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Increased testosterone and proinflammatory cytokines in patients with polycystic ovary syndrome correlate with elevated GnRH receptor autoantibody activity assessed by a fluorescence resonance energy transfer-based bioassay

Hongliang Li[1], **Yankai Guo**[1], **Jielin Deng**[1], **Hayley Fischer**[1], **Elizabeth A. Weedin**[2], **Heather R. Burks**[2], **LaTasha B. Craig**[2], **Xichun Yu**[1]

[1]Section of Endocrinology and Diabetes, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

[2]Section of Reproductive Endocrinology & Infertility, Department of Obstetrics & Gynecology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Abstract

Purpose: The recently identified agonistic autoantibodies (AAb) to the gonadotropin-releasing hormone receptor (GnRHR) are a novel investigative and therapeutic target for polycystic ovary syndrome (PCOS). In this study, we used a new cell-based fluorescence resonance energy transfer (FRET) bioassay to analyze serum GnRHR-AAb activity and examine its relationship with testosterone and proinflammatory cytokines in patients with PCOS.

Methods: Serum samples from 33 PCOS patients, 39 non-PCOS ovulatory infertile controls and 30 normal controls were tested for GnRHR-AAb activity and proinflammatory cytokines in a FRET-based bioassay and multiplex bead-based immunoassay, respectively. Correlation was analyzed using the Spearman's correlation test.

Results: Serum GnRHR-AAb activity was significantly higher in the PCOS patients than for the ovulatory infertile ($p < 0.05$) and normal ($p < 0.01$) controls. GnRHR-AAb were positive in 39% of PCOS patients, 10% of ovulatory infertile controls, and 0% of normal controls. PCOS IgG-induced GnRHR activation was specifically blocked by the GnRHR antagonist cetrorelix. Serum levels of proinflammatory cytokines interleukin-2, interleukin-6, interferon-γ and tumor necrosis factor-α were significantly increased in PCOS patients compared with ovulatory infertile and normal controls ($p < 0.01$). Correlation analysis demonstrated positive correlations of GnRHR-AAb activity with testosterone and proinflammatory cytokine levels in the PCOS group.

Correspondence to: Xichun Yu, Section of Endocrinology and Diabetes, Department of Medicine, University of Oklahoma Health Sciences Center, 1122 NE 13th St, ORB 331, Oklahoma City, OK 73117, USA. xichun-yu@ouhsc.edu.

Conflict of interest: The authors have no conflict of interest to disclose.

Ethical approval: This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Board and conforms to the US Federal Policy for the Protection of Human Subjects.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Conclusion: Elevated GnRHR-AAb activity, as assessed by a new FRET assay, is associated with increased testosterone and proinflammatory cytokines in PCOS, suggesting autoimmune activation of GnRHR may contribute to the pathogenesis of this common disorder.

Keywords

Agonistic autoantibodies; GnRH receptor; Inflammatory cytokines; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disorder and the leading cause of anovulatory infertility [1,2]. The underlying etiology of PCOS remains largely unknown but likely involves a combination of genetic, environmental, and lifestyle factors [3,4]. Gonadotropin-releasing hormone (GnRH) plays a key role in regulating the neuroendocrine control of reproductive function. The pulsatile secretion of hypothalamic GnRH into the anterior pituitary triggers the synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then act on the ovaries to regulate steroidogenesis, folliculogenesis and ovulation [5,6]. GnRH pulsatility is disrupted in PCOS, causing deranged release of LH and FSH [7–9]. A hallmark of PCOS is persistently increased GnRH and LH pulsatility with elevated testosterone and relatively decreased FSH [10]. These abnormalities may occur in early puberty and manifest from the first menstrual cycle even though some PCOS complications do not arise until later [9,11]. Since an excessive immune-inflammatory response has long been recognized as a major driving force in many diseases, dysregulation of the hypothalamic-pituitary-gonadal axis resulting from immune-inflammatory-mediated alterations in GnRH or its receptor could be a plausible underlying mechanism for reproductive dysfunction. However, this has not yet been investigated for causal association with PCOS.

