Diagnostic value of total testosterone and free androgen index measured by LC–MS/MS for PCOS and insulin resistance

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Abstract

Background: The objective of the study was to explore the clinical significance of steroid hormones in the diagnosis of PCOS and PCOS-related insulin resistance through liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) and chemiluminescent immunoassay (CLIA).

Methods: The study included 114 patients with PCOS and 100 controls. Steroid hormone levels in serum were measured using LC–MS/MS and CLIA. The Bland–Altman method was used to check the consistency between the two methods. The diagnostic value of the LC–MS/MS method for female hyperandrogenemia and PCOS was evaluated.

Results: Women with PCOS were younger than controls on average ($p < 0.001$). PCOS patients had higher luteal hormone (LH, $p < 0.001$), insulin ($p = 0.002$), estradiol (E2, $p < 0.001$), total testosterone (TT, $p < 0.001$), free androgen index (FAI, $p = 0.021$), dehydroepiandrosterone sulfate (DHEA, $p = 0.021$), insulin resistance index (HOMA-IR) ($p = 0.034$), and fasting glucose ($p = 0.017$) levels than controls as measured by CLIA. The diagnostic value of TT was the best, and the area under the AUC curve was 0.766. Women with PCOS had higher androstenedione (A2, $p < 0.001$), FAI ($p < 0.001$), TT ($p < 0.001$), and 17-hydroxyprogesterone (17-OHP, $p < 0.001$) levels than controls as measured by LC–MS/MS. The ROC curve showed that the diagnostic efficacy of A2, TT, and 17-OHP was 0.830, 0.851, and 0.714, respectively. The consistency of TT detected by LC–MS/MS and CLIA was poor according to the Bland–Altman method. Detected TT by LC–MS/MS had the highest diagnostic efficiency for PCOS. The diagnostic power of the LC–MS/MS results for PCOS-related insulin resistance was analyzed. The results showed that the FAI had the highest diagnostic power, with an ROC curve of 0.789.

Conclusion: LC–MS/MS is more sensitive and accurate than CLIA in the determination of serum TT and FAI. TT is more effective for the diagnosis of PCOS, whereas FAI is more valuable in the diagnosis of insulin resistance.
INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a common endocrine system disorder affecting 5−15% of fertile women worldwide. The symptoms of PCOS are often hyperandrogenism, hyperinsulinemia, menstrual dysfunction, hirsutism, and infertility. PCOS patients need early detection and early treatment; if not treated in time, PCOS may lead to diabetes, hyperlipidemia, cardiovascular diseases, and even endometrial cancer, which have a substantial influence on women’s physical and mental health.

Although the etiology of PCOS is not clear, many views believe that hyperandrogenism plays a key role in the pathogenesis and progression of PCOS. On the one hand, hyperandrogenism inhibits the maturation of follicles and causes multiple follicular cysts; on the other hand, the increase in androgen causes dysfunction of the hypothalamic-pituitary-gonadal axis; thus, a vicious cycle of excessive androgen and continuous anovulation is formed. Research shows that approximately 60%−80% of PCOS patients have hyperandrogenemia. At present, clinical antiandrogen therapy has not achieved satisfactory results, which is caused by the complicated mechanisms of androgen production. The diagnosis of hyperandrogenemia plays a positive role in the early diagnosis, treatment, and long-term prevention of PCOS.

Insulin resistance is the most serious complication in PCOS patients and can lead to lipid metabolism disorder, vascular endothelial damage, and diabetes. Studies show that 75% of obese PCOS patients have insulin resistance; even if their weight is normal, they may also have hyperinsulinemia and abnormal blood glucose. Therefore, the diagnosis of insulin resistance in PCOS is a key step in the treatment of the long-term risks of PCOS.

At present, the level of steroid hormones is mainly detected by chemiluminescence immunoassay (CLIA). CLIA is relatively safe (no radiation hazard), stable, rapid, and easily automated, and the detection level can reach ng/ml. However, CLIA is mainly based on antigen antibody immune reactions, and nonspecific binding is inevitable; CLIA may overestimate androgen levels, resulting in poor accuracy and sensitivity. Increasing evidence has shown that liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) is more precise than CLIA. LC-MS/MS methods have higher accuracy and lower uncertainty than immunoassays, especially when the concentration of testosterone is relatively low. In recent years, with the continuous maturity of technology, an increasing number of institutions have used LC-MS/MS to detect testosterone. It is believed that precise measurement of total testosterone is very important for the diagnosis, treatment, and prevention of PCOS.

