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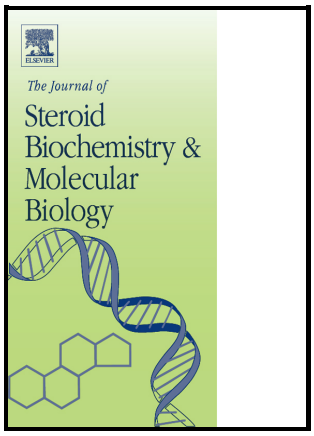

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The serum steroid signature of PCOS hints at the involvement of novel pathways for excess androgen biosynthesis

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Abstract

Context: Polycystic ovary syndrome (PCOS) is defined by androgen excess and ovarian dysfunction in the absence of a specific physiological diagnosis. The best clinical marker of androgen excess is hirsutism, while the best biochemical parameter is still a matter of debate. Current consensus guidelines recommend, among other hormones, serum free testosterone as an important serum parameter to measure androgen excess. Recently, however, novel active androgens and androgen metabolic pathways have been discovered.

Objective: To assess the contribution of novel androgens and related steroid biosynthetic pathways to the serum steroid pool in PCOS women in comparison to healthy controls.

Design: This is a case control study, wherein PCOS was diagnosed according to the AE-PCOS 2009 criteria. Serum steroid profiling was performed by liquid chromatography high-resolution mass spectrometry.

Setting: Yeditepe University and associated clinics in Istanbul, Turkey, together with Bern University Hospital Inselspital, Bern, Switzerland.

Participants: 42 PCOS women and 42 matched, healthy control women.

Main outcome measures: Assessment of 34 steroids compartmentalized in four androgen related pathways: the classic androgen pathway, the backdoor pathway, the C11-oxy backdoor pathway, and the C11-oxy (11β-hydroxyandrostenedione) pathway.

Results: Metabolites of all four pathways were identified in healthy and PCOS women. Highest concentrations were found for progesterone in controls and androstenedione in PCOS. Lowest levels were found for 11-ketotestosterone in controls compared to PCOS, and for 20α-hydroxyprogesterone in PCOS compared to controls. PCOS also had higher serum testosterone levels compared to the controls. PCOS women had overall higher levels of steroid metabolites of all four androgen pathways compared to healthy controls.

Conclusions: Novel alternative pathways contribute to the androgen production in healthy and PCOS women. Hyperandrogenism in PCOS is characterized by an overall increase of serum androgens in the classic, backdoor and C11-oxy pathways. While monogenetic disorders of steroid biosynthesis can be recognized by a specific pattern in the steroid profile, no diagnostic pattern or classifier was found in the serum for PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is the most frequent endocrine disorder in women, characterized by androgen excess and ovarian dysfunction (oligo- or anovulation and polycystic ovaries) in the absence of related disorders such as congenital adrenal hyperplasia (Azziz et al., 2009; Escobar-Morreale, 2018). In addition, associated metabolic and cardiovascular disorders are substantial health risks for PCOS patients (Bazarganipour et al., 2015; Kempegowda, Melson, Manolopoulos, Arlt, & O'Reilly, 2020; Li et al., 2019), and thus, a diagnosis of PCOS carries a large psychosocial and socioeconomic burden (Azziz et al., 2009; Cooney & Dokras, 2018).

The disease mechanism(s) of PCOS in general are still unknown and hyperandrogenism, which is one of its main characteristics, is specifically also difficult to trace to only one origin. Several hypotheses have been tested so far, mostly focusing on insulin resistance and androgen excess (Dapas & Dunaif, 2022; Rosenfield & Ehrmann, 2016; Wagner et al., 2022). However, a complex pathomechanism including genetic, epigenetic, microbiotic and environmental factors seems likely (D. H. Abbott, Dumesic, & Levine, 2019; D.H. Abbott, Greinwald, & Levine, 2022; Batra, Bhatnager, Kumar, Suneja, & Dang, 2022; Bruni, Capozzi, & Lello, 2022; de Zegher, Lopez-Bermejo, & Ibanez, 2018; Niinuma, Lubbad, Lubbad, Moin, & Butler, 2023; Tennilä et al., 2021). In females, androgens and precursors are synthesized in the ovarian theca cells and the zona reticularis of the adrenal cortex, from where they may be secreted into the circulation and further converted into (more) active androgens in peripheral tissues (Andersen & Ezcurra, 2014; Naamneh Elzenaty, du Toit, & Fluck, 2022).

In PCOS, studies have shown some specific changes in the hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes. Compared to controls, the HPA axis seems to be hyperreactive to adrenocorticotropic hormone (ACTH) stimulation (Azziz, Black, Hines, Fox, & Boots, 1998), while in the HPG axis an increase in luteinizing hormone (LH) is found in many women (Liao, Qiao, & Pang, 2021). Peripheral conversion of circulating androgens to testosterone (T) in adipose tissue is also increased in some women with PCOS (Rosenfield & Ehrmann, 2016; Rosenfield, Mortensen, Wroblewski, Littlejohn, & Ehrmann, 2011; Wagner et al., 2022). Moreover, anti-Müllerian hormone (AMH) levels are typically higher in PCOS and block follicle maturation in the ovaries (Bongrani et al., 2022; Rudnicka et al., 2021). Neurometabolic factors or the ‘neuronal-reproductive-metabolic circuits’ have also been described in PCOS, underscoring neuroendocrine factors, including gonadotropin-releasing hormone (GnRH) and neuropeptides, and their influence on reproductive and metabolic disorders in PCOS (Liao et al., 2021).

Furthermore, numerous genetic studies have been performed trying to identify underlying genes which explain the PCOS phenotypes (Almawi et al., 2023; Marti et al., 2017; Shukla, Mukherjee, Patil, & Joshi, 2023), and although some large genome-wide association studies

(GWAS) studies have revealed some hints (Day et al., 2018; Hayes et al., 2015; Zhang et al., 2020), conclusive results are still missing (Hiam et al., 2019).