The GnRH receptor (GnRHR) is a member of the seven-transmembrane G protein-coupled receptor (GPCR) family [12]. GPCRs mediate cellular responses to a variety of stimuli and are involved in diverse physiological and pathological processes. Over the past two decades, there has been growing evidence of a new type of autoimmunity associated with functional autoantibodies against GPCRs [13,14]. These autoantibodies primarily target the second extracellular loop (ECL2) of the receptors and act as allosteric receptor agonists or antagonists, leading to continuous uncontrolled or blocked receptor signaling and thus contributing to disease pathogenesis. We have recently demonstrated in a retrospective study [15] that agonistic autoantibodies (AAb) directed to the GnRHR ECL2 are present in PCOS patients and may be pathophysiologically relevant, given their potential to persistently activate GnRHR. Like other GPCR-AAb, GnRHR-AAb binding to and stimulation of GnRHR are not expected to lead to receptor downregulation [16]. These autoantibodies were detected by ELISA, and their activity was measured in a limited number of subjects using a cell-based calcium flux assay. There are inherent disadvantages associated with calcium flux assays. These assays require special automation and signal recording with sufficient temporal resolution due to the extremely transient nature of calcium flux [17,18]. Automated analysis for a large volume of time-series data also remains a challenge.

PCOS is a proinflammatory state, and emerging evidence supports a role for chronic inflammation in the development of metabolic abnormalities and ovarian dysfunction in this disorder [19]. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), two important proinflammatory cytokines that may impact ovarian reproductive function [20], are elevated in the serum and follicular fluid of infertile women with PCOS compared with body mass index (BMI)-matched controls [21]. There is also evidence of a dominant type 1 T helper (Th1) immune inflammatory response in PCOS [22,23]. We therefore focused on TNF-α, IL-6, and two other proinflammatory Th1 cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) to investigate their possible association with GnRHR-AAb.

In the present study, we expanded the functional screening for GnRHR-AAb in PCOS patients and controls from the previous cohorts using a new cell-based fluorescence resonance energy transfer (FRET) bioassay, analyzed the functional activity of GnRHR-AAb, and evaluated the relationship of GnRHR-AAb activity with LH, testosterone and proinflammatory cytokines to provide further evidence for an autoimmune component for the etiopathogenesis of PCOS. Serum levels of GnRH were not measured in this study.

Materials and Methods

Study design:

A case-control study was performed on 33 patients with polycystic ovary syndrome (PCOS), 39 ovulatory infertile controls (OIC), and 30 normal controls (NC) to analyze serum GnRHR-AAb activity and its relationship with serum levels of LH, testosterone and proinflammatory cytokines.

Study subjects:

Sera from prior retrospective cohorts [15] of 40 age- and BMI-matched pairs of PCOS patients and OIC subjects were studied. Thirty-three PCOS patients and 39 OIC subjects had samples available for further testing. The NC group in the original study was expanded to include a total of 30 healthy women with regular periods and no history of hirsutism, infertility or autoimmune disease. These normal subjects were recruited and consented under the auspices of an Institutional Review Board-approved program seeking normal subjects with a minimal history of autoimmune-related diseases. Some were enrolled as students in the various colleges and others as employees on campus. This group was similar to the other two matched groups for race/ethnicity and BMI ± 5 but were slightly younger in age (−6 years on average). All serum samples were de-identified and sent frozen to the research laboratory. This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Board (IRB #4351), and all subjects provided written informed consent.

GnRHR autoantibody activity assay:

Serum-induced GnRHR activation was assessed in GnRHR-NFAT-*bla* CHO-K1 cells using the GeneBLAzer FRET-based β-lactamase reporter assay (K1854, Invitrogen) according to the manufacturer's instructions. Briefly, cells were plated in 384-well plates and incubated overnight. The individual serum (1:20) or IgG samples were then added and incubated for 5 hours, followed by incubation with the β-lactamase substrate CCF4-AM (LiveBLAzer-

FRET B/G Loading Kit, Invitrogen) for 2 hours. The plates were read on a Hidex Sense microplate reader (Hidex, Finland).

Serum IgG randomly selected from 8 PCOS patients with elevated GnRHR-AAb activity and 8 NC subjects were purified using the NAb Protein A/G Spin Kit (Thermo Fisher Scientific) and tested for dosage response (10-150 μg/mL) and inhibition with the GnRHR antagonist cetrorelix (0.1 μM).

All samples were tested in triplicate. Negative (buffer) and positive (GnRH) controls were included in each assay. Data were calculated as the ratio of the emissions 460/530 nm (blue/green) after subtraction of the background values and expressed as fold increase over buffer baseline to normalize the individual values. The intra- and inter-assay coefficients of variation were 8.9% and 5.7%, respectively.

LH and total testosterone (T) assays:

Serum levels of LH and total testosterone in 30 PCOS patients, 26 OIC and all 30 NC subjects were measured using the human LH (ENZKIT107-0001) and total T (ADI-901-176) ELISA kits as described previously [15]. Both kits were obtained from Enzo Life Sciences (Farmingdale, NY).