In this study, we analyzed and compared the total testosterone (TT) and free testosterone index (FAI) levels of PCOS patients and control women detected by LC-MS/MS and CLIA and evaluated the diagnostic value of the LC/MS method for female hyperandrogenemia and PCOS.

MATERIALS AND METHODS

Study subjects

A total of 114 patients with PCOS and 100 controls were recruited at Nanjing Maternal and Child Health Care Hospital. All procedures were performed with written informed consent. PCOS is diagnosed when at least two of the following criteria are met: (i) oligomenorrhea and/or anovulation, (ii) clinical and/or biochemical hyperandrogenism and (iii) polycystic ovaries according to the Rotterdam 2003 criteria. None of the women took hormonal drugs for at least 3 months before our study.

Hormone measurement by CLIA

Hormone levels were tested in the Clinical Laboratory of Nanjing Maternal and Child Health Care Hospital. As given in Table 1, lutetial hormone (LH), follitropin (FSH), prolactin (PRL), progesterone (PROG), cortisol, insulin, estradiol (E2), TT, dehydroepiandrosterone sulfate (DHEA) were measured by a Beckman automated chemiluminescence immunoanalyzer 7500 (Beckman). Sex hormone-binding globulin (SHBG) were tested by Cobas 6000 (Roche). Uric acid, total protein, total cholesterol, glutamic-pyruvic transaminase (GPT), glutamic oxiracetam transaminase (GPT), and fasting glucose were test by Beckman Coulter AU5800 (Beckman). All the reagents required for the test were matching reagents, which were used according to the specified requirements. The remaining serum was stored at −80°C for LC-MS/MS analysis.

Homeostatic model assessment of insulin resistance (HOMA-IR) is an indicator used to evaluate insulin resistance. HOMA-IR is equal to fasting blood glucose (FBG)×fasting insulin (fin)/22.5. The FAI is equal to 100×total testosterone (TT)/sex hormone-binding globulin (SHBG). Insulin resistance (IR) was defined as HOMA-IR>2.14 and fin ≥12.6 μU/ml.

Steroid hormone measurement by LC-MS/MS

As given in Table 2, aldosterone (ALD), 18-hydroxycorticosterone (18-OH-B), cortisone, cortisol/hydrocortisone, 21-deoxycortisol (21-DOC), corticosterone, 11-deoxycortisol, androstenedione (A2), 11-deoxycorticosterone, total testosterone 17-hydroxyprogesterone (17-OHP), 17-hydroxy pregnenolone, DHT,
PROG, androsterone, pregnenolone, and HHEA-S were assayed by LC–MS/MS.

Firstly, 10 mg of each pure calibrator was dissolved in 10 ml methanol (Merck Millipore) to obtain a 1 mg/ml solution, which was stored at −80°C. The stock solutions were diluted with methanol and mixed to obtain preworking solutions containing the 18 calibrators (Cambridge Isotope Laboratories). Then, the samples were serially diluted to obtain a standard curve intermediate. The calibrator working solution was prepared with 5% methanol/water and stored in the refrigerator at −20°C. Secondy, each internal standard (IS) was weighed and dissolved in methanol to obtain solutions of different concentrations. Each internal standard mother solution was diluted and mixed with acetonitrile (Merck Millipore, USA) to obtain the internal standard working solution and stored at −20°C. Then, steroid hormones in serum were treated by protein precipitation combined with solid phase extraction (SPE). The specific steps are as follows: A 200 μl serum sample was mixed with 200 μl internal standard solution. The mixture was rotated for 3 min. Then, 500 μl of deionized water was added, followed by 1 min of shaking. After centrifugation at 15,000 × g for 10 min, the mixture was transferred to a well on a Waters Oasis Prime HLB μElution column plate for SPE. A 700 μl extraction supernatant was added to the 96-well Oasis Prime HLB μElution column plate, the balance was activated with 200 μl methanol and 200 μl ddH2O, and the supernatant slowly flowed through the SPE plate and into the waste container by positive pressure. Then, 200 μl methanol/ddH2O (15:85, v/v) was added to the SPE plate in sequence. Then, 30 ml acetonitrile was added to the SPE plate twice, and the filtrate was collected. The filtrate was diluted with 60 μl methanol/ddH2O (5:95, v/v) and vortexed. The dilution filtrates were collected for LC–MS/MS analysis.

