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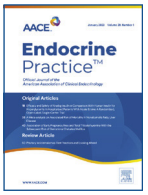

Original Article

Discriminatory Value of Steroid Hormones on Polycystic Ovary Syndrome and Clustering of Hyperandrogenism and Metabolic Factors

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ABSTRACT

Objective: We determined (1) if 11-oxygenated androgens better identify polycystic ovary syndrome (PCOS) diagnosis in women with obesity compared to total or free testosterone (T) and free androgen index; (2) how biochemical hyperandrogenism and metabolic factors cluster in a cohort of women with infertility and obesity.

Methods: Women with obesity and PCOS comprised the study group ($N = 132$). Ovulatory women with obesity and idiopathic, tubal or male factor infertility were the control group ($N = 83$). Steroid hormones were measured by means of liquid chromatography tandem mass spectrometry. Receiver operating characteristic curves and principal component analysis were used.

Results: Women with obesity and PCOS had higher 11-ketotestosterone (11 KT) (1.22 nmol/L [0.84; 1.65] vs 1.05 [0.78; 1.35], $P = .04$) compared to controls, but not 11β-hydroxyandrostenedione 4.30 [2.87; 5.92] vs 4.06 [3.22; 5.73], $P = .44$). 11-ketotestosterone (area under the curve: 0.59) did not better discriminate PCOS in women with obesity compared to: total T (0.84), free T (0.91), and free androgen index (0.85). We identified 4 principal components (PCs) in the PCOS group (72.1% explained variance): (1) insulin resistance status; (2) blood pressure; (3) obesity; (4) androgen status and 4 PCs in the control group (68.7% explained variance) with variables representing metabolism being dispersed in component 2, 3, and 4.

Conclusions: Eleven-oxygenated androgens do not aid in the diagnosis of PCOS in women with obesity. Insulin resistance is the strongest PC in the PCOS group. There is no major dominant characteristic that defines obese non-PCOS women.

Introduction

Polycystic ovary syndrome (PCOS) is a common cause of infertility, with hyperandrogenism as one of the defining features.[1] Generally, it affects up to 20% of women in reproductive age worldwide.[1,2] There is a bidirectional interaction between PCOS and obesity,[3] supported by a Mendelian randomization study demonstrating that high bioavailable serum testosterone (T) levels in woman may be both a cause and consequence of obesity.[4] The mechanism behind this is far from clear. Obesity in women with PCOS might be driven by a decreased postprandial thermogenesis and an impaired regulation of gut hormones that control

Abbreviations: A4, androstenedione; AUC, area under the curve; BMI, body mass index; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; FAI, free androgen index; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; 11 KT, 11-ketotestosterone; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLE, liquid-liquid extraction; 11OHA4, 11β-hydroxyandrostenedione; PCA, principal component analysis; P, principal component; PCOS, polycystic ovary syndrome; ROC, receiver operating characteristic; SHBG, sex hormone binding globulin; T, testosterone.

The study was registered in the Netherlands Trial Registry (NTR 1530).

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appetite.[5,6] Obesity can exacerbate hyperandrogenism by enhancing adverse metabolic effects, such as insulin resistance, metabolic syndrome, and dyslipidemia.[7] In turn, hyperinsulinemia in women with PCOS stimulates ovarian androgen synthesis and reduces sex hormone binding globulin (SHBG) production in the liver, resulting in an increase in free and/or bioavailable androgen levels,[8,9] leading to clinical signs of hyperandrogenism.

Serum total or free T, followed by free androgen index (FAI), and dehydroepiandrosterone sulfate (DHEA-S) are the most widely used biomarkers for the diagnosis of PCOS.[10] However, recent research has shown that the diagnosis of biochemical hyperandrogenism should not be exclusively based on the quantification of just 1 androgenic steroid hormone but rather the quantification of several steroid hormones simultaneously.[11,12] Moreover, the focus has shifted to include the analysis of 11-oxygenated C19 steroids, originating from the adrenal gland, as it has been demonstrated that 11-oxygenated androgens represent the majority of circulating androgens in PCOS.[13,14] Of the 11-oxygenated C19 steroids, 11-ketotestosterone (11 KT) is the most clinically relevant and active steroid hormone since it activates the androgen receptor in a similar manner to T .[13,14] 11β-hydroxyandrostenedione (11OHA4) is a major product of adrenal steroidogenesis, the second most abundant C19 steroid after DHEA-S. It is produced by zona reticularis of the adrenal glands and the precursor for the active steroids 11 KT.[12]

The complexity and heterogeneity of PCOS explains the existence of several different diagnostic criteria such as National Institutes of Health Criteria, Rotterdam criteria, and Androgen Excess Society Criteria.[15] Although adiposity and metabolic factors do not form any part of the accepted diagnostic criteria of PCOS, they may be clinically important in identifying etiological background in PCOS. Principal component analysis (PCA) is a technique that objectively reflects the clustering of variables in a given population, allowing us to investigate the main components (principal components [PCs]) of the variables included in the model, which may imply different etiological factors.[16] Data from women with PCOS have been reported by means of PCA in previous studies. Stuckey et al[17] clustered metabolic and cardiovascular risk factors in 378 women with PCOS with a broad range of body mass index (BMI) level. They identified that insulin resistance constitutes the main component, explaining 37% of variance within the population. In another cohort with young women with PCOS, in line with Stuckey et al's study, "insulin resistance and adiposity" is the first component.[18] The results of both studies imply that insulin resistance to be the central etiological factor in PCOS. However, obesity, which is common in PCOS, complicates the interpretation of results as it is difficult to distinguish between the effects of obesity or effects of PCOS independently. Moreover, these 2 studies did not examine the PCs of a control group (women without PCOS). Thus, whether the above-mentioned findings are relevant in ovulatory women with infertility and obesity is unclear.