Cytokine assay:

The levels of proinflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α in the sera from 16 PCOS patients, 12 OIC and 10 NC subjects randomly selected from the three groups were measured using the Bio-Plex Pro™ magnetic bead-based immunoassay on a Bio-Plex platform (Bio-Rad Laboratories) according to the manufacturer's instruction. Briefly, samples and standards were incubated with the beads in the assay plate for 30 min. Detection antibodies were then added and incubated for 30 min, followed by incubation with streptavidin-phycoerythrin (SA-PE) for 10 min. The plate was read using the MAGPIX™ system and the generated multiplex data were analyzed using Bio-Plex Manager™ software version 6.0.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism (version 8.4.3). Continuous variables were tested for normality with the D'Agostino & Pearson test. Data were expressed as mean ± standard deviation (SD) or median and interquartile range, where appropriate. Group comparisons were performed using Student's t test or Mann-Whitney test for comparison of two groups and one-way ANOVA or Kruskal-Wallis test for multiple group comparisons. Categorical variables were compared using the χ^2 or Fisher's exact test. Autoantibody positivity was defined as values above the mean + 2SD from the NC group. Correlations were analyzed using the Spearman's correlation test. Statistical significance was set at $p < 0.05$.

Results

Subject characteristics:

The characteristics of the study subjects were described previously [15]. The age (23.9 ±2.1 yrs), BMI (24.8 ± 4.2 kg/m^2), serum LH (6.7 ± 2.7 mIU/mL) and testosterone (204.1 ± 86.1 pg/mL) for the expanded NC group were similar to those for the 14 normal controls included in the original study. They were younger ($p < 0.01$), but similar in BMI compared to either the PCOS group ($p = 0.173$) or OIC group ($p = 0.178$). Serum LH and testosterone levels were not significantly different from the OIC group ($p = 0.102$ and 0.709, respectively), but were significantly lower than the PCOS group ($p < 0.001$ and 0.01, respectively).

GnRHR autoantibody activity:

A GnRHR-transfected cell-based FRET assay was used to detect presence of GnRHR-AAb in sera from 33 PCOS patients, 39 OIC and 30 NC subjects. The PCOS group showed a significantly elevated mean activity value of GnRHR-AAb compared with the OIC group ($p < 0.05$) and NC group ($p < 0.01$) (Fig. 1a). Thirteen of the 33 PCOS patients (39%) and 4 of the 39 OIC subjects (10%) were positive for GnRHR-AAb. None of the 30 NC subjects showed GnRHR-AAb positivity (Fig. 1b). It is of interest that two of the OIC subjects showed high values despite no other characteristics being identified to distinguish them from the remainder of this phenotypically heterogeneous group. Nonetheless, there were no significant differences in GnRHR-AAb positivity and mean activity values between the OIC and NC groups ($p = 0.20$ and 0.72, respectively).

To examine the dose response of GnRHR-AAb, serum IgG from 8 AAb-positive PCOS patients and 8 NC subjects (AAb-negative control) were purified and tested at 4 different concentrations (10, 50, 100, and 150 μg/mL) for their activation potential in the FRET assay (Fig. 2a). There was a significant dose effect of PCOS IgG on GnRHR activation and the maximal effect occurred at 100-150 μg/mL of IgG. No significant dose effect was observed for the NC IgG. Receptor-specific activity of GnRHR-AAb in purified IgG (100 μg/mL) was tested with the selective GnRHR blocker cetrorelix. As shown in Fig. 2b, cetrorelix markedly suppressed PCOS IgG-induced GnRHR activation (from 2.15 ±0.17 to 1.50 ± 0.09 fold increase, $p < 0.01$). No significant change in GnRHR activity with cetrorelix was found in the NC IgG group (1.45 ± 0.15 vs 1.43 ± 0.13 fold increase, $p = 0.596$).

Serum levels of proinflammatory cytokines:

The concentrations of proinflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α in the sera from 16 PCOS patients, 12 OIC and 10 NC subjects were measured with a multiplex bead-based immunoassay. There were significant increases in the levels of all these cytokines in the PCOS group compared to either the OIC group or NC group. The cytokine levels were slightly higher in the OIC group compared to the NC group but did not reach significance ($p > 0.05$). Details of the cytokine levels and group comparisons are presented in Table 1.

