Androgen levels in women with various forms of ovarian dysfunction: associations with cardiometabolic features

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STUDY QUESTION: Are differences in androgen levels among women with various forms of ovarian dysfunction associated with cardiometabolic abnormalities?

SUMMARY ANSWER: Androgen levels differed substantially between women with and without ovarian dysfunction, and increased androgen levels were associated with impaired cardiometabolic features in all women irrespective of their clinical condition.

WHAT IS KNOWN ALREADY: Sex steroid hormones play important roles in the development of cardiovascular diseases (CVD). Extremes of low as well as high androgen levels have been associated with increased CVD risk in both men and women.

STUDY DESIGN, SIZE, DURATION: This cross-sectional study included 680 women with polycystic ovary syndrome (PCOS), premature ovarian insufficiency (POI), natural post-menopausal women (NM), or regular menstrual cycles (RC) (170 women per group).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Measurements of serum testosterone, androstenedione and dehydroepiandrosterone sulfate were performed using liquid chromatography-tandem mass spectrometry. Assessments were taken of body mass index (BMI), blood pressure, lipid profiles, glucose, insulin and SHBG, and the bioactive fraction of circulating testosterone was calculated using the free androgen index (FAI).

MAIN RESULTS AND THE ROLE OF CHANCE: PCOS women were hyperandrogenic [median FAI = 4.9 (IQR 3.6–7.4)], and POI women were hypoandrogenic [FAI = 1.2 (0.8–1.7)], compared with RC women [FAI = 1.7 (1.1–2.8)], after adjustment for age, ethnicity, smoking and BMI (P < 0.001). After adjustment for age, there were no significant differences in androgens between POI and NM (P = 0.15) women and between POI and NM (P = 0.27) women, the latter indicating that chronological aging rather than ovarian aging influences the differences between pre- and post-menopausal women. A high FAI was associated with elevated triglycerides (β log FAI for PCOS: 0.45, P < 0.001, POI: 0.25, P < 0.001, NM: 0.20, P = 0.002), insulin (β log FAI for PCOS: 0.77, POI: 0.44, NM: 0.40, all P < 0.001), HOMA-IR (β log FAI for PCOS: 0.82, POI: 0.46, NM: 0.47, all P < 0.001) and mean arterial pressure (β log FAI for PCOS: 0.05, P = 0.002, POI: 0.07, P < 0.001, NM: 0.04, P = 0.04) in all women; with increased glucose (β log FAI for PCOS: 0.05, P = 0.003, NM: 0.07, P < 0.001) and decreased high-density lipoprotein (β log FAI for PCOS: −0.23, P < 0.001, NM: −0.09, P = 0.03) in PCOS and NM women; and with increased low-density lipoprotein (β log FAI for POI: 0.083, P = 0.041) in POI women. Adjustment for BMI attenuated the observed associations. Associations between FAI and cardiometabolic features were the strongest in PCOS women, even after adjustment for BMI.

LIMITATIONS, REASONS FOR CAUTION: Associations between androgen levels and cardiometabolic features were assessed in PCOS, POI and NM women only, due to a lack of available data in RC women. Due to the cross-sectional design of the current study, the potential associations between androgen levels and actual future cardiovascular events could not be assessed.

† These authors contributed equally.
Androgens and cardiometabolic features in women

**WIDER IMPLICATIONS OF THE FINDINGS:** This study affirms the potent effect of androgens on cardiometabolic features, indicating that androgens should indeed be regarded as important determinants of women’s health. Future research regarding the role of androgens in the development of CVD and potential modulatory effects of BMI is required.

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**Key words:** PCOS / POI / menopause / androgens / cardiovascular risk factors

**Introduction**

Sex steroid hormones are recognized to play a crucial role in the development of cardiovascular disease (CVD) in men and women (Vitale et al., 2009). Although CVD is more prevalent amongst men, the incidence of CVD in women increases steadily beyond 50 years of age (Go et al., 2013). Consequently, CVD currently represents the world’s leading cause of death in women (WHO, 2015). This rise in CVD incidence in women has been previously attributed to a decline in premenopausal estrogen levels following the menopausal transition (Villablanca et al., 2010). More recently, research has extended our understanding of the potential role of androgens in the development of CVD in both men and women.

Various studies in both sexes have proposed that extremes of low as well as high androgen concentrations are associated with increased CVD risk (Patel et al., 2009; Laughlin et al., 2010; Soisson et al., 2013). In men, low androgens have been associated with dyslipidemia, increased body mass index (BMI), diabetes, hypertension and CVD mortality (Hyde et al., 2012; Srinath et al., 2015). Several studies in post-menopausal women have also reported an inverse relation between endogenous androgen levels, dyslipidemia and atherosclerosis (Bernini et al., 2001; Khatibi et al., 2007; Quyang et al., 2009). Furthermore, improvements in lipid profiles following estradiol/testosterone replacement therapy have been reported in post-menopausal women (Castelo-Branco et al., 2007; Britto et al., 2012).