In this study, we aim to explore if 11-oxygenated androgens derived from adrenal better identify PCOS diagnosis compared total T, free T, and FAI measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) in a cohort of women with infertility and obesity. Further, we identify and compare clusters of characteristics of women with obesity and infertility with and without PCOS to elucidate the involvement of hyperandrogenism and metabolic factors in the pathogenesis of PCOS by means of PCA.

Materials and Methods

Study Population

We used the baseline data and samples originating from a multicenter randomized controlled trial that examined whether a 6-month lifestyle intervention prior to infertility treatment in women with obesity improved live birth rate, compared to first-line initiation of infertility treatment. In total, 577 infertile women with obesity were randomized between 2009 and 2012. The inclusion criteria for the infertile women were being between 18 and 39 years of age with a BMI $\geq$29 kg/m^2. Infertility was defined as unsuccessful conception for at least 12 months. Women with severe endometriosis, premature ovarian failure, endocrinopathy, untreated preexisting hypertension, or women with hypertension-related complications in previous pregnancy were excluded. The study was conducted according to the Declaration of Helsinki and approved by the medical ethics committee of the University Medical Center Groningen (METc code: 2008/284), in combination with approval of the board of directors from the other participating hospitals. All women included in the study provided written informed consent. The study was registered in the Netherlands Trial Registry (NTR 1530). The study protocol and main outcomes, including cardiometabolic outcomes, have been published previously.[19-21]

For this study, women with PCOS (based on the Rotterdam criteria[22]) comprised the study group. Ovulatory women with idiopathic, tubal or male factor infertility were the control group. Due to financial constraints, we decided to include a maximum number of controls in this study. From the group of women with unexplained infertility samples were randomly selected, and from the group of women with male or tubal infertility all samples were initially included.

Study Procedures (Baseline Characteristics)

Weight (kg), height (cm), waist circumference (cm), hip circumference (cm), and systolic and diastolic blood pressure (mmHg) were measured by trained research nurses at baseline. Fasting blood samples were obtained through venipunctures into 1 serum and 1 sodium fluoride vacutainer tube. Serum samples were kept at room temperature for a minimum of 30 minutes to allow for coagulation. Afterward, it was centrifuged at 1700*g* for 10 minutes at 4 °C to obtain serum and plasma, which were stored at −80 °C. The steroid hormones progesterone, 17-hydroxyprogesterone, androstenedione (A4), total T, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), and DHEA-S were measured using LC-MS/MS.[23] The method was slightly adjusted to include DHEA and DHEA-S in the analysis. Changes included the use of ammonium fluoride in the solvent and a different analytical column (Luna Omega C18, 2.1 × 100 mm 1.6 μm, Phenomenex). 11OHA4

Highlights

- Eleven-oxygenated androgens do not discriminate polycystic ovary syndrome (PCOS).
- Insulin resistance is the strongest principle component in the PCOS group.
- There is no major dominant characteristic that defines obese non-PCOS women.

Clinical Relevance

Insulin resistance is the first component in women with polycystic ovary syndrome (PCOS) and obesity. This underlines the importance of a broad range of therapeutic interventions to reduce insulin resistance in order to improve general health in women with PCOS and obesity.

and 11 KT were analyzed using liquid-liquid extraction (LLE) followed by LC-MS/MS.[14] The use of LLE is instead of supported liquid extraction as mentioned in the paper; however, both LLE and supported liquid extraction had transferable assay performance characteristics. An additional change was the use of a D_4-11OHA4 internal standard, instead of a D_7-11OHA4 internal standard (Isosciences). High-sensitivity C-reactive protein was measured with an immuno-turbidimetric assay (catalog number 04956923190). Triglycerides (catalog number 11730711216), total cholesterol (catalog number 11491458216), high-density lipoprotein cholesterol (HDL-C) (catalog number 04713214190) and low-density lipoprotein cholesterol (catalog number 04711220190) concentrations were measured using (enzymatic) colorimetric assays. Fasting plasma glucose was measured with an enzymatic UV test (hexokinase method). All previously described assays were produced by Roche Modular P or COBAS 6000 (Roche, Manheim). Insulin and SHBG were measured with the Architect manufactured by Abbott Diagnostics (reagent kit 8K41-27), using a chemiluminescent micro particle immunoassay. Insulin resistance was quantified using the homeostatic model assessment of insulin resistance (HOMA-IR). The HOMA-IR was calculated by multiplying the concentration of fasting insulin (μU/L) with the concentration of fasting glucose (mmol/L) divided by 22.5.[24] Free T was calculated applying the formula of Vermeulen et al, making use of the total T, albumin and SHBG levels.[25] The FAI was calculated by dividing the total T level by the SHBG level and multiplying this by 100. The presence of metabolic syndrome was assessed if the women met at least 3 of the following criteria: glucose ≥5.6 mmol/L, HDL-C <1.3 mmol/L, triglycerides ≥1.7 mmol/L, waist circumference ≥ 88 cm or blood pressure ≥ 130/85 mmHg, based on the criteria of the National Cholesterol Education Program Adult Treatment Panel III.[26]