Ten microliters of filtrate from SPE was added to the Waters® ACQUITY UPLC® System – XevoTM TQS MS for LC–MS/MS analysis. The auto sampler temperature was 10°C. The binary mobile phase consisted of 2 mM ammonium acetate (Sigma-Aldrich, USA) + 0.1% formic acid (Sigma-Aldrich) + ddH2O (A) and 2 mM ammonium acetate + 0.1% formic acid + methanol (B). The column temperature was kept at 45°C. The optimized parameters for MS/MS were as follows: capillary voltage, 3.2 kV; cone voltage, 50 V; source temperature, 150°C; desolvation temperature, 650°C; cone gas flow, 150 L/h; desolvation gas flow, 800 L/h; and collision gas flow, 0.15 ml/min. The steroid profile was based on electrospray ionization-mass spectrometry (ESI-MS) data combined with tandem mass spectrometry in the positive ion mode or negative ion mode. Quantitation by multiple reaction monitoring (MRM) analysis was performed in the positive ion mode for analytes and in the negative ion mode for DHEAs.

### TABLE 1 Baseline characteristics and hormone levels by CLIA in control and PCOS patients

| Characteristics          | Control (100) | PCOS (114) | p^a  |
|--------------------------|---------------|------------|------|
| Age (year)               | 26.53 ± 3.30  | 24.19 ± 4.98 | <0.001 |
| Height (m)               | 1.62 ± 0.05   | 1.63 ± 0.05 | 0.276 |
| Weight (Kg)              | 63.15 ± 10.23 | 65.94 ± 13.54 | 0.088 |
| BMI (kg/m^2)             | 24.01 ± 3.55  | 24.84 ± 4.57 | 0.14  |
| SHBG (mmol/L)            | 34.16 ± 33.69 | 32.25 ± 32.10 | 0.673 |
| LH (mIU/ml)              | 4.90 ± 4.21   | 13.86 ± 6.99 | <0.001 |
| FSH (mIU/ml)             | 7.67 ± 12.40  | 6.84 ± 1.88  | 0.483 |
| Prolactin (ng/ml)        | 13.55 ± 5.62  | 12.32 ± 6.80 | 0.174 |
| Progesterone (ng/ml)     | 1.36 ± 5.86   | 1.18 ± 2.54  | 0.763 |
| Cortisol (mg/ml)         | 10.44 ± 4.87  | 11.03 ± 5.07 | 0.388 |
| Insulin (μIU/ml)         | 8.58 ± 7.52   | 13.53 ± 14.97 | 0.002 |
| HOMA-IR                  | 1.18 ± 2.20   | 3.15 ± 4.00  | 0.034 |
| E2 (pg/ml)               | 44.00 ± 15.54 | 75.19 ± 49.61 | <0.001 |
| Total testosterone (ng/ml)| 0.56 ± 0.27 | 0.75 ± 0.23 | <0.001 |
| FAI                      | 0.019 ± 0.027 | 0.032 ± 0.027 | 0.021 |
| DHEA (ng/ml)             | 6.89 ± 0.26   | 7.91 ± 3.16  | 0.021 |
| Uric acid (μmol/L)       | 223.19 ± 149.87 | 283.64 ± 151.93 | 0.004 |
| Total protein (g/L)      | 53.55 ± 34.52 | 62.91 ± 28.56 | 0.033 |
| Total cholesterol (mmol/L)| 3.29 ± 2.14  | 4.13 ± 1.81  | 0.002 |
| GPT (U/L)                | 17.02 ± 21.06 | 28.10 ± 29.84 | 0.002 |
| GOT (U/L)                | 16.92 ± 15.04 | 22.28 ± 13.99 | 0.008 |
| Fasting glucose (mmol/L) | 4.04 ± 2.34  | 4.71 ± 1.64  | 0.017 |

Abbreviations: FSH, follicle-stimulating hormone; GOT, glutamic oxiracetam transaminase; GPT, glutamic-pyruvic transaminase; LH, luteal hormone; SHBG, sex hormone-binding globulin.