Recently, the biochemistry behind human androgen biosynthesis has risen as a hot topic in PCOS research, due to newer chromatographic and mass spectrometric methods (Keevil, 2019; Olesti, Boccard, Visconti, Gonzalez-Ruiz, & Rudaz, 2021; Storbeck et al., 2019), novel pathways and active androgen metabolites which have been (re-)discovered in diseases and disorders (Saito et al., 2016; Turcu, Rege, Auchus, & Rainey, 2020). So far, clinical guidelines recommend assessing androgen excess in PCOS biochemically through the measurement of T, LH, follicle-stimulating hormone, estradiol (E2), dehydroepiandrosterone (DHEA)-sulfate (DHEA-S), androstenedione (A4), steroid-hormone binding globulin, 17α-hydroxyprogesterone (17OHP4), and prolactin levels, together with thyroid function tests (Cussen et al., 2022).However, these measurements only reflect the classical pathway of androgen biosynthesis and neglects other alternative pathways that have been shown to contribute to androgen excess in PCOS and other disorders (O'Reilly et al., 2017; Swart et al., 2021; Taylor et al., 2022; Torchen et al., 2020; Tosi et al., 2022; Walzer et al., 2022; Yoshida et al., 2018).

In this study we performed comprehensive serum steroid profiling of PCOS women in comparison to healthy controls, with the aim of describing steroid levels in multiple metabolic pathways which lead to active androgen production. We also performed complex data analysis in search for a diagnostic marker or algorithm as previously found for 24h urine steroid profiling of PCOS (Dhayat et al., 2018).

Methods

Patient characterization and sample collection

Ethical approval for the study was obtained from the Ethical Committee of Yeditepe University, Istanbul, Turkey (Decision No: 1149). Informed consent was obtained from all participants. Subject characterization was completed during a regular clinical visit, and the PCOS diagnosis concluded according to the AE-PCOS 2009 criteria (Azziz et al., 2009), which included the completion of a standardized questionnaire. Participants were more than 2 years post-menarchal. Essential clinical criteria for participation in the study were: a) the history of menstrual cycles, defined as irregular if the patients presented with polymenorrhea, defined as more than one menstruation in a cycle, or amenorrhea, defined as no menstruation in two to three cycles; b) a physical exam, including the assessment of signs of hirsutism and acne and obesity according to body mass index (BMI) measurements; and c) an ultrasound of the ovaries. Infertility was defined as unsuccessful pregnancy following one year of unprotected sexual intercourse. Exclusion criteria included: other diagnosable diseases other than PCOS

and regular intake of any medications (especially hormonal or antidiabetic drugs). All participants provided an un-timed blood sample, which was collected in a gel containing tube and immediately processed to serum. Serum samples were aliquoted and immediately stored at -80^{0}C. Thereafter, samples were transported on dry ice to the steroid laboratory in Bern, Switzerland, and batch-analyzed.

Steroid profiling and anti-Müllerian hormone (AMH) measurement

Serum E2 was measured with the ADVIA Centaur Enhanced Estradiol Assay kit (Catalog # 10490889, RRID:AB_2895133) on an Immulite 2000 (Siemens Healthcare GmbH, Münich, Germany). AMH was measured with the AMH Gen II ELISA kit (Catalog # A79765, RRID:AB_2800500) (Beckman Coulter, Pasedena, CA, USA). Thirty-three serum steroids were measured by an *in-house* liquid chromatography high-resolution mass spectrometry (LC-HRMS) method as previously described and validated (Andrieu, du Toit, Vogt, Mueller, & Groessl, 2022). Briefly, 550 µL serum were spiked with 38 µL of a mixture of internal standards (3.8 nM each), a protein precipitation step using zinc sulfate and methanol followed and steroids were extracted using solid-phase extraction with an OasisPrime HLB 96-well plate. Samples were resuspended in 100 µL 33% methanol in water and 20 µL injected into the LC-HRMS instrument (Vanquish UHPLC coupled to a QExactive Orbitrap Plus, Thermo Fisher Scientific) using an Acquity UPLC HSS T3 column (Waters). Data from the mass spectrometer was processed using TraceFinder 4.0 (Thermo Fisher). Enzyme activities were estimated by calculating product to precursor ratios as previously reported (Rumsby, Woodward, & SpringerLink, 2019). Steroid pathway involvement was estimated by calculating ratios of specific precursor and downstream metabolites.

Statistical analyses

Statistical analyses were performed with SPSS Statistics software Version 22 (IBM Corp., Armonk, NY, USA) and programmed with Python using established statistical packages. Normality of data was examined with Shapiro-Wilk and D'Agostino and Pearson's tests, statistical differences between groups were analysed either by Mann-Whitney U test, Student's *t*-test, Chi-squared test or Fisher's Exact test, where appropriate. Data was visualized using GraphPad Prism v9.4.1 and correlation heatmaps generated using Metaboanalyst 5.0 online software (Pang et al., 2021).

Results

In our study, we profiled 42 PCOS patients matched with 42 healthy controls and compared their serum steroid panels. The patient demographic characteristics are summarized in Table 1. Our PCOS group differed significantly from the control group in age, weight/BMI, menstrual

irregularities, signs of hirsutism and acne, infertility, AMH levels and reported prior high serum T diagnostic measurements. As our control group was slightly older than the PCOS women, we tested whether there was a potential correlation between age and AMH levels in both the controls and the PCOS individuals, however no significant correlations (p>0.05) were found for age and AMH levels (Fig. S1).

Thirty-three steroids were quantified and compared between our PCOS group and controls as summarized in Table 2. Etiocholanolone and 21-deoxycortisol were only detected in a few samples and not measured above the limit of accurate quantification (LOQ) in either group (Table S1), and were therefore not included in Table 2 and further analysis. A4, T, androsterone (AST) and 11-ketotestosterone (11KT) were significantly higher in our PCOS group compared to the controls, while progesterone (P4) and 20α-hydroxyprogesterone (20αOHP4) were significantly lower in our PCOS group compared to the controls (Fig. 1). From these metabolites, all were detected and quantified in all samples, except for P4 and 20αOHP4 which were quantified above their LOQ in 95% and 98% of samples, respectively (Table S1). P4 circulates in low levels and cycles with the highest levels reported during the secretory (luteal) phase (Andrieu et al., 2022; Schiffer et al., 2023). P4 levels measured in the PCOS and control groups crowded around the minimum reported levels for the luteal phase and the maximum reported levels for the follicular (menstruation and proliferative) phase (Fig. S2). It should be noted that most of our PCOS patients had menstrual irregularities and did not cycle normally (PCOS inclusion criteria). As our control group was slightly slimmer than the PCOS women, we tested whether there was a potential correlation between BMI and statistically different metabolite levels in both the controls and the PCOS individuals, however no significant correlations were found for BMI and steroid levels (data not shown). In total we quantified eight downstream progesterone metabolites, including C11-oxy progesterones and C11-oxy androgens in our PCOS cohort. 11β-Hydroxyandrostenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-ketoandrostenedione (11KA4) and 11KT were quantified in our PCOS cohort and their levels are corroborated by previous publications reporting these androgens in PCOS (O'Reilly et al., 2017; Swart et al., 2021; Taylor et al., 2022; Torchen et al., 2020; Tosi et al., 2022; Walzer et al., 2022; Yoshida et al., 2018), but we additionally quantify 11-ketoandrostanolone (also known as 11-ketodihydrotestosterone; 11KDHT) and 5α-androstanetrione (11K5αDIONE) in our cohort.