Statistical Analysis

The data are presented as mean ± SD, as median [IQR] or as number (percentage). Normal distribution was tested with histograms combined with the Kolmogorov-Smirnov test. The baseline characteristics were compared between women with PCOS and the controls. Normal distributed data was compared using Student *t* test and non-normal distributed data was analyzed using Mann-Whitney *U* Test.

Those steroid hormones (ratios) which showed statistically significant differences between the PCOS group and the control group were examined for their discriminatory value based on receiver operating characteristic (ROC) curves. Sensitivity was plotted against (1-specificity) at each level, and area under the curve (AUC) was computed. The discriminatory performance was classified according to AUC values.[27] AUC values closest to 1 indicate a very good discriminatory ability, with a very small portion of overlapping values between the groups and, thus, low false positive and false negative ratios. AUC values ≥0.80 indicate a good discriminating performance. AUC values between 0.70 and 0.79 indicate fair discriminating performance, and AUC values ≤0.69 indicate poor or no discriminating ability. Among the markers with crude AUC values ≥0.70, we further adjusted for confounders,[28] identified as those showing statistically significant demographic differences between groups. The incremental value of the marker in addition to the confounder were calculated and presented.

PCA was performed to determine the PCs. All factors with eigenvalues exceeding 1 were retained for analysis. Varimax rotation was used to produce interpretable factors. Variables with the coefficient ≥0.5 were regarded as influential within each PC. PCs are ranked according to their explained variance, so that the first component always has the highest explained variance. Missing values of variables were handled with pairwise deletion. Variables for inclusion were carefully chosen, given the small sample size, to ensure parsimony of the final model. Variable BMI (adiposity) was included. T and FAI are 2 variables with high discriminatory value in PCOS diagnosis in this population and thus were selected to represent hyperandrogenism. Variables to represent metabolic factors were arbitrarily selected based on the basic features of the metabolic syndrome (2001 revised criteria of the National Cholesterol Education Program Adult Treatment Panel III[26]) including waist circumference; blood pressure; glucose; HDL-C and triglycerides. In addition, HOMA-IR was also included as an important indicator of insulin resistance. Associations between the considered variables were calculated by Pearson correlation coefficients.

Statistical analyses were performed with SPSS statistics version 27.0 (IBM). A P value ≤ .05 was considered statistically significant.

Results

The flowchart of the selection of current study groups is shown in Fig. 1. In total, 574 women were available for the analysis. At the randomization, 201 women were diagnosed as PCOS based on the Rotterdam criteria. The numbers of infertile couples with unexplained, male factor and tubal factor were 163, 118, and 25, respectively. The financial constraints or exclusion of missing blood samples or when blood sampling took place in the luteal phase led to 132 women in the PCOS group and 83 in the ovulatory control group.

Clinical and metabolic characteristics of women with PCOS and obesity and non-PCOS obese ovulatory controls are shown in Table 1. The mean age was 28.1 years in the PCOS group and 30.9 years in the control group (P < .001) and was corrected in the ROC analysis. BMI was not significantly different (36.0 ± 3.2 vs 35.7 ± 3.5, P = .51) and waist-hip ratio was statistically significant higher in the PCOS group than the ovulatory control group

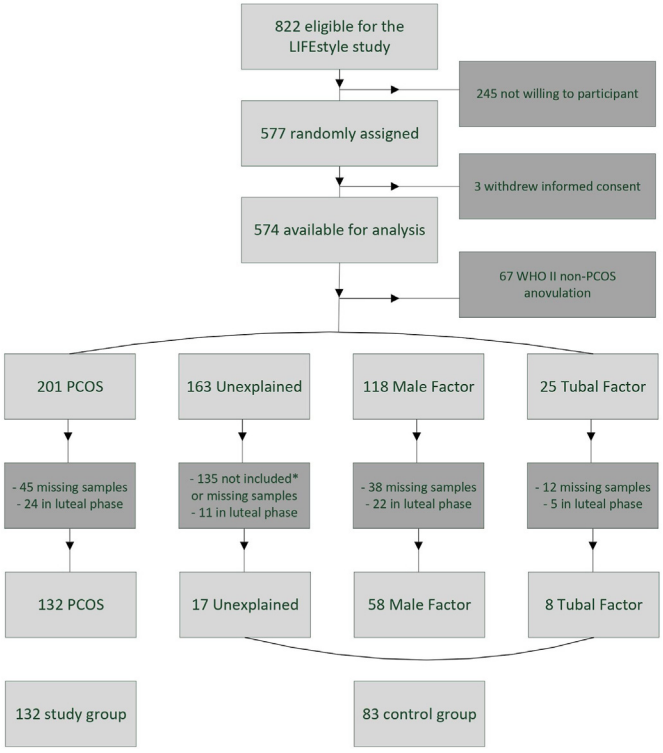

Fig. 1. Flowchart of the study. Women were excluded from the study when blood sampling took place in the luteal phase. *Financial constraints limited the number of samples. *PCOS* = polycystic ovary syndrome; *WHO* = World Health Organization.