^aP values were determined by two tailed student’s t-test.
2.4 | Statistical analysis

SPSS 20.0 was used for analysis. The variables are presented as the mean ± standard deviation. Independent sample t tests were used for intergroup comparisons. The differences detected by LC–MS/MS and CLIA between the PCOS group and the control group were compared by interaction analysis. p < 0.05 was considered statistically significant. Spearman rank correlation analysis was used to analyze the correlation between total testosterone as detected by the LC–MS/MS method and by the CLIA method. The chi square test was used to compare the rates between groups. The Bland–Altman method was used to measure the consistency of the two methods. ROC curves were used to analyze the PCOS diagnostic efficiency.

3 | RESULTS

3.1 | Baseline characteristics and hormone levels between the control and PCOS groups

There was a significant difference in age between the groups (control vs. PCOS, 26.53 ± 3.30 vs. 24.18 ± 4.98, p < 0.001). However, there was no difference in height (control vs. PCOS, 1.62 ± 0.05 vs. 1.63 ± 0.05, p = 0.276), weight (control vs. PCOS, 63.15 ± 10.23 vs. 65.94 ± 13.54, p = 0.088) or BMI (control vs. PCOS, 24.01 ± 3.55 vs. 24.84 ± 4.57, p = 0.140). The main baseline characteristics of the subjects are given in Table 1.

As given in Table 1, there were differences between the control group and the PCOS group in luteal hormone (LH) (4.90 ± 4.21 vs. 13.86 ± 6.99, p < 0.001), insulin (8.58 ± 7.52 vs. 13.53 ± 14.97, p = 0.002), E2 (44.00 ± 15.54 vs. 75.19 ± 49.61, p < 0.001), HOMA-IR (1.18 ± 2.20 vs. 3.15 ± 4.00, p = 0.034), TT (0.56 ± 0.27 vs. 0.75 ± 0.23, p < 0.001), FAI (0.019 ± 0.027 vs. 0.032 ± 0.027, p < 0.021), DHEA (6.89 ± 0.26 vs. 7.91 ± 3.16, p = 0.021), uric acid (223.19 ± 149.87 vs. 283.64 ± 151.93, p = 0.004), total protein (53.55 ± 34.52 vs. 62.91 ± 28.56, p = 0.033), total cholesterol (3.29 ± 2.14 vs. 4.13 ± 1.81, p = 0.002), GPT (17.02 ± 21.06 vs. 28.10 ± 29.84, p = 0.002), GOT (16.92 ± 15.04 vs. 22.28 ± 13.99, p = 0.008), and fasting glucose (4.04 ± 2.34 vs. 4.71 ± 1.64, p = 0.017) levels as measured by CLIA (Table 1).

Since the LC–MS/MS method has higher accuracy and lower uncertainty, LC–MS/MS was used to detect steroid hormones in both groups. As given in Table 2, women with PCOS had higher androstenedione (5.04 ± 1.68 vs. 3.08 ± 1.28, p < 0.001), FAI (2.17 ± 1.64 vs. 0.94 ± 1.19, p < 0.001), TT (0.53 ± 0.21 vs. 0.30 ± 0.14, p < 0.001), and 17-OHP (1.08 ± 0.71 vs. 0.69 ± 0.44, p < 0.001) levels than those in the controls, whereas there were no differences in serum