GnRHR autoantibody correlations with LH, testosterone and cytokines:

To examine the relationship of GnRHR-AAb activity with LH, testosterone and proinflammatory cytokines, values available for all these variables from 38 subjects (16

PCOS patients, 12 OIC and 10 NC subjects) were analyzed with Spearman's correlation coefficient for the presence of any correlations. There was a significant positive correlation between GnRHR-AAb activity and testosterone when all three groups were combined (Fig. 3a). This significant correlation was also observed in the PCOS group ($r = 0.61$, $p = 0.0130$), but not in the OIC ($r = 0.45$, $p = 0.1404$) or NC ($r = -0.10$, $p = 0.7850$) groups. Although not statistically significant, there was a tendency for GnRHR-AAb activity to positively correlate with LH in the combined groups (Fig. 3b). The PCOS group showed a similar trend of correlation ($r = 0.46$, $p = 0.0758$). This weak correlation may reflect the fact that only basal LH levels were assessed, not LH pulsatility. No significant correlation was found in the OIC ($r = -0.27$, $p = 0.3912$) or NC ($r = -0.16$, $p = 0.6567$) groups.

When proinflammatory cytokine levels were compared with GnRHR-AAb activity, a significant overall correlation was observed for the measured cytokines in the combined groups (Fig. 4). IL-2, IL-6, IFN-γ and TNF-α were all strongly positively correlated with GnRHR-AAb activity ($p < 0.001$) and demonstrated a similar linear relationship, suggesting inflammation may be associated with GnRHR-AAb. This positive correlation was also found in the PCOS group (IL-2: $r = 0.75$, $p = 0.0012$; IL-6: $r = 0.58$, $p = 0.0193$; IFN-γ: $r = 0.81$, $p = 0.0002$; TNF-α: $r = 0.77$, $p = 0.0007$), but not in the OIC group (IL-2: $r = 034$, $p = 0.2721$; IL-6: $r = 0.17$, $p = 0.5964$; IFN-γ: $r = 0.39$, $p = 0.2031$; TNF-α: $r = 0.13$, $p = 0.6947$) or NC group (IL-2: $r = 0.47$, $p = 0.1711$; IL-6: $r = 0.20$, $p = 0.5837$; IFN-γ: $r = 0.58$, $p = 0.0816$; TNF-α: $r = 0.01$, $p > 0.9999$).

Discussion

The objective of this study was to extend previous work on identification of pathophysiologically relevant GnRHR-AAb in PCOS by expanding their functional screening and evaluating their relationship with hyperandrogenism and inflammation associated with PCOS. Using a new cell-based FRET assay, we replicated previous findings that GnRHR-AAb from PCOS patients act as an allosteric agonist to induce GnRHR activation even in the absence of an orthosteric agonist. The activation potential of circulating GnRHR-AAb was confirmed using purified serum IgG from PCOS patients, which demonstrated a significant dose effect on GnRHR activation in the FRET assay. This IgG-induced GnRHR activation was effectively suppressed with the GnRHR antagonist cetrorelix, thus providing an estimate of receptor-specific activity of GnRHR-AAb.

The GeneBLAzer GPCR assay uses a mammalian-optimized *bla* reporter gene combined with a FRET-enabled substrate to provide reliable detection of cellular responses to stimuli. With a better signal to noise ratio, cell-based reporter gene assays offer the advantage of wide linearity and sensitivity that allow detection of weak GPCR agonists or allosteric modulators [24,25]. They are also easy to set up and can be scaled down to 1536- or 3456-well formats, making them suitable for high-throughput screening for GPCR ligands and modulators. Our studies support the potential usefulness of this assay platform in screening larger number of subjects with PCOS for the presence of functional allosteric GnRHR-AAb.

PCOS has been an enigma since it was first identified as Stein-Leventhal syndrome in 1935 [26]. Although it is considered a heterogeneous disorder, the true etiology remains unclear,

which elevates the importance of further research in this area to seek identification of improved diagnostic testing and therapeutic options for patients impacted by this syndrome. Hyperandrogenism appears to be a common and major factor in humans and in most animal models [4]. The origin and timing of these androgens are a major unanswered question. An autoimmune mechanism has been proposed for some cases of PCOS, wherein an increased prevalence of organ-specific and non-specific autoantibodies has been observed [27,28]. However, the clinical significance of these autoantibodies is not yet clearly defined. It is known that autoimmune diseases are more prevalent in women than men [29,30]. In general, women have stronger immune responses to infection, vaccination, and trauma with enhanced antibody production [31]. Unlike classical autoantibodies, certain GPCR-directed autoantibodies are capable of producing pathophysiological effects by triggering or blocking receptor-specific signaling pathways. The term “functional autoantibody diseases” has been coined to refer to diseases associated with such functionally active autoantibodies [32]. The well-established presence of TSH receptor agonistic antibody as causative in Graves’ disease is perhaps the best example. In contrast to natural physiological ligands, these autoantibodies are unable to induce GPCR desensitization, and consequently cells fail to retain this important protective mechanism against autoantibody-mediated receptor overstimulation. This loss of desensitization, first documented for autoantibodies against the β1-adrenergic and M2 muscarinic receptors, has now been well recognized as a characteristic feature of GPCR-AAb [32,16]. The identification of functional GnRHR-AAb in a significant percentage of PCOS patients suggests that PCOS may be another member of the “functional autoantibody diseases” family. We have confirmed the specific activity of GnRHR-AAb from PCOS patients using two different functional assays. The uncontrolled prolonged effect of GnRHR-AAb in vivo would presumably disrupt the pulsatile stimulation of pituitary GnRHR and produce the hormonal characteristics of PCOS, viz., persistently increased LH and testosterone. This is compatible with our observation that GnRHR-AAb activity, which is significantly increased in the PCOS group, is positively correlated with serum levels of testosterone. Further studies are needed to clarify how these autoantibodies promote the preferential release of LH over FSH by pituitary gonadotropes. There were a few elevated GnRHR-AAb values in the OIC group. It is possible that these two clinically abnormal control subjects with high values were incorrectly identified or they were exposed to GnRHR-AAb but expressed a “subclinica” manifestation of the full pathophysiology of PCOS [15].