Table 1
Clinical and Metabolic Characteristics of Women With Polycystic Ovary Syndrome and Obesity and Nonpolycystic Ovary Syndrome Obese Controls

Parameters	PCOS (n = 132)	Controls (n = 83)	P value
Demographics			
Age (y)	28.1 ± 4.3	30.9 ± 4.2	<.001
Weight (kg)	103.7 ± 12.7	101.6 ± 13.2	.24
BMI	36.0 ± 3.2	35.7 ± 3.5	.51
Waist circumference (cm)	108.7 ± 9.1	106.3 ± 9.3	.06
Hip circumference (cm)	124.2 ± 8.8	124.3 ± 9.2	.98
Waist-hip ratio	0.88 ± 0.07	0.86 ± 0.06	.02
Systolic blood pressure (mmHg)	125 (116.5; 134)	125 (120; 135)	.25
Diastolic blood pressure (mmHg)	80 (74; 85)	80 (75; 87)	.07
Current smoker	34 (26%)	25 (30%)	.51
Hirsutism	29 (24%)	9 (11%)	.02
Acne	17 (14%)	4 (5%)	.04
Metabolic measures			
hs-CRP (mg/L)	4.7 (2.2; 8.7)	4.0 (1.9; 7.6)	.25
Triglycerides (mmol/L)	1.2 (0.9; 1.7)	1.1 (0.8; 1.6)	.93
Total cholesterol (mmol/L)	4.8 ± 1.0	4.8 ± 0.9	.77
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.3	.57
LDL-C (mmol/L)	3.1 ± 0.8	3.1 ± 0.8	.84
Glucose (mmol/L)	5.4 ± 0.7	5.4 ± 0.7	.43
Insulin (pmol/L)	102 (77.1; 136)	72.2 (56.9; 106)	<.001
HOMA-IR	3.3 (2.5; 4.6)	2.5 (1.9; 3.6)	<.001
Metabolic syndrome	66 (55%)	49 (62%)	.33
Serum hormone measures			
SHBG (nmol/L)	26.9 (18.8; 38.9)	31.2 (25.0; 45.8)	.005
P (nmol/L)	0.24 (0.16; 0.42)	0.33 (0.17; 0.89)	.02
17-OHP (nmol/L)	1.64 (1.19; 2.34)	1.03 (0.67; 1.82)	<.001
A4 (nmol/L)	7.21 ± 2.81	4.45 ± 1.83	<.001
Total T (nmol/L)	1.66 (1.30; 2.20)	0.94 (0.73; 1.27)	<.001
DHT (nmol/L)	0.33 ± 0.19	0.28 ± 0.15	.047
DHEA (nmol/L)	17.6 (11.1; 25.9)	15.2 (10.5; 20.4)	.40
DHEA-S (nmol/L)	4.88 (3.46; 6.57)	5.38 (3.81; 6.69)	.29
11 KT (nmol/L)	1.22 (0.84; 1.65)	1.05 (0.78; 1.35)	.04
11OHA4 (nmol/L)	4.30 (2.87; 5.92)	4.06 (3.22; 5.73)	.44
Free T (nmol/L)	0.035 (0.028; 0.046)	0.016 (0.013; 0.020)	<.001
FAI	6.45 (4.67; 8.79)	2.95 (2.10; 4.22)	<.001
T/DHT ratio	5.72 (4.41; 7.56)	3.78 (2.79; 5.86)	<.001
T/A4 ratio	0.26 ± 0.07	0.24 ± 0.07	.047
A4/17-OHP ratio	4.14 ± 1.36	3.92 ± 1.55	.28
DHEA-S/DHEA ratio	0.29 (0.21; 0.40)	0.30 (0.22; 0.45)	.18

Abbreviations: A4 = androstenedione; BMI = body mass index; DHEA = dehydroepiandrosterone; DHEA-S = dehydroepiandrosterone sulfate; DHT = dihydrotestosterone; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; hs-CRP = high-sensitivity C-reactive protein; 11 KT = 11-ketotestosterone; LDL-C = low-density lipoprotein cholesterol; 11OHA4 = 11β-hydroxyandrostenedione; 17-OHP = 17-hydroxyprogesterone; PCOS = polycystic ovary syndrome; SHBG = sex hormone binding globulin; T = testosterone.
The baseline characteristics were compared between women with PCOS and the controls. Normal distributed data was compared using Student t test and non-normal distributed data was analyzed using Mann-Whitney U Test.