| Steroid hormone level by LC–MS in control and PCOS patients | Control (100) | PCOS (114) | p² |
|-------------------------------------------------------------|--------------|------------|----|
| 18-OH-B (ng/ml)                                             | 0.28 ± 0.10  | 0.290 ± 0.10 | 0.625 |
| ALD (ng/ml)                                                 | 0.07 ± 0.05  | 0.06 ± 0.05  | 0.125 |
| Cortisone (ng/ml)                                           | 21.71 ± 5.12 | 21.60 ± 5.09 | 0.883 |
| Cortisol/hydrocortisone (ng/ml)                             | 125.50 ± 46.06 | 131.04 ± 52.68 | 0.417 |
| 21-DOC (ng/ml)                                              | 0.10 ± 0.07  | 0.11 ± 0.08  | 0.297 |
| Corticosterone (ng/ml)                                      | 5.21 ± 4.99  | 5.36 ± 4.40  | 0.818 |
| 11-deoxycortisol (ng/mL)                                    | 0.21 ± 0.21  | 0.22 ± 0.19  | 0.602 |
| Androstenedione/A2 (ng/ml)                                  | 3.08 ± 1.28  | 5.04 ± 1.68  | <0.001 |
| 11-deoxycorticosterone (ng/ml)                              | 0.04 ± 0.03  | 0.04 ± 0.03  | 0.631 |
| FAI                                                         | 0.94 ± 1.19  | 2.17 ± 1.64  | <0.001 |
| Total testosterone (ng/ml)                                  | 0.30 ± 0.14  | 0.53 ± 0.21  | <0.001 |
| DHA/DHEA (ng/ml)                                            | 6.39 ± 3.61  | 6.75 ± 3.19  | 0.437 |
| 17-OHP (ng/ml)                                              | 0.69 ± 0.44  | 1.08 ± 0.71  | <0.001 |
| 17-hydroxy pregnenolone (ng/ml)                             | 5.84 ± 5.51  | 5.42 ± 4.11  | 0.534 |
| DHT (ng/ml)                                                 | 0.01 ± 0.01  | 0.01 ± 0.01  | 0.728 |
| PROG (ng/ml)                                                | 1.10 ± 4.31  | 0.88 ± 3.31  | 0.665 |
| Androsterone (ng/ml)                                        | 0.35 ± 0.40  | 0.39 ± 0.32  | 0.478 |
| Pregnenolone (ng/ml)                                        | 2.00 ± 1.55  | 1.77 ± 1.34  | 0.25  |
| DHEA-S (μg/ml)                                              | 2.42 ± 1.27  | 2.61 ± 1.15  | 0.251 |

Abbreviations: 17-OHP, 17-hydroxyprogesterone; 18-OH-B, 18-hydroxycorticosterone; 21-DOC, 21-deoxycortisol; ALD, aldosterone; DEHA-S, dehydroepiandrosterone sulfate; FAI, free androgen index.

²P values were determined by two tailed student’s t-test.
concentrations of the steroids DHEA-S, cortisol, cortisone, corticosterone, and 11-deoxycortisol.

3.2 | Diagnostic value of TT detected by LC–MS/MS for PCOS

The diagnostic values of hormones with significant differences between the control and PCOS groups were evaluated. As shown in Figure 1A, E2, TT, and DHES had good diagnostic value for PCOS detected by CLIA. Moreover, TT had the highest diagnostic potency for PCOS in the CLIA group, with an area under the curve (AUC) of 0.766. A2, TT and 17-OHP tested by LC–MS/MS were further analyzed for their diagnostic value for PCOS. The AUCs of A2, TT, and 17-OHP were 0.83, 0.851, and 0.714, respectively (Figure 1B). These results supported that TT had a better diagnostic value for PCOS not only detected by CLIA but also by LC–MS/MS. Moreover, TT detected by LC–MS/MS had a higher diagnostic value than TT detected by CLIA.

Since TT detected by LC–MS/MS had a higher diagnostic value than TT detected by CLIA, the agreement between the two methods was analyzed. The results suggested that the consistency between the results of the two methods was poor, and the stability of TT detected by LC–MS/MS was higher than that of TT detected by CLIA (Figure 2).

3.3 | Diagnostic value of FAI detected by LC–MS/MS for insulin resistance of PCOS

Insulin resistance is the most serious complication of PCOS, and the diagnostic value of steroid hormones detected by CLIA and LC–MS/MS for insulin resistance was tested. First, Pearson's correlation was used to test the steroid hormones associated with insulin resistance. A2 (R = 0.201, p = 0.003) and 17-OHP (R = 0.232, p = 0.001) had better positive relationships with HOMA-IR than TT, detected by both CLIA (R = 0.149, p = 0.03) and by LC–MS/MS (R = 0.149, p = 0.029) (Figure 3). The diagnostic efficacy of all differential indicators was analyzed for insulin resistance among PCOS patients. The AUCs of TT (CLIA), A2, TT (LC–MS/MS), and 17-OHP were 0.587, 0.611, 0.602, and 0.572, respectively (Figure 4).