In recent years, chronic low-grade inflammation, despite its unknown origin, has emerged as an important contributor to the pathogenesis of PCOS [33,34,19]. Inflammatory markers such as C-reactive protein, IL-6, IL-18 and TNF-α are frequently elevated in PCOS patients, especially those with obesity, insulin resistance and other metabolic risk factors [35–37]. In line with these observations, our studies demonstrated significantly increased levels of circulating proinflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α in PCOS patients compared to BMI-matched ovulatory infertile and normal controls. Furthermore, these proinflammatory cytokines positively correlated with GnRHR-AAb activity, raising an intriguing link between GnRHR-AAb and inflammation. GnRH and its agonists have been reported to induce a proinflammatory Th1 shift in T cell immunity, which stimulates production of the signature Th1 cytokine IFN-γ both in vitro and in vivo [38,39],

possibly by activating the GnRHR present in peripheral blood mononuclear cells [40]. A similar immune cell-mediated proinflammatory effect would be expected for GnRH-like GnRHR-AAb. Since androgens, adipose tissue, and inflammation are closely interlinked [19,37], it is also conceivable that GnRHR-AAb may promote inflammation through a hyperandrogenism-mediated imbalance in the production of pro- and anti-inflammatory adipokines.

The question as to how GnRHR autoantibodies are generated in women with PCOS is yet to be answered. Since the receptor extracellular loops are in the ectodomain, these autoantibodies may occur as a consequence of tissue injury from inflammation, ischemia, or exposure to toxic or infectious substances, which shed and release potential autoantigens into the circulation to trigger an autoimmune response [41]. Molecular mimicry between antigenic determinants from GnRHR ECL2 and antigenic proteins from infectious agents, if identified, can also lead to GnRHR-AAb production. This has proven to be the case for autoantibodies to the β1-adrenergic receptor and angiotensin II type 1 receptor, which share high homology of their ECL2 epitope sequences with a protein component of Trypanosoma cruzi and parvovirus B19, respectively [42,43].

To establish an autoimmune etiology for a disease, reproduction of the disease in experimental animals is required in addition to identification of a corresponding autoantigen and an autoimmune response in the form of an autoantibody or cell-mediated immunity [44,45]. This was confirmed in our recently developed rat model of GnRHR autoantibody-induced PCOS. These rats produced potent functional GnRHR-AAb after immunization with the GnRHR ECL2 peptide, and exhibited PCOS-like phenotypic changes, including increased LH secretion, hyperandrogenemia, and follicular atresia [46]. These data provide compelling evidence for a pathogenic role of GnRHR-AAb in the development of PCOS.

Identification and confirmation of pathological GnRHR-AAb will ultimately transform PCOS from a disease of unknown etiology to an autoimmune-linked disease. The development and validation of a reliable assay to detect GnRHR-AAb as a potential diagnostic test would improve early diagnosis and management of PCOS. We have identified an epitope target of GnRHR-AAb on the ECL2 of GnRHR [46], and we anticipate blocking these autoantibodies with specific epitope-mimetic peptide inhibitors that we have successfully developed for inhibiting other GPCR-AAb [47,48]. Once confirmed, these blocking agents could then be used in the treatment of PCOS.

In conclusion, using a new cell-based fluorescence resonance energy transfer (FRET) bioassay, we replicated previous findings that GnRHR-AAb from PCOS patients are an activator of GnRHR. These autoantibodies appear to be associated with hyperandrogenism and inflammation in PCOS. Our discovery of potentially causative GnRHR-AAb in PCOS raises the possibility of a paradigm shift in our understanding of the pathogenesis, diagnosis and potential treatment of a large subgroup of subjects with PCOS.