(0.88 ± 0.07 vs 0.86 ± 0.06, P = .02). As for metabolic measures, insulin and HOMA-IR were statistically significantly higher in the PCOS group than the ovulatory control group. There were no statistically significant differences in other parameters. Among the tested steroid hormones and their ratios, SHBG, A4, total and free T, DHT, 11 KT, FAI, T/DHT ratio, and T/A4 ratio showed statistically significant differences between the PCOS and the ovulatory control group. DHEA, DHEA-S, 11OHA4 did not differ between the groups and thus we did not test their discriminatory value with ROC curves. The comparisons of baseline characteristics between women included in the analysis and women not included, separately for PCOS and non-PCOS are shown in Supplementary Table 1.[29] There were no statistically significant differences between women included and not included in the current analysis.

The crude AUCs for representing PCOS diagnosis of free T (0.91), FAI (0.85), T (0.84), A4 (0.81), T/DHT ratio (0.70), SHBG (0.62), T/A4 ratio (0.58), and DHT (0.57) respectively in ROC analysis are shown in Table 2. 11 KT (0.59) does not have a promising discriminatory

Table 2
Discriminatory Value of Serum Hormone Measures on Polycystic Ovary Syndrome Diagnosis With Receiver Operating Characteristic Curves Analyses

Parameters	AUC	95% CI	P value
SHBG	0.62	0.54-0.69	.005
P	0.59	0.51-0.68	.02
17-OH progesterone	0.67	0.59-0.75	<.001
A4	0.81	0.75-0.87	<.001
Total T	0.84	0.79-0.89	<.001
DHT	0.57	0.49-0.65	.08
11 KT	0.59	0.51-0.67	.04
Free T	0.91	0.85-0.96	<.001
FAI	0.85	0.79-0.90	<.001
T/DHT ratio	0.70	0.63-0.78	<.001
T/A4 ratio	0.58	0.51-0.66	.04

Abbreviations: A4 = androstenedione; AUC = area under the curve; DHT = dihydrotestosterone; FAI = free androgen index; 11 KT = 11-ketotestosterone; 17-OHP = 17-hydroxyprogesterone; P = progesterone; SHBG = sex hormone binding globulin; T = testosterone.

value for PCOS diagnosis. Among the markers with crude AUC values ≥0.70, the incremental value of the marker A4 (AUC difference: 0.14, 95% CI: 0.07-0.21), total T (0.18, 95% CI: 0.10-0.25), free T (0.21, 95% CI: 0.11-0.32), and FAI (0.18, 95% CI: 0.10-0.25) in addition to age (confounder) showed significant improvement, but T/DHT ratio (0.02, 95% CI: −0.01 to 0.04) did not (Fig. 2).

With respect to the PCA results, we identified four PCs in the PCOS group (Table 3). In the first component, the dominant variables were triglycerides, HOMA-IR, glucose, and HDL-C, explaining 30.2% of variance. We may call it "insulin resistance" component. The second component ("blood pressure") contained systolic and diastolic blood pressure (17.2% explained variance). Component 3 ("obesity and visceral adiposity"): BMI and waist circumference (14.1% explained variance). Component 4 ("biochemical hyperandrogenism"): T and FAI (10.6% explained variance). These components together explain 72.1% of variance in the PCOS group. The PCA results of the ovulatory obese control group are shown in Table 4. The first component contained T and FAI (23.2% explained variance). Component 2: systolic and diastolic blood pressure and HOMA-IR (18.5% explained variance). Component 3: BMI and waist circumference (15.6% explained variance). Component 4 contained triglycerides and HDL-C (11.4% explained variance). These components describe in 68.7% the variance in the ovulatory obese control group. Fig. 3 is the visualization of PCA results in both groups, which depicts the influence of each variable within each PC either as a positive or negative influence. Supplementary Table 2 and Table 3,[29] show the correlation matrix for the variables used in the analyses, in the PCOS group and the ovulatory control group, respectively.

Discussion

In this post hoc cross-sectional analysis, we investigated the discriminatory value of various androgen steroid hormones secreted by both the ovaries and adrenal glands and their ratios for the diagnosis of PCOS in women with infertility and obesity. We found 11-oxygenated androgens derived from adrenal do not serve as promising discriminatory biomarkers for PCOS in women with obesity. We further examined specific clusters of the well-known diagnostic and associated variables for adiposity, biochemical hyperandrogenism, and metabolic syndrome in this cohort. We have identified four PCs explaining 72.1% of variance within the PCOS and four PCs explaining 68.7% of variance within the ovulatory obese women.

