as the synthetic ligand leuprolide. We then established a qualitatively similar dosage response with IgG from PCOS subjects. We were able to suppress this IgG-induced GnRHR activation with the GnRHR antagonist cetrorelix in sera from a subgroup of PCOS subjects to provide an estimate of the specific receptor activity of the PCOS patients' AAb. By contrast, this selective antagonist did not suppress the mean GnRHR-AAb activity in IgG from a subgroup of OIC controls. This supports the concept that there was little if any *specific* autoimmune activation of the receptor in these infertile subjects. There were a few elevated ELISA values in the OIC group. The 5 subjects with ELISA values in the top-mode of the OIC group were examined in more detail. Three of these 5 had relatively low GnRHR-AAb activity and 3 showed *no significant response* to cetrorelix blockade in vitro. Two had measurable GnRHR-AAb activity, 1 with a significant decrease of activity to cetrorelix blockade while the other had no response. It seems apparent that the initial elevated ELISA values for the 3 OIC subjects with no evidence for cetrorelix suppression of specific GnRHR-AAb activity represent a "false positive" ELISA with interaction(s) with the target 28 amino acid ECL2 peptide probably separate from the specific epitope associated with GnRHR activation. The possibility exists the other 2 controls were incorrectly identified or they were exposed to the autoimmune activation of GnRHR but expressed a "subclinical" manifestation of the full pathophysiology of PCOS.

A limitation of the present study is its retrospective nature. This purpose is to examine the likelihood for the presence of GnRHR-AAb and to study their potential for a pathophysiological role in PCOS. These relationships are currently being examined in larger and well-defined cohorts of PCOS subjects recruited for the PPCOSII trial compared with ovulatory controls with unexplained infertility recruited for the AMIGOS trial, both sponsored by the

Reproductive Medicine Network [44, 45]. The potential interaction of the GnRHR-AAb activity and AMH are being considered in detail in that study.

The criteria for demonstrating an autoimmune basis for a disease has been summarized by Witebsky [46, 47]. Three major criteria include a candidate autoantibody, demonstration that the antibody will produce a measurable and predictive change in its putative target tissue (cells containing the active receptor), and evidence that passive or active transfer of the autoantibody to an animal model will stimulate the receptor containing cells and reproduce the equivalent findings of the parent disease. We believe our data are sufficiently convincing as to satisfy the first 2 criteria. We have successfully performed active animal immunization studies to confirm these 3 criteria [48].

The pathophysiology of PCOS is complex and requires careful attention to the pulsatile nature of GnRH and its impact on its target receptor. The GnRHR is also present in the hypothalamus and may participate in a form of feedback modulation of the hypothalamic kisspeptin/neurokinin B/dynorphin (KNDy) cell-sensitive control of GnRH release from the hypothalamus [49-51]. This hypothalamic GnRHR may alter hypothalamic GnRH or kisspeptin control by direct activation or by an allosteric activation of the normal ligand for the neuron–receptor interactions. Although the direct presence of GnRHR on the KNDy cell complex has not yet been confirmed, indirect input via the complex hypothalamic cellular structures has not been excluded. The question of whether GnRHR-ECL2-AAbs can access this hypothalamic space would need to be considered. This impact of GnRHR-AAb also would be expected on the GnRHR response to GnRH in the anterior pituitary. A peripheral impact on GnRHR in target tissues such as the ovary and endometrial cells is also possible [8]. We have demonstrated the copresence of PCOS serum IgG *appears to accentuate* the agonist effect of GnRH in the GnRHR bioassay (see Fig. 6). These GPCR-AAb fail to desensitize their target receptor activity in contrast to the effect of the receptor's normal ligand. This is important for the understanding that these AAb when present will not suppress GnRHR activity and may enhance this through both orthosteric and allosteric activity. It is not yet clear how they increase the pulsatility but this has been present in our animal data. We are further exploring the possibility that AAb to the kisspeptin receptor may produce similar alterations in GnRH and LH pulsatility observed in PCOS. There is great need for a biochemical assay that is predictive or diagnostic of PCOS. The ELISA study provides some predictive value but the overlap of values is restrictive. The ELISA technique using synthetic peptides has limitations in sensitivity since the peptide stereochemistry is frequently different from the native structure. Since the clinical diagnosis of PCOS is inherently imprecise, based on the presence of just 2 of 3 general clinical features, it is possible that 1 or more of the infertile subjects could represent false negatives for PCOS. ELISA-based assays fail to measure activity of the AAb and this likely is the more important property of the AAb in vivo. The cell-based bioassay of AAb activity and the ability to demonstrate suppression of activity using a competitive inhibitor in those possessing the GnRHR-AAb appears promising.

In conclusion, we have identified an activating autoantibody to the GnRHR that may contribute to the pathophysiology in a subgroup of PCOS subjects. We have confirmed specificity of this AAb. We now have evidence that induction of the autoantibody in a rat animal model will reproduce the equivalent findings. We are also working on identifying the specific epitopes of the ECL2 of GnRHR that bind the AAb. With this information we anticipate blocking the AAb with suitable specific decoy peptides. These research avenues provide promise for future diagnostic tools and potential treatments.

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Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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