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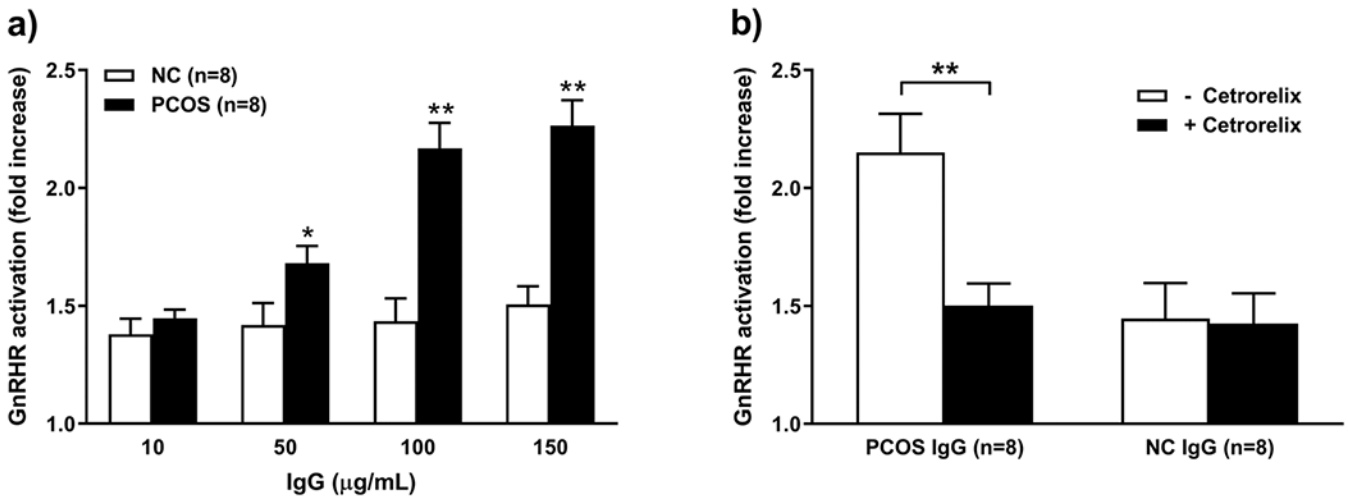

Fig. 1.

Serum autoantibody-induced GnRHR activation in cell-based FRET assay, **a**, Individual and mean values from 33 PCOS patients, 39 ovulatory infertile controls (OIC) and 30 normal controls (NC) are presented. Values are expressed as fold increase over buffer baseline. The dashed line represents the threshold for elevated GnRHR autoantibody activity (defined as 2 SD above the mean normal control value), **b**, The percentage of PCOS, OIC and NC subjects with elevated GnRHR autoantibody activity is shown. All serum samples were tested at 1:20 dilution. *$p < 0.05$, **$p < 0.01$

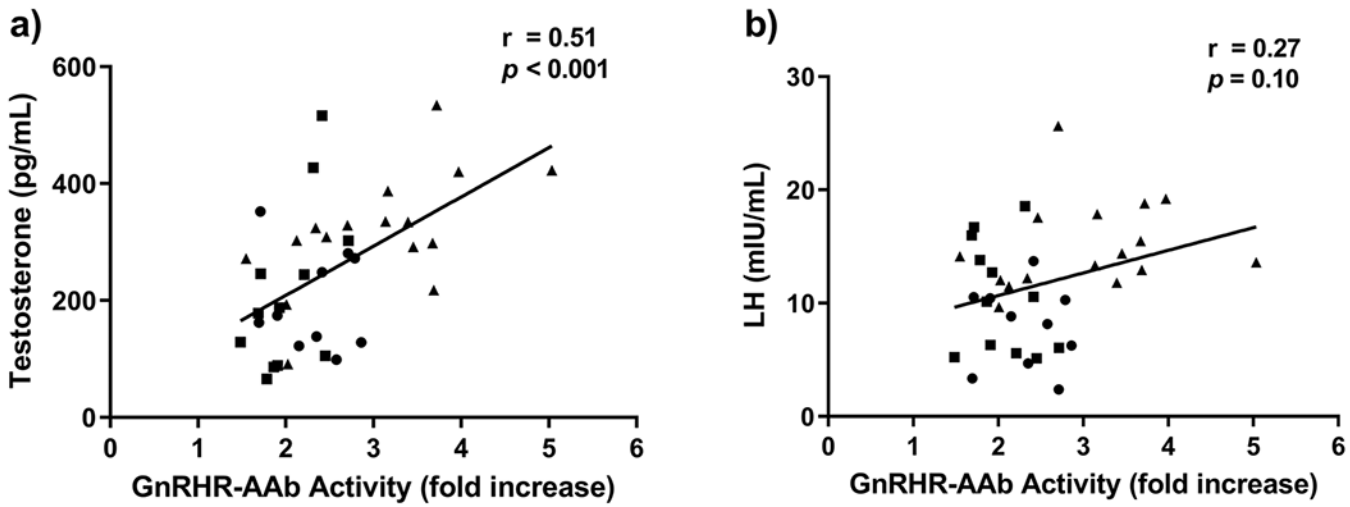

Fig. 2.