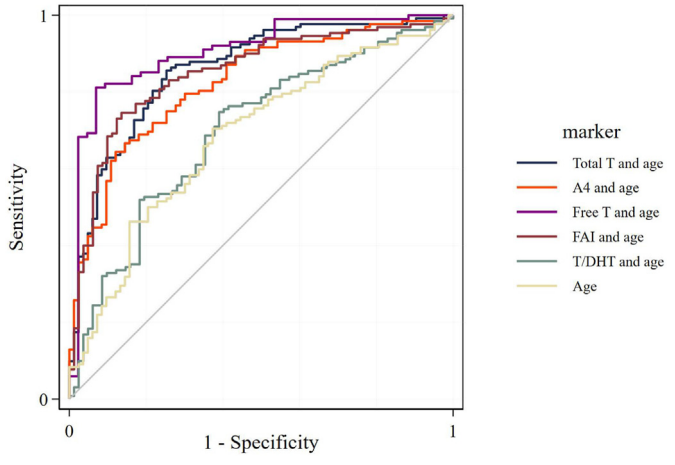

Fig. 2. Assessment of the discriminatory performance of androstenedione, total and free testosterone, free androgen index, and testosterone/dihydrotestosterone ratio in addition to age using receiver operating characteristic curves. *A4* = androstenedione; *DHT* = dihydrotestosterone; *FAI* = free androgen index; *T* = testosterone.

Table 3
Variable Loadings and Percent of Variance Explained by Each Principal Component in the Polycystic Ovary Syndrome Group

Parameters	First PC (30.2%)	Second PC (17.2%)	Third PC (14.1%)	Fourth PC (10.6%)
T	0.015	−0.014	0.088	**0.862**
FAI	0.294	−0.136	0.057	**0.76**
BMI	−0.028	0.103	**0.901**	0.013
Waist circumference	0.245	−0.075	**0.824**	0.15
Triglycerides	**0.849**	−0.15	0.064	0.001
HDL-C	**−0.637**	−0.077	−0.07	−0.077
Glucose	**0.812**	0.039	−0.053	0.098
HOMA-IR	**0.753**	−0.017	0.277	0.274
Systolic blood pressure	0.02	**0.884**	0.041	−0.092
Diastolic blood pressure	−0.023	**0.912**	−0.005	−0.038

Abbreviations: BMI = body mass index; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; PC = principal component; T = testosterone.
Variables with an absolute value of at least 0.5 are shown in bold. Positive signs indicate that higher values of the variable are influential in the component whilst negative signs indicate the influence of lower values.

The research conducted on the role of 11-oxygenated androgens in the diagnosis of PCOS has not yet yielded a consensus.[13,30,31] The variations in PCOS phenotypes, diverse populations, and wide range of BMI levels could potentially account for these discrepancies, although the exact mechanism behind these conflicting findings remains unclear. In our cohort consisting solely of women with obesity, both 11 KT and 11OHA4 do not show promise as markers for diagnosing PCOS. Whether this accounts for various PCOS phenotypes[32,33] needs to be established, since we did not phenotype the PCOS in this respect. It is worth noting that 11-oxgenated steroids levels in our study are lower than those reported by others.[13,31] The most likely explanation for this deviation is the use of different analytical methods and difference in calibration. Although both studies used LC-MS/MS to analyze the steroids, there can still be considerable difference between LC-MS/MS methods. We call for interlaboratory studies to try and address issues of assay standardization, particularly for the 11-oxygenated steroids. In addition, there is still a great deal of controversy as to whether the ovary or the adrenal are the main site of production resulting in hyperandrogenism in PCOS.[34] Our findings show no significant difference in DHEAS, DHEA, or 11OHA4 derived from adrenal between PCOS and controls imply that the adrenal might

Table 4
Variable Loadings and Percent of Variance Explained by Each Principal Component in the Control Group

Parameters	First PC (23.2%)	Second PC (18.5%)	Third PC (15.6%)	Fourth PC (11.4%)
T	**0.879**	0.072	−0.078	0.037
FAI	**0.848**	0.047	0.156	0.216
BMI	0.048	0.054	**0.906**	−0.025
Waist circumference	−0.002	0.22	**0.892**	0.06
Triglycerides	0.211	0.129	−0.016	**0.777**
HDL-C	−0.032	0.154	−0.036	**−0.736**
Glucose	−0.46	0.498	0.008	0.423
HOMA-IR	−0.2	**0.629**	0.014	0.39
Systolic blood pressure	0.121	**0.715**	0.103	−0.052
Diastolic blood pressure	0.124	**0.729**	0.202	−0.179

Abbreviations: BMI = body mass index; FAI = free androgen index; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; PC = principal component; T = testosterone.
Variables with an absolute value of at least 0.5 are shown in bold. Positive signs indicate that higher values of the variable are influential in the component whilst negative signs indicate the influence of lower values.