Based on the measured steroid metabolites, apparent enzyme activities were estimated by calculating product-to-substrate ratios (Fig.2 and Table S2). These calculations showed that the catalytic activity of 3β-hydroxysteroid dehydrogenase (3βHSD), cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), adrenal cytochrome P450 11β-hydroxylase/cytochrome P450 aldosterone synthase (CYP11B), cytochrome P450 aromatase (CYP19A1), reductive

17β-hydroxysteroid dehydrogenase (17βHSD) and hepatic 20α-hydroxysteroid dehydrogenase (AKR1C1) and cytochrome P450 3A4 (CYP3A4) were statistically different between the PCOS group and the controls (Fig. 2). These differences were driven by lower P4 levels for 3βHSD, CYP17A1, CYP11B, AKR1C1 and CYP3A4 activities, by higher T levels for 3βHSD and CYP19A1 and by higher 11KT levels for 17βHSD in PCOS patients. All other apparent enzyme ratios were not significantly different between the PCOS group and the controls (Table S2).

We were especially interested in differences in steroid metabolic pathways between the PCOS and control groups. To investigate this, we calculated product-to-substrate ratios which are representative of steroid metabolites in respective steroid metabolic pathways (Table 3). From our analysis, the backdoor pathway to AST and dihydrotestosterone (DHT) biosynthesis was significantly more active in PCOS women, signified by lower P4 levels and higher AST levels resulting in a higher ratio in our PCOS group compared to the controls. All other steroid metabolic pathways ratios were not statistically different between the PCOS group and the controls. Furthermore, while we also wished to include ratios for the C11-oxy backdoor pathways to 11KDHT (L. Barnard, Gent, van Rooyen, & Swart, 2017; D. van Rooyen, Gent, Barnard, & Swart, 2018; Desmaré van Rooyen, Yadav, Scott, & Swart, 2020), unfortunately the steroid intermediate metabolites in these pathways are not commercially available or were not detected in our cohort and these pathways could therefore not be fully investigated. When we however considered the ratios of 11KDHT levels over the levels of P4 and 17OHP4 or the levels of P4 and 11-ketoprogesterone (11KP4), these ratios were significantly different between our PCOS cohort and the controls ($p<0.05$). This result hints at the involvement of these pathways and that they should be investigated in future analyses.

Considering the sum of steroid classes (Fig. 3), while not significantly different, our PCOS cohort had lower mean z-scores for the C_{21} steroids, combined and when this class of steroids were divided into glucocorticoids (Fig.3A) and mineralocorticoids (Fig.3B). Glucocorticoids were also the dominant class of C_{21} steroids measured in our cohort, while mineralocorticoids and progesterones were measured at 8-fold lower levels. We similarly investigated the sum of C_{19} steroids in our PCOS group, and the z-scores were not significantly different in our PCOS group compared to our controls (Fig.3C). When this class of androgens are further divided into the classic C_{19} steroids and the C11-oxy C_{19} steroids (Fig.3D), the classic C_{19} steroids showed a lower mean z-score in the PCOS group, while the sum of the C11-oxy C_{19} steroids were similar in both groups. Our correlation heatmaps show stronger correlations in our PCOS group for androgen metabolites, while a large correlation cluster of androgen metabolites, including the C11-oxy androgens, together with progesterone metabolites, pregnenolone, 11-

deoxycortisol and 11-deoxycorticosterone were also identified in the PCOS group, which did not show such strong correlations in the control group (Fig. S3).

Finally, univariate and multivariate ROC analyses, sPLS-DA and hierarchical clustering analyses were performed with the steroid data in search for a classification model for PCOS against controls. However, all these analyses did not reveal a strong classifier that would suffice for an improved diagnostic test (data not shown). Furthermore, principal component analysis confirmed the importance of quantifying progesterone metabolites in PCOS patients, as P4, 20αOHP4 and dihydroprogesterone were identified as principal components in our dataset (Fig.S4). Finally, a summary of our results is depicted in Figure 4.

Discussion

We have investigated steroid levels and androgen metabolic pathways in PCOS and were focused on identifying the involvement of alternate androgen pathways in PCOS.

Increased levels of A4, AST, T and 11KT, together with decreased levels of P4 and 20αOHP4 in our cohort were in parallel with published literature. It is known that the classical androgen pathway comprising A4 and T, and the backdoor pathway (in this study represented by the metabolite AST) are linked to androgen excess in women with PCOS, thus leading to the hyperandrogenic characteristics of PCOS (Marti et al., 2017). Additionally, two more recently described alternate pathways of active androgen synthesis, the C11-oxy pathway and the C11-oxy C_{21} backdoor pathway potentially also play major roles in hyperandrogenism in PCOS (O'Reilly et al., 2017; Swart et al., 2021; Yoshida et al., 2018). The C11-oxy pathway and the C11-oxy C_{21} backdoor pathway are both androgenic pathways with 11-keto androgens as end products (L. Barnard, du Toit, & Swart, 2021). 11KT and 11KDHT are potent androgens with their androgenic activity comparable to T and DHT (Storbeck et al., 2013). Nevertheless, some early intermediates of the C11-oxy and C11-oxy C_{21} backdoor pathways including 11β-hydroxy5αDIONE, 11K5αDIONE, 11OHT and 11β-hydroxydihydrotestosterone also have concentration dependent partial or full androgenic activity (Bloem et al., 2015). Therefore, our data inform on the involvement of all pathways for producing the hyperandrogenic state observed in PCOS, with the backdoor pathway contributing significantly more when compared to healthy control women in our cohort.