Dose effect of PCOS IgG on GnRHR activation (**a**) and effect of GnRHR blockade with cetrorelix on IgG-induced GnRHR activation (**b**) in cell-based FRET assay. Data are means and SD for serum IgG purified from 8 antibody-positive PCOS subjects and 8 normal controls (NC). **a**, *$p < 0.05$, **$p < 0.01$ vs NC; **b**, IgG (100 μg/mL) was tested with and without cetrorelix (0.1 μM). **$p < 0.01$

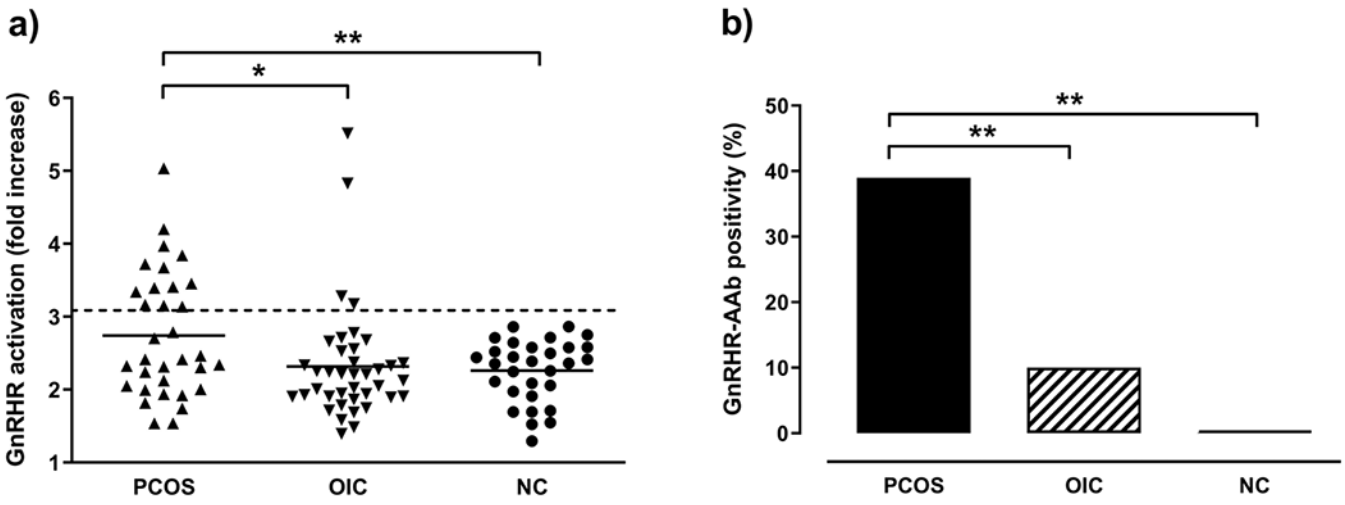

Fig. 3.

Correlations of GnRHR autoantibody activity with testosterone (**a**) and luteinizing hormone (LH) (**b**). Values from a total of 38 subjects including 16 PCOS patients (closed upward triangles), 12 ovulatory infertile controls (closed squares) and 10 normal controls (closed circles) were analyzed. GnRHR autoantibody activity values are expressed as fold increase over buffer baseline. Spearman correlation coefficient (r) values and significance (*p*) are shown