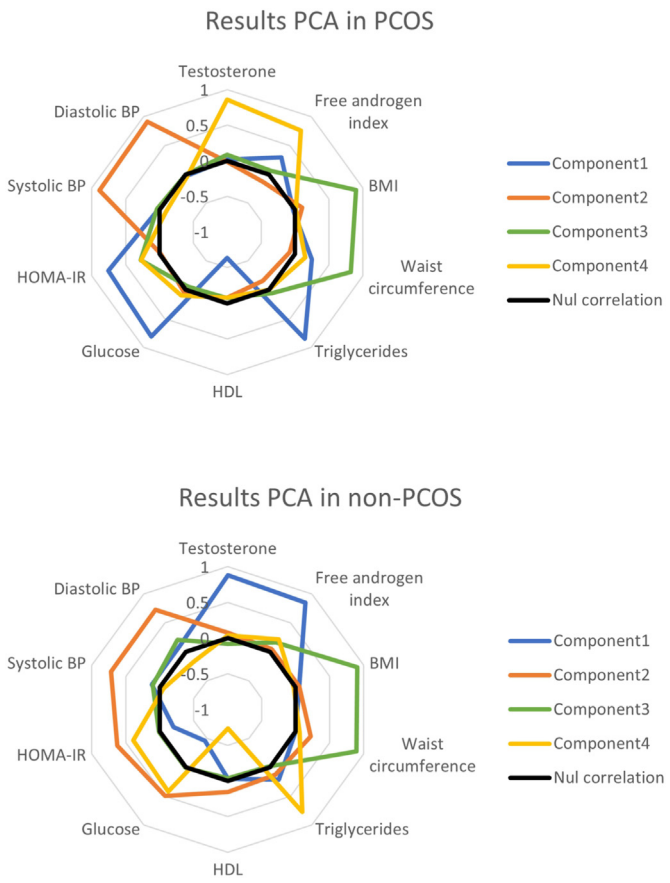

Fig. 3. Radar graph of principal components. For each component the distance from the nul (0) represents the influence of that variable in each component. *BMI* = body mass index; *BP* = blood pressure; *HDL* = high-density lipoprotein cholesterol; *HOMA-IR* = homeostatic model assessment of insulin resistance; *PCA* = principal component analysis; *PCOS* = polycystic ovary syndrome.

has little contribution this group of women with obesity and PCOS and most likely points to the ovary instead.

Understanding the pathophysiological components specific for women with PCOS and obesity compared to obese non-PCOS women may identify the role of obesity in the etiological background of PCOS. The first component yielded by PCA in the PCOS group aggregated metabolic variables including triglycerides, HDL-C, glucose, and HOMA-IR as dominant variables. We may call it the "insulin resistance" component. Insulin resistance has been considered to be the central etiological factor in PCOS.[9,35] Compensatory hyperinsulinemia causes hyperandrogenism by stimulating ovarian androgen production in theca cells and inhibiting hepatic production of SHBG.[9,35] Hyperinsulinemia and hyperandrogenism results in increased catecholamine-induced lipolysis and release of free fatty acids into circulation, and ultimately leads to hypertriglyceridemia.[36,37] Insulin resistance leads to a decrease in HDL-C levels, presumably as a consequence of increased hepatic lipase activity. This in turn leads to elevated breakdown of HDL particles and reduced activity of muscle lipoprotein lipase activity.[38] Insulin resistance, represented by HOMA-IR in our cohort is well-known to be related to each of these variables and confirmed by the significant correlations in statistical analysis. The analysis reveals that the "insulin resistance" component dominates the variance in a cohort of women with PCOS and obesity, which is in line with previous PCA in PCOS women with a broad range of BMI level.[17,18] Thus, it is reasonable to assume that insulin resistance is the leading causal factor in the pathogenesis of women with PCOS. Since only women with obesity constituted our study group, our findings about the associations between insulin resistance and PCOS were not confounded by obesity.

Systolic and diastolic blood pressure is the second PC in our PCOS group. Hypertension is prevalent in PCOS.[39-43] In a follow-up study of a Dutch PCOS population, the prevalence of hypertension in PCOS group was 2.5 times greater than that of an age-matched group.[39] However, obesity, which is common in PCOS, complicates the interpretation of studies since this is an important risk factor for hypertension. While one study demonstrated that obesity is the major determinant of the abnormalities in blood pressure found in young women with PCOS[44]; In another study, women with PCOS were 40% more likely to have elevated blood pressure than non-PCOS women, independent of age, BMI, and other confounders.[45] It remains unclear whether there is a role (or to what extent) for obesity in the association found between PCOS and hypertension. Our data indicate that blood pressure is an individual PC independent of insulin resistance or BMI, which highlights its important impact on women with PCOS and obesity. It suggests that treatment should also target antihypertensive strategies in women with PCOS and obesity, independent of insulin sensitizing strategies used for component 1.

Beyond expectations, BMI and waist circumference are the third PC independent of "insulin resistance" and "blood pressure" components, which is in contrast with previous studies which show that BMI and/or waist circumference are central variables in the first component.[17,18,46] The explanation for the difference between our results and other studies might be straightforward. The above-mentioned studies included a wide range of BMI (from underweight to obesity), while we only included women with obesity with a BMI $\geq$29. Therefore, the effect of BMI explaining the variance of the PCOS women is obscured in our PCA. Besides, the study of Stuckey et al[17] used the National Institutes of Health Criteria to define PCOS, dominating by hyperandrogenism, while we used Rotterdam criteria. Different diagnostic criteria for PCOS may lead to inconsistency in PCA results and needs to be verified in more PCOS cohorts.