In a previous study which profiled the steroid signature of PCOS from 24h urine, we found that androstanediol was increased in PCOS patients compared to controls, and this metabolite proved to be the best discriminative marker steroid of PCOS (Dhayat et al., 2018). This steroid is metabolised in the backdoor pathway to DHT. In this same study urinary AST and T were also increased, as was urinary 11β-hydroxyandrosterone, which highlights the involvement of

the C11-oxy pathway. Thus, all data match and underscore the role of the backdoor pathway and of 11-oxygenated androgens in PCOS.

The quantification of higher levels of circulating 11KT levels in our PCOS group, could be due to increased AKR1C3 mediated conversion of 11KA4 to 11KT by adipocytes (Fig.4), as our PCOS group measured at a higher BMI value (M. Barnard et al., 2018; Davio et al., 2020; Paulukinas, Mesaros, & Penning, 2022; Quinkler et al., 2004; Schiffer et al., 2023). Similar to 11KT, increased T levels measured in our PCOS cohort could also be due to AKR1C3-mediated adipocyte metabolism. Recent work further reported that serum C11-oxy androgens were unrelated to body fat distribution when normal-weight BMI-matched PCOS women were compared to healthy women, while serum total/free T and A4 levels were higher in PCOS women, concurrent with a greater android/gynoid fat mass ratio compared to controls (Dumesic et al., 2023). Notably, many research groups report increased androgen levels in non-obese PCOS women, and it is rather that the adipocytes are dysfunctional in some regard. It is clear that in future studies the role of adipose tissue in the development of hyperandrogenism in the PCOS phenotype will be important. Moreover, the subsequent interaction of increased sex-steroids on adipose tissue development will also warrant in-depth investigation as modulated adipocyte differentiation and function due to sex-steroids leads to dysfunctional metabolic and endocrine pathways in PCOS women (de Medeiros, Rodgers, & Norman, 2021; Manneras-Holm, Benrick, & Stener-Victorin, 2014).

Concerning the question of adrenal or ovarian origin of androgen excess in PCOS, the literature and our data suggest that both endocrine organs may contribute. Higher AMH levels measured in our cohort of PCOS women compared to controls (as in many other studies) show dysregulated ovarian cycling function, which in literature is also correlated to higher intrafollicular androgen levels (Bongrani et al., 2022). Nevertheless, higher A4, T and especially 11KT, which requires the involvement of CYP11B and 11β-hydroxysteroid dehydrogenase enzyme activities expressed in the adrenals but not ovaries, suggest the involvement of the adrenals followed by their peripheral conversion in, for example, adipose tissue (Paulukinas et al., 2022) (Fig.5).

Another characteristic of PCOS is the disruption of the menstrual cycle with constantly low P4 levels comparable to levels in control women in the follicular phase of a normal cycle (Andrieu et al., 2022; Schiffer et al., 2023). In line with that, we found significantly lower P4 levels in our PCOS cohort and decreased levels of 20αOHP4, the single step inactive metabolite of P4 (Beranic, Gobec, & Rizner, 2011) (Fig.5). This profile of low P4 with excess androgens seems very characteristic for PCOS.

In contrast to our previous study investigating 24h urinary steroid profiles of PCOS (Dhayat et al., 2018), we found no discriminating, diagnostic biomarker for PCOS compared to controls in their serum steroid profiles. The androgen composition of serum is active androgens and their precursors which can be a narrow and very fluctuating representation of the whole androgen pool compared to a 24h urine collection (Schiffer et al., 2019). However, a metabolite cluster analysis of the serum steroid data revealed specific correlations for PCOS compared to controls. In PCOS, androgens correlated more strongly, while progesterone metabolites had weaker correlations.

By contrast, combining steroid metabolomics and machine learning on a single serum sample has been shown to discriminate PCOS from non-classic 21-hydroxylase deficiency with 100% sensitivity and specificity, two entities which are clinically indistinguishable (Bachelot et al., 2023).

Our study has some limitations. Timing of blood sampling was unfortunately not fixed. Also, while the control group were close in age to the PCOS group, the age-difference was significant. Additionally, the control group was not cycled, nor could the PCOS group be accurately cycled to determine where each woman was in her menstrual cycle. In addition, as our PCOS group represents the classic phenotype of PCOS (including hirsutism, acne, infertility, menstrual irregularities and higher BMI) this study does not allow the identification of specific steroids related to only one physiological consequence of the syndrome, but rather provides a comprehensive steroid profile representing the whole PCOS phenotype. Finally, our pathway analysis is hindered by missing intermediate metabolite levels, as these steroids are not yet commercially available to be included in analytical methods.

PCOS is a complex, multifaceted disorder for which androgen excess is an important but evidently not a fully discriminating feature. Subtypes of the disorder have been phenotypically determined, but underlying pathomechanisms remain unsolved. Ongoing next-generation sequencing efforts reveal that PCOS is a highly heritable complex trait and that distinct etiologies of PCOS may deconstruct the syndrome in the near future providing a specific molecular diagnosis and precise targets for treatment (Dapas & Dunaif, 2022).

In conclusion, our findings show the involvement of alternate steroid pathways and androgen and progesterone metabolites in the clinical phenotype of hyperandrogenism in PCOS. Evaluation of the serum steroid profiles, androgenic pathways and steroidogenic enzyme activities showed that there is a shift towards the androgen backdoor pathway and the production of C11-oxy steroids in PCOS women, which may drive the clinical manifestations of the hyperandrogenic characteristics in PCOS.

Acknowledgements

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Figures and Tables:

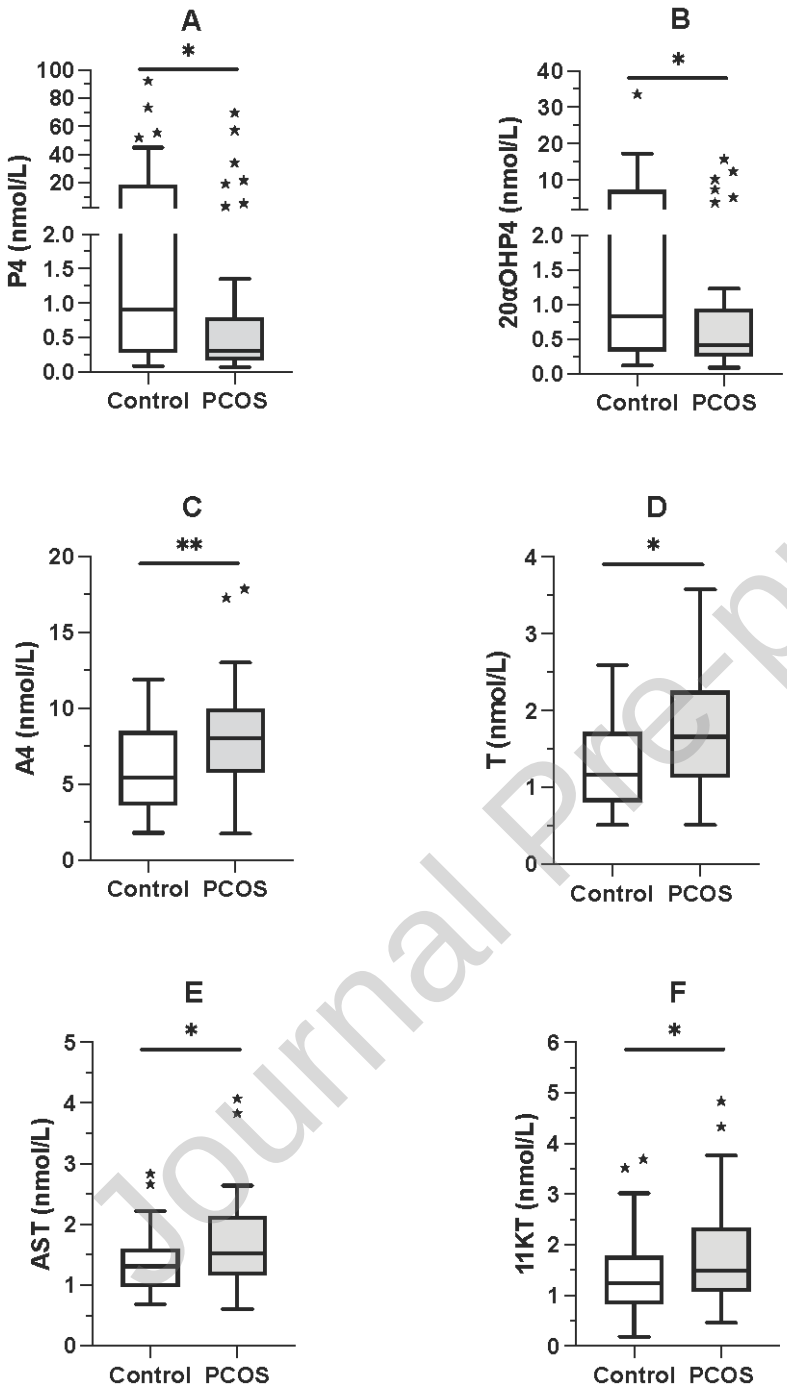

Figure 1. Serum steroid metabolite levels which were different in PCOS women (n=42) compared to control women (n=42). Data are shown as box plots with median values and 25%-75% ranges as indicated. Minimum and maximum values are indicated with T-bars. Statistical comparison was performed by a Mann-Whitney U test and p values are shown above the plots (*$p<0.05$; **$p<0.01$). A. P4; B. 20αOHP4; C. A4; D. T; E. AST and F. 11KT.

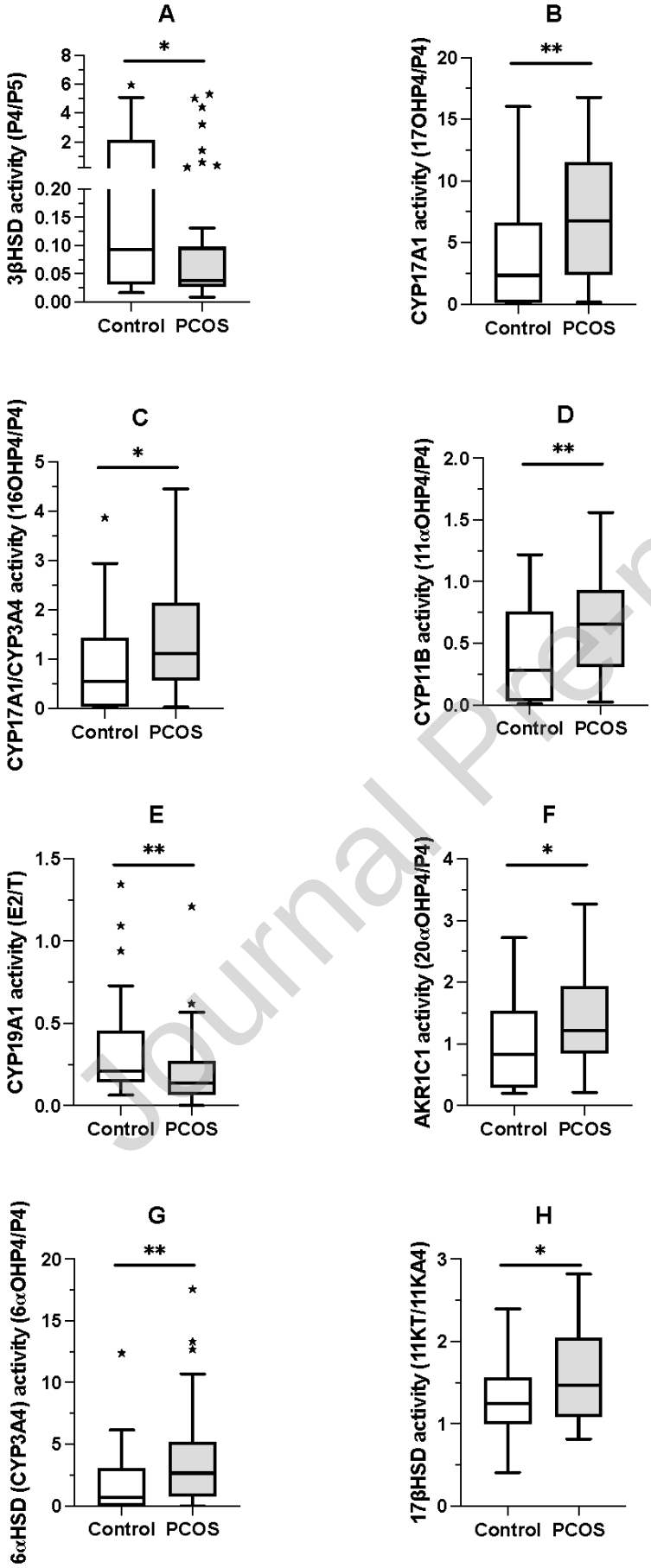

Figure 2. Apparent steroid enzyme activities in PCOS women (n=42) compared to control women (n=42). Activities were calculated as product-to-substrate ratios. Data are shown as box plots with median values and 25%-75% ranges as indicated. Minimum and maximum values are indicated with T-bars. Statistical comparison was performed by a Mann-Whitney test for all except for the AKR1C1 activity for which a Student's t-test was used and p values are shown above the plots A. 3βHSD activity (P4/P5); B. CYP17A1 activity (17OHP4/P4); C. CYP17A1/CYP3A4 activity (16OHP4/P4); D. CYP11B activity (11αOHP4/P4); E. CYP19A1 activity (E2/T); F. AKR1C1 activity (20αOHP4/P4); G. 6αHSD (CYP3A4) activity (6αOHP4/P4) and H. 17βHSD activity (11KT/11KA4).