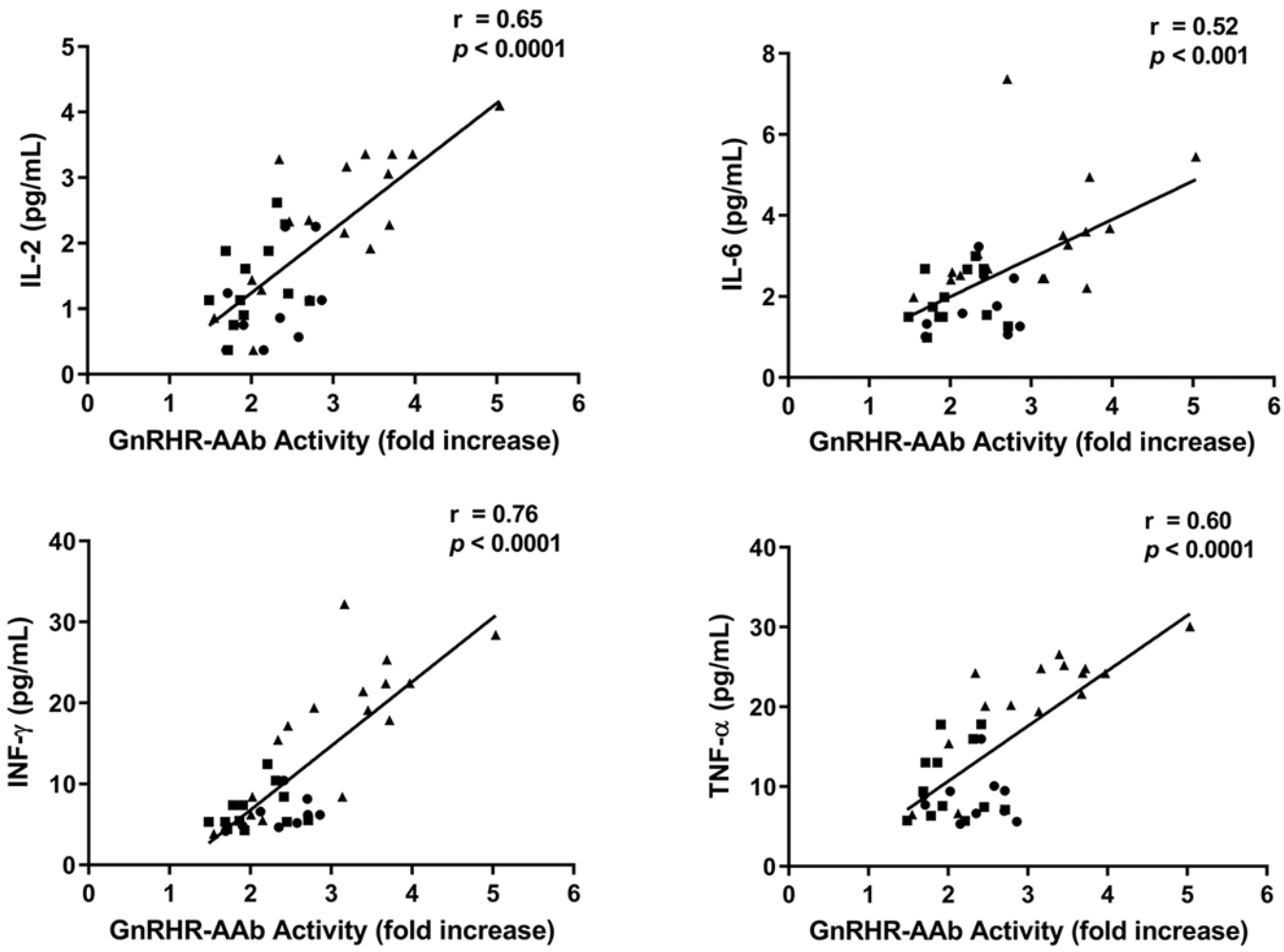

Fig. 4.

Correlations of GnRHR autoantibody activity with proinflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α. Values from a total of 38 subjects including 16 PCOS patients (closed upward triangles), 12 ovulatory infertile controls (closed squares) and 10 normal controls (closed circles) were analyzed. GnRHR autoantibody activity values are expressed as fold increase over buffer baseline. Spearman correlation coefficient (r) values and significance (*p*) are shown

Table 1

Serum levels of proinflammatory cytokines in subjects with polycystic ovary syndrome (PCOS), ovulatory infertile controls (OIC) and normal controls (NC)

Variables (pg/mL)	PCOS (n = 16)	OIC (n = 12)	NC (n = 10)	*p* value PCOS vs OIC	*p* value PCOS vs NC	*p* value OIC vs NC
IL-2	2.44 (1.88-3.34)	1.13 (0.75-1.77)	1.13 (0.52-1.39)	< 0.001	< .001	0.999
IL-6	3.03 (2.47-3.66)	1.86 (1.50-2.58)	1.43 (1.21-1.82)	0.004	< .001	0.971
IFN-γ	18.51 (10.43-22.44)	5.82 (4.93-7.38)	5.25 (4.53-5.68)	< 0.001	< .001	0.884
TNF-α	22.91 (18.21-24.83)	9.40 (6.42-14.80)	7.28 (6.28-9.04)	0.001	< .001	0.999

Data are median (interquartile range). *P* values are derived from Kruskal-Wallis test with Dunn's correction