TT in the body is either combined with SHBG or free in serum. It is generally thought that only free testosterone in serum, called FAI, could play an important role in PCOS. The diagnostic efficacy of FAI by CLIA and LC–MS/MS was analyzed. According to the results, the AUCs of FAI detected by CLIA and LC–MS/MS were 0.788 and 0.798, respectively (Figure 4). Therefore, FAI detected by LC–MS/MS had the best diagnostic value for insulin resistance among PCOS patients.

4 | DISCUSSION

At present, most clinical labs use the chemiluminescence method based on antigen antibody reaction (CLIA) or enzyme-linked immunosorbent assay to detect androgens. Due to the large amount of impurities in serum, the specificity of the test results is not high. This study compared the application of LC–MS/MS and CLIA to detect TT in PCOS patients and healthy women control, which provided new clinical data for the study of female hyperandrogenemia.

As expected, the mean TT level, FAI, measured by both LC–MS/MS and CLIA, was significantly higher in the PCOS group than in the control group. In women with PCOS, DHEA detected by CLIA was elevated compared with controls. Interestingly, the levels of androstenedione and 17-OHP detected by LC–MS/MS were elevated in women with PCOS compared to control women. It is well known that
FIGURE 2  Comparison of TT concentration between CLIA and LC-MS/MS. (A). The concentration of TT between control women and PCOS women by CLIA or LC-MS/MS. The concentration of TT in LC-MS/MS was lower and more stable than the CLIA. ***indicated \( p < 0.001 \). (B). The Bland–Altman method was used to detected the consistency of TT between two methods. The showed poor consistency between CLIA and LC-MS/MS.

FIGURE 3 Correlation between the different hormones which were difference between NC and women with PCOS and HOMA-IR. (A). Correlation between TT detected by CLIA and HOMA-IR in women with PCOS (\( R = 0.149, p = 0.03 \)); (B) Correlation between testosterone detected by LC-MS/MS and HOMA-IR in women with PCOS (\( R = 0.149, p = 0.029 \)); (C). Correlation between androstenedione detected by LC-MS/MS and HOMA-IR in women with PCOS (\( R = 0.201, p = 0.003 \)); (D). Correlation between 17α-hydroxyprogesterone detected by LC-MS/MS and HOMA-IR in women with PCOS (\( R = 0.232, p = 0.001 \)).
TT is not the only androgen elevated in women with PCOS. There is a view that androgens are not equal in women with PCOS, and testosterone has a much greater affinity with the androgen receptor than other androgens. A study carried out by Keefe C, in which LC–MS/MS was used to measure 13 steroids, found that women with PCOS had higher testosterone, androstenedione, and 17-OH progesterone levels than controls, but no differences in DHEAS, cortisol, corticosterone or their 11-deoxy precursors. In Cao, Z et al.’s study, the authors developed a simultaneous quantitation LC–MS/MS method for testosterone (T), androstenedione (A4), dehydroepiandrosterone sulfate (DHEAS), dihydrotestosterone (DHT), and 17-hydroxyprogesterone (17-OHP) in female serum, which was validated with good performance. The results provided better diagnostic power for PCOS with the combination of T, A4, DHEAS, and DHT measurements. Another study also confirmed that T, A4, and DHT together might contribute to a more accurate diagnosis of PCOS.

Among women with PCOS, increased HOMA-IR was also observed. These results were consistent with two other studies reported by Stepto NK and Kogure GS. Stepto NK reported that the IR of women with PCOS was higher than that of the BMI-matched control group. IR was present in 62% of the overweight control group and 95% of the overweight PCOS group. Kogure GS found that women with PCOS had higher serum levels of total testosterone and androstenedione and higher HOMA-IR scores than the control group.