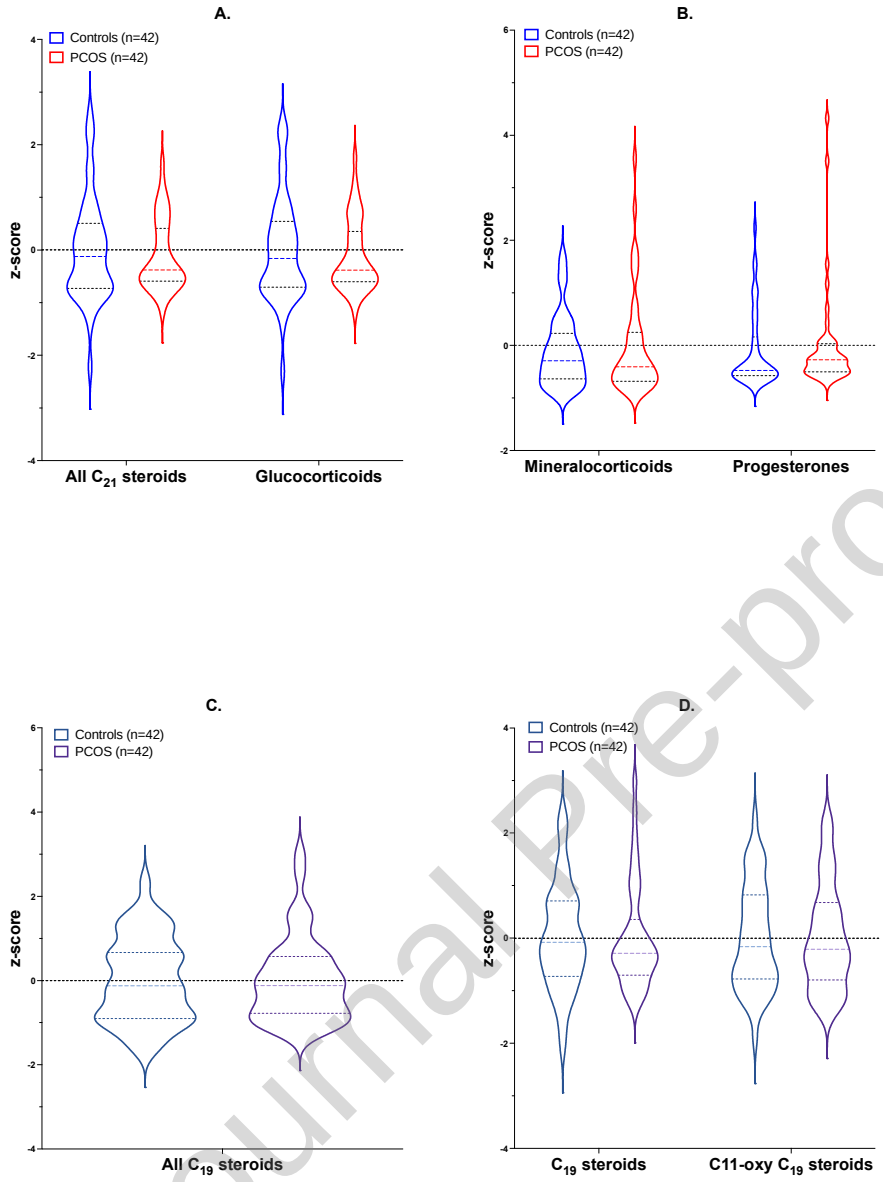

Figure 3. The concentration distribution of C_{21} steroids (A and B) and C_{19} steroids (C and D) quantified in PCOS patients compared to controls. A. The distribution of the sum of all the C_{21} steroids and glucocorticoid measurements; B. The distribution of the mineralocorticoid and progesterone measurements. Glucocorticoids (P5, P4, 17OHP4, S, F, E); mineralocorticoids (P5, P4, 11-DOC, CORT, ALDO); progesterones (P4 and its metabolites). C. The distribution of all the C_{19} steroids together, including DHEA-S measurements; D. the distribution of C_{19} steroids (excluding the DHEA-S measurements; including DHEA, A5, A4, T, DHT, AST, 5αDIONE, Etio) and the C11-oxy C_{19} steroids (11OHA4, 11OHT, 11KA4, 11KT, 11K5αDIONE, 11KDHT). Dotted lines within the violin plots represent the median, while the thinner dotted lines represent the 25th (bottom) and 75th (top) percentile of the measurement distribution.

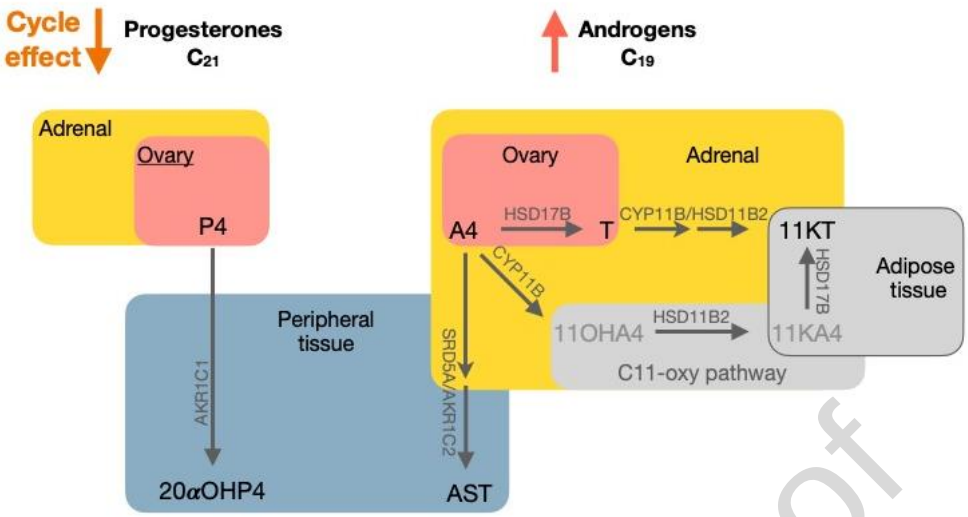

Figure 4. Schematic summary of hyperandrogenism in our PCOS cohort. Steroid metabolites in bold were statistically different between the PCOS group and the controls.