Finally, in component 4 of the PCOS group, T and FAI were the most influential variables explaining 10.6% of variance. Total T and FAI have the highest discriminatory value for PCOS. In a way, it is surprising to see that FAI was not related to the first PC, knowing the great impact of insulin resistance on SHBG and influence of insulin resistance on androgen production in theca cells.[47,48] FAI seems to be a specific marker of hyperandrogenism in women with PCOS and obesity, separate from the influence of adiposity and metabolic factors. This finding contrasts with a previous study showing that FAI is in the first component and strongly associated with metabolic status and insulin resistance.[46] However, although both studies used Rotterdam criteria to define PCOS, differences in BMI range (36 vs 25) and different variables included in PCA might explain the heterogeneity of the results. Of note, our results are in line with the results of Stuckey et al,[17] who also showed that T was in the last component, despite the difference in BMI levels between studies.

Furthermore, we explored the main components of the non-PCOS obese ovulatory control group. The order of the components 1 and 4 were reversed, while components 2 and 3 were rather similar compared to the PCOS group. "T and FAI" is the first component in non-PCOS obese women, which explains 23.2% of variance in this population, followed by "blood pressure and HOMA-IR," "BMI and waist circumference," and "triglycerides and HDL-C." It is worth noting that although four PCs were identified, the proportion of variance explained by each component is relatively close and the variables representing metabolism are dispersed in component 2, 3, and 4. It is reasonable to assume that there is no major dominant characteristic that defines obese non-PCOS women. Despite this, a recent Mendelian randomization study indicating that elevated bioavailable serum T might be a cause of obesity in women,[4] is

aligned with our finding that "T and FAI" is the first component in obese ovulatory women even without PCOS well. Given that PCA was conducted in 2 distinct study populations, 1 with PCOS and 1 without, direct statistical comparisons between them are not be feasible. Nevertheless, we did notice a key difference: in the group without PCOS, unlike the PCOS group, insulin resistance did not emerge as the strongest PC. This indicates that insulin resistance is a distinctive feature of PCOS, rather than being primarily associated with obesity.

Knowing which variables are influential in the components may facilitate clinicians to adapt appropriate treatment of these components in women with PCOS and obesity. Insulin resistance is the strongest component in the PCOS group. This underlines the importance of a broad range of therapeutic interventions to reduce insulin resistance in order to improve general health in women with PCOS and reduce long-term consequences. Lifestyle coaching targeted at 5% to 10% weight loss and/or using of weight-reducing drugs and/or insulin resistance-lowering drugs as adjunctive treatment for infertility may improve reproductive and cardiometabolic outcomes in women with PCOS and obesity.[10]

While highlighting the strengths of our study including the well-defined cohort which allowed us to avoid the absolute confounding factor of BMI and reliable measuring methods for steroids using LC-MS/MS, several limitations need to be mentioned. First, although we used the Rotterdam criteria consistently to diagnose PCOS, we did not divide the subtypes of PCOS.[32,33] Evaluation of different PCOS phenotypes will help to elucidate the pathophysiological role of androgens, being adrenal derived or ovarian derived in this syndrome. Second, the PCOS and control groups were not matched for age. However, we do not think that this slight difference in mean age could have blunted results in the control group. Indeed, when including the women's age in the variable set, the androgen variables remained the first component in the control group, indicating that aging had no significant effect on androgen status in reproductive age. Third, owing to the focus of the original study design, the investigation of role of other factors associated with PCOS, which can affect metabolic and hormonal status, such as abnormal aldosterone levels or gut microbiome was not feasible. The study design allows to verify associations but precludes a cause-and-effect direction. Moreover, due to financial constraints and lack of blood samples, we had fewer PCOS and non-PCOS samples in the current analysis than in the original trial. We compared baseline characteristics between women included and not included to see if the current sample is representative. Finally, as all women with PCOS in this analysis were infertile and obese, our results cannot be generalized to other populations of women with PCOS who are not infertile and obese.

Conclusions

In conclusion, we find that 11-oxygenated androgens derived from the adrenals do not serve as promising discriminatory biomarkers for PCOS in women with obesity and infertility. Whether it applies to lean women with PCOS or women with PCOS without infertility needs further investigation. PCA yields objectively the classification/ranking of variables with the metabolic and androgen status in women with obesity and with or without PCOS. Insulin resistance is the strongest PC in the PCOS group. There is no major dominant characteristic that defines obese non-PCOS women.

Data Availability

Data analyzed in the present study are not publicly available due to privacy but are available from the corresponding author on reasonable request.

Disclosure

A.H. reports consultancy for development and implementation of a lifestyle App MyFertiCoach developed by Ferring Pharmaceutical Company.

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Author contributions

J.M.H., B.G.K., I.P.K., and A.H. designed the study; Z.W. and M.V.F. analyzed and interpreted the data; Z.W. and H.G. contributed to statistical analysis; Z.W. and A.V.d.V contributed to writing - original draft; M.V.F., H.G., A.E.P.C., A.V.O., J.M.H., B.G.K., I.P.K., and A.H. contributed to writing - review & editing.

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