Insulin resistance is the pathophysiological basis of PCOS. HOMA-IR is one of the indices used to evaluate insulin resistance. It has been reported that approximately 70% of women with PCOS are insulin resistant. In our study, testosterone detected by CLIA and androstenedione, testosterone and 17α-hydroxyprogesterone detected by LC–MS/MS were positively correlated with HOMA-IR. FAI detected by LC–MS/MS had the highest diagnostic value, with an ROC curve of 0.798. Sezai Sahmay found that BMI and free testosterone were positively correlated with HOMA-IR. Soulmaz Shorakae reported that testosterone and FAI were both correlated with insulin resistance in women with PCOS. A study reported that insulin resistance was highly related to serum free testosterone but weakly related to total testosterone or other androgens. A recent study has shown that insulin clearance was significantly damaged in women with PCOS compared with controls. Insulin can stimulate ovarian theca cells to secrete androgens. IR stimulates the production of androgens in the ovaries and reduces SHBG levels in the liver, which increases free testosterone. It is believed that although the diagnostic criteria of PCOS do not distinguish between different androgens in detail, androgens are not equal in action. Testosterone and DHT have a much greater affinity for the androgen receptor than androstenedione and DHEA.

In this study, TT detected by LC–MS/MS had the highest diagnostic efficacy, with an AUC of 0.851, which was better than the AUC of 0.766 for CLIA. The consistency between the two methods was poor, which suggested that there were differences between them. Joëlle Taieb found that the immunoassay method was unreliable for the measurement of serum from children and women, especially when their testosterone concentration was low (<1.7 nmol/L). Urszula Ambroziak concluded that LC–MS/MS was more reliable in the measurement of serum 17-OHP and androgens than immunoassays in women with hyperandrogenism. Željko Debeljak assessed the analytical bias of six serum steroid hormones using LC–MS/MS, and the results showed that there was a large difference between all immunoassays and the LC–MS/MS assay. In a longitudinal study of healthy children, the author demonstrated the importance of testing serum testosterone by LC–MS/MS, especially at low levels in children. Due to its high specificity and high sensitivity, LC–MS/MS can produce more accurate and reliable detection results, which are more suitable for complex biological samples, and its sensitivity can reach the pg/ml level, which is especially useful for low-concentration hormone detection, so it can provide better services for clinical diagnosis and is increasingly favored by clinical detection.

Although the LC–MS/MS method requires an expensive apparatus and long operation procedures, it has been used as a reference method for hormone measurements by professional societies. In conclusion, our study revealed a close relationship between HA and IR by LC–MS/MS measurement of TT.

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**CONFLICT OF INTEREST**

The authors have declared that no competing interests exist.

**DATA AVAILABILITY STATEMENT**

All relevant data are within the paper.
ETHICS STATEMENT
The experimental protocols were approved by the Ethics Committee of the Nanjing Maternity and Child Health Care Hospital. The methods were performed in accordance with the approved guidelines by the Ethics Committee of the Nanjing Maternity and Child Health Care Hospital.