Table 1. Demographic characteristics of the cohort.

	Control (n=42)			PCOS (n=42)			
	Median	**Percentile 25th**	**75th**	**Median**	**Percentile 25th**	**75th**	**p Value**
Age (Years)	27,5	23,8	33,3	23	19	27,3	**<0,0001******
Height (cm)	163	159	165,3	163	158	165,3	ns
Weight (kg)	60	53	74,8	69,5	55,8	85,3	**<0,05***
BMI (kg/m^2)	22,6	19,9	28,7	28,2	22,6	35,5	**<0,05***
AMH (ng/mL)	4	2,1	6,1	6,8	3,9	11,9	**<0,01****
	% positive association			**% positive association**			
Obesity	8 (19%)			15 (35,7%)			ns
Menstrual Irregularities	11 (26,2%)			34 (81%)			**<0,0001******
Hirsutism	1 (2,4%)			26 (61,9%)			**<0,00001******
High Testosterone	0 (0%)			8 (19%)			**<0,01****
Acne	3 (7,1%)			20 (47,6%)			**<0,0001******
Infertility	10 (32,2%)			11 (68,7%)			**<0,001*****
Familial PCOS	2 (4,8%)			6 (14,3%)			ns
Familial Diabetes	15 (35,7%)			21 (50%)			ns

n = Number of samples, values marked with stars and in bold mark statistical significance (p<0.05), ns = not significant. Student's t-test was used to investigate differences between groups for age, height, weight and BMI. Mann-Whitney U test was used to test for a difference of serum AMH levels between groups. Chi-Square test was applied to test for differences between groups for obesity, menstrual irregularities, hirsutism, acne, infertility, and familial history of diabetes. Fisher's Exact test was applied to test for differences between groups for reported prior high testosterone measurements and familial history of PCOS.

Table 2. Serum steroid metabolites in nmol/L.

		Control (n=42)			PCOS (n=42)			
Steroid metabolite		**Median**	**Percentile 25th**	**75th**	**Median**	**Percentile 25th**	**75th**	**p Value**
Trivial name	**Abbreviation**							
Pregnenolone	P5	8,02	5,82	11,73	7,01	4,85	10,15	ns
Progesterone	P4	0,90	0,28	18,27	0,30	0,17	0,79	**<0,05 ***
17α-Hydroxypregnanolone	17OHTHP	*0,77*	*0,12*	*1,33*	*0,74*	*0,17*	*2,16*	ns
17α-Hydroxyprogesterone	17OHP4	2,39	1,43	4,48	2,23	1,44	3,76	ns
17α ,20α-Dihydroxyprogesterone	17α,20α-diOHP4	1,14	0,73	1,74	1,18	0,76	1,74	ns
11α-Hydroxyprogesterone	*11αOHP4*	*0,24*	*0,15*	*0,62*	*0,22*	*0,11*	*0,30*	*ns*
11-Ketoprogesterone	11KP4	0,13	0,09	0,16	0,14	0,11	0,24	ns
16α-Hydroxyprogesterone	16αOHP4	0,55	0,31	0,95	0,43	0,24	0,82	ns
5α/β-Dihydroprogesterone	*DHP4*	*0,69*	*0,36*	*3,35*	*0,49*	*0,18*	*1,30*	*ns*
20α-Hydroxyprogesterone	20αOHP4	0,82	0,33	7,26	0,41	0,26	0,94	**<0,05 ***
5α/β-Pregnan-3α-20α-diol	20αOHTHP	*3,38*	*2,04*	*4,75*	*3,77*	*2,04*	*5,50*	ns
6α-Hydroxyprogesterone	6αOHP4	0,63	0,40	1,05	0,77	0,49	1,26	ns
Pregnanetriol	*Ptriol*	*4,91*	*3,43*	*8,71*	*4,76*	*2,16*	*7,50*	*ns*
Androstenedione	A4	5,40	3,63	8,47	8,02	5,77	9,94	**<0,01 ** **
Dehydroepiandrosterone	DHEA	18,49	10,63	25,92	21,39	13,76	30,11	ns
Dehydroepiandrosterone sulfate	DHEA-S	5512,51	3458,93	7678,39	6945,64	4196,05	9800,92	ns
Androstenediol	A5	*10,24*	*7,42*	*13,58*	*9,01*	*7,19*	*12,37*	ns
Testosterone	T	1,16	0,81	1,71	1,65	1,12	2,25	**<0,05 ***
Dihydrotestosterone	DHT	0,42	0,28	0,62	0,50	0,32	0,84	ns
Androsterone	AST	1,30	0,97	1,59	1,51	1,16	2,13	**<0,05 ***

11-Ketoandrostanolone	11KDHT	1,90	1,15	2,59	2,17	1,43	3,07	ns
11β-Hydroxyandrostenedione	11OHA4	5,38	3,75	8,21	6,36	4,15	8,95	ns
11-Ketoandrostenedione	11KA4	0,98	0,74	1,37	1,01	0,79	1,44	ns
11β-Hydroxytestosterone	11OHT	0,41	0,24	0,55	0,46	0,30	0,65	ns
11-Ketotestosterone	11KT	1,23	0,82	1,77	1,49	1,08	2,33	**<0,05 ***
5α-androstane-3,17-dione	5αDIONE	*0,50*	*0,19*	*0,78*	*0,54*	*0,15*	*0,95*	ns
5α-Androstanetrione	11K5αDIONE	49,30	34,43	75,46	58,26	38,24	82,24	ns
Corticosterone	CORT	9,40	4,97	16,41	7,65	4,51	20,24	ns
11-Deoxycorticosterone	11-DOC	0,17	0,07	0,25	0,11	0,06	0,18	ns
Cortisol	F	304,83	223,96	438,76	280,76	212,79	466,19	ns
11-Deoxycortisol	S	0,89	0,39	1,47	0,90	0,46	1,45	ns
Cortisone	E	58,55	47,20	74,51	59,63	44,01	72,93	ns
Aldosterone	ALDO	0,32	0,17	0,51	0,33	0,15	0,52	ns
Estradiol	E2	0,27	0,16	0,49	0,19	0,10	0,45	ns

n= Number of samples, values marked with stars and bold are showing statistically significance ($p<0.05$, FDR<0.05), ns = not significant. Mann-Whitney U test was used to calculate the difference of all metabolites between the groups, except for cortisone for which a Student's t-test was used to calculate the difference between the groups. Values in italics represent steroid measurements >LOD and <LOQ (Table S1).