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REFERENCES
1. Cesta CE, Månsson M, Palm C, Lichtenstein P, Iliadou AN, Landén M. Polycystic ovary syndrome and psychiatric disorders: comorbidity and heritability in a nationwide Swedish cohort. Psychoneuroendocrinology. 2016;73:196-203.
2. Qiao J, Feng HL. Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. Hum Reprod Update. 2011;17(1):17-33.
3. Norman RJ, Dewaillly D, Legro RS, Hickey TE. Polycystic ovary syndrome. Lancet (London, England). 2007;370(9588):685-697.
4. Walters KA, Handelsman DJ. Role of androgens in the ovary. Mol Cell Endocrinol. 2018;465:36-47.
5. Nisenblat V, Norman RJ. Androgens and polycystic ovary syndrome. Curr Opin Endocrinol Diabetes Obes. 2009;16(3):224-231.
6. Ye W, Xie T, Song Y, Zhou L. The role of androgen and its related signals in PCOS. J Cell Mol Med. 2021;25(4):1825-1837.
7. Moghetti P. Insulin resistance and polycystic ovary syndrome. Curr Pharm des. 2016;22(36):5526-5534.
8. Goodman NF, Cobin RH, Futterweit W, et al. American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and PCOS Society disease state clinical review: guide to the best practices in the evaluation and treatment of polycystic ovary syndrome - part 2. Endocr Pract. 2015;21(11):1291-1300.
9. Yang Y, Ouyang N, Ye Y, et al. The predictive value of total testosterone alone for clinical hyperandrogenism in polycystic ovary syndrome. Reprod Biomed Online. 2020;41(4):734-742.
10. Kanakis GA, Tsametis CP, Goulis DG. Measuring testosterone in women and men. Maturitas. 2019;125:41-44.
11. Taysi S, Polat MF, Sari RA, Bakan E. Serum adenosine deaminase and cytokine deaminase activities in patients with systemic lupus erythematosus. Clin Chem Lab Med. 2002;40(5):493-495.
12. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab. 2007;92(2):405-413.
13. La'uulu SL, Kalp KJ, Straseski JA. How low can you go? Analytical performance of five automated testosterone immunoassays. Clin Biochem. 2018;58:64-71.
14. Hultenieni IT, Tajar A, Lee DM, et al. Comparison of serum testosterone and estradiol measurements in 3174 European men using platform immunoassay and mass spectrometry; relevance for the diagnostics in aging men. Eur J Endocrinol. 2012;166(6):983-991.
15. McCartney CR, Marshall JC. Clinical practice. Polycystic ovary syndrome. N Engl J Med. 2016;375(1):54-64.
16. Mooradian AD, Morley JE, Korenman SG. Biological actions of androgens. Endocr Rev. 1987;8(1):1-28.
17. Keeffe CC, Goldman MM, Zhang K, Clarke N, Reitz RE, Welt CK. Simultaneous measurement of thirteen steroid hormones in women with polycystic ovary syndrome and control women using liquid chromatography-tandem mass spectrometry. PloS One. 2014;9(4):e93805.
18. Cao Z, Lu Y, Cong Y, et al. Simultaneous quantitation of four androgens and 17-hydroxyprogesterone in polycystic ovarian syndrome patients by LC-MS/MS. J Clin Lab Anal. 2012;26(12):585-592.
19. Wang Z, Wang H, Peng Y, et al. A liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay to profile 20 plasma steroids in endocrine disorders. Clin Chem Lab Med. 2020;58(9):1477-1487.
20. Stepto NK, Cassar S, Joham AE, et al. Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulinaemic clamp. Hum Reprod. 2013;28(3):777-784.
21. Kogure GS, Ribeiro VB, Gennaro FGO, Miranda-Furtado CL, Reis RMD. Physical performance regarding hand grip strength in women with polycystic ovary syndrome. Rev Bras Ginecol Obstet. 2020;42(12):811-819.
22. Sahmay S, Aydogan Mathyk B, Sofiyeva N, Atakul N, Azemi A, Erel T. Serum AMH levels and insulin resistance in women with PCOS. Eur J Obstet Gynecol Reprod Biol. 2018;224:159-164.
23. Tosi F, Fiers T, Kaufman JM, et al. Implications of androgen assay accuracy in the phenotyping of women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2016;101(2):610-618.
24. Tosi F, Dal Molin F, Zamboni F, et al. Serum androgens are independent predictors of insulin clearance but not of insulin secretion in women with PCOS. J Clin Endocrinol Metab. 2020;105(1):e1981-e1989.
25. Garg D, Tai R. Inositol treatment and ART outcomes in women with PCOS. Int J Endocrinol. 2016;2016:1979654-1979659.
26. Sørensen AE, Udesen PB, Wissing ML, Englund ALM, Dalgaard LT. MicroRNAs related to androgen metabolism and polycystic ovary syndrome. Chem Biol Interact. 2016;259( Pt A):8-16.
27. Debeljuk Ž, Markovčič I, Pavela J, et al. Analytical bias of automated immunoassays for six serum steroid hormones assessed by LC-MS/ MS. Biochem Med. 2020;30(3):e230701.
28. Mouritsen A, Seeborg T, Johanssen TH, et al. Longitudinal changes in circulating testosterone levels determined by LC-MS/MS and by a commercially available radioimmunoassay in healthy girls and boys during the pubertal transition. Horm Res Paediatr. 2014;82(1):12-17.
29. Kushnir MM, Rockwood AL, Roberts WL, Yue B, Bergquist J, Meikle AW. Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. Clin Biochem. 2011;44(1):77-88.

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