Table 3. **Investigated steroid metabolic pathways.**

		Control (n=42)			PCOS (n=42)			
Steroid metabolic pathways	**Product-to-substrate ratios**	**Median**	**Percentile 25th**	**75th**	**Median**	**Percentile 25th**	**75th**	**p Value**
Adrenal mineralocorticoid pathway	ALDO/(P5+P4+11-DOC+CORT)	0,01	0,01	0,02	0,02	0,01	0,03	ns
Adrenal glucocorticoid pathway	F/(17OHP4+S)	86,24	50,26	148,41	93,77	68,94	154,25	ns
Alternative pathway to DHT	DHT/(DHEA+A4+5αDIONE)	0,02	0,01	0,03	0,02	0,01	0,02	ns
Classic pathway to DHT	DHT/(DHEA+A4+T)	0,02	0,01	0,02	0,02	0,01	0,02	ns
Backdoor pathway to AST and DHT	(AST+DHT)/(P4+17OHP4+DHP4)	0.41	0.08	0.73	0.70	0.48	0.94	**<0,01****
C11-oxy pathway to 11KT	11KT/(11OHA4+11OHT+11KA4)	0,18	0,14	0,22	0,19	0,16	0,27	ns
C11-oxy pathway to 11KDHT	11KDHT/(11OHA4+11OHT+11KA4+11KT)	0,23	0,19	0,27	0,22	0,18	0,26	ns
C11-oxy pathway to 11KT (including C_{19} precursors)	11KT/(DHEA+A4+T+11OHA4+11OHT+11KA4+11KT)	0,04	0,03	0,05	0,04	0,03	0,06	ns
C11-oxy pathway to 11KDHT (including C_{19} precursors)	11KDHT/(DHEA+A4+T+11OHA4+11OHT+11KA4+11KT)	0,06	0,04	0,07	0,05	0,04	0,07	ns

Steroid metabolic pathways in PCOS women and in controls, depicted as ratios calculated from steroid precursors serving as the entry points into these pathways over steroid end-products of these pathways. Differences between our groups were analyzed using the Mann-Whitney U test for all except for the backdoor pathway to AST and DHT ratio which was analyzed using the Student's t-test. n= Number of samples, values marked with stars and bold are showing statistically significance (p<0.05), ns = not significant.

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EMA Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - Original Draft, Funding acquisition

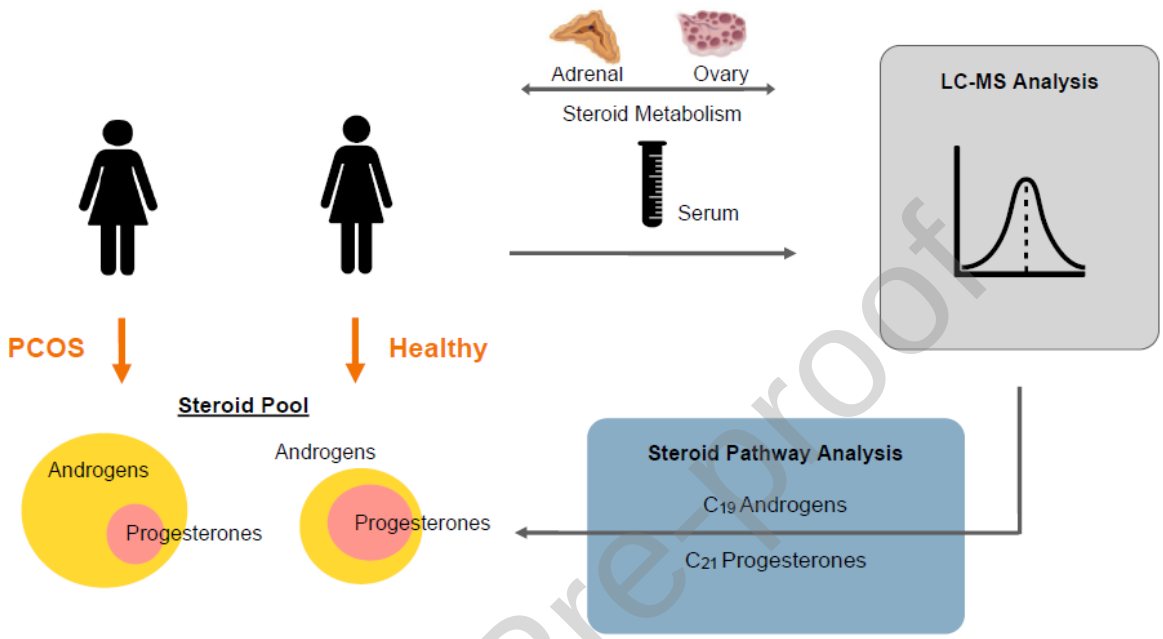
TdT Methodology, Validation, Formal analysis, Visualization, Writing - Original Draft, Funding acquisition

OS Investigation, Resources

RA Investigation, Resources

MG Investigation, Resources, Formal analysis,

CEF Conceptualization, Methodology, Validation, Formal analysis, Writing - Review & Editing, Supervision, Project administration, Funding acquisition



Highlights

- Classical androgen levels, androstenedione and testosterone, were higher in PCOS
- The downstream androgen metabolite, androsterone, was higher in PCOS
- 11-Ketotestosterone, produced in the C11-oxy androgen pathway, was higher in PCOS
- Active androgen metabolic pathways were identified in PCOS
- Steroid pathway analysis hints at the involvement of even more androgen pathways