In contrast, increased androgen concentrations have been shown to impair cardiovascular health in both men and women (Rexrode et al., 2003; Xu et al., 2013). Women with polycystic ovary syndrome (PCOS), especially those with hyperandrogenism, exhibit an increased prevalence of dyslipidemia, insulin resistance, obesity and CVD (Toulis et al., 2011; Daan et al., 2014). Moreover, supplementation of androgens in post-menopausal women has been associated with decreased insulin sensitivity and with dyslipidemia (Zang et al., 2006). A chronically induced hyperandrogenic state appears to cause atherogenicity through inflammation, as demonstrated in female to male transsexuals treated with testosterone (Gooren et al., 2014).

In women, androgens are produced in the ovaries, in the adrenal cortex and through peripheral conversion of precursor hormones (Davison et al., 2005). Decreased levels of circulating androgens have been reported in women with premature ovarian insufficiency (POI), who experience menopause before the age of 40 years (Kalantaridou et al., 2006; Janse et al., 2012). POI has been identified as a risk factor for the development of CVD (Roeters van Lennep et al., 2014). In this study, we aimed to compare androgen levels measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and assess their associations with cardiometabolic features in women under different clinical conditions: PCOS (associated with hyperandrogenism), POI (associated with hypoandrogenism), women who experienced natural menopause (NM) and women of reproductive age with regular menstrual cycles (RC).

**Materials and Methods**

**Study population**

Four groups of women were included in this study: women previously diagnosed with PCOS, women previously diagnosed with POI, women who experienced natural menopause (NM) and women of reproductive age with regular menstrual cycles (RC).

The included women with PCOS or POI participated in a large prospective cohort study on menstrual cycle disturbances within the reproductive outpatient clinic of the University Medical Center Utrecht between November 2004 and July 2011. Women were screened according to a standardized protocol consisting of medical/reproductive history, anthropometric measurements, transvaginal ultrasoundography and an extensive fasting endocrine/metabolic laboratory evaluation. Spare serum samples were collected and stored at −20 °C. The screening procedure has been previously described in detail elsewhere (Broekmans et al., 2006; Valkenburg et al., 2008). This study was conducted with approval of the local institutional ethical review board, and all participants provided written informed consent. The study was registered on www.clinicaltrials.gov with trial number NCT0230904.

PCOS was diagnosed according to the Rotterdam criteria, if at least two of the following characteristics were present: ovulatory dysfunction, androgen excess and/or polycystic ovarian morphology (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Ovulatory dysfunction was defined as an average menstrual cycle length of 35 days to 6 months (oligomenorrhea) or absence of a menstrual bleeding for ≥ 6 months (amenorrhea). For the current study, we selected women with an anticipated hyperandrogenic PCOS phenotype, based on the primary clinical and biochemical assessment of hyperandrogenism. Clinical and biochemical hyperandrogenism were defined as a Ferriman–Gallway score > 8, and/or a free androgen index (FAI) > 4.5 [FAI: (Testosterone/SHBG) × 100] (Veitmeulen et al., 1999). POI was defined as secondary amenorrhea ≥ 4 months occurring...
before the age of 40 years with accompanying FSH levels above 40 IU/L (Coulam et al., 1986). Women with POI were excluded if they had a history of past ovarian surgery and/or gonadotoxic treatment such as chemotherapy or radiation therapy.

Included NM women were selected from the Rotterdam Study. The Rotterdam Study is a large prospective population-based cohort study of men and women of 45 years of age and older, which was initiated in 1990 in Ommoord, a suburb of Rotterdam, the Netherlands. This study has been designed to investigate the incidence and risk factors for various chronic illnesses such as cardiovascular and endocrine diseases, as has been previously described in detail (Hofman et al., 2013). For the current study, women who experienced natural menopause after the age of 45 years, with a history of previous regular menstrual cycles throughout their reproductive life were selected.

Included RC women were participants in a preconceptional cohort study in women starting IVF/ICSI treatment within the reproductive outpatient clinic of the University Medical Center Utrecht between October 2006 and November 2013. This study was registered on www.clinicaltrials.gov with trial number NCT02309073. For the current study, we included women undergoing IVF/ICSI treatment with the indication of severe male infertility, since these women were clinically evaluated and exhibited no signs of female reproductive dysfunction. Severe male infertility was defined as a semen analysis volume × concentration × motility of <2.0 million. Included women reported a regular mean menstrual cycle length between 21 and 35 days.

None of the women included in this study were using any form of hormonal therapy/contraception for at least 6 weeks prior to the moment of blood withdrawal. The current study was conducted with institutional ethical review board permission and all included women provided written informed consent.

Endocrine and metabolic assessment

In all women testosterone, dehydroepiandrosterone sulfate (DHEAS) and androstenedione were measured with LC-MS/MS in serum samples that were previously stored at −20°C. All steroid hormones were measured simultaneously with an LC-MS/MS method using the CHS®MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The Steroids Kit uses a combined solvent extraction and protein precipitation method with acetonitrile containing the deuterated internal standards 2H₅-H₃-testosterone, 2H₅-17α-hydroxyprogesterone and 2H₅-testosterone. The internal standards undergo processing identical to the analytes. The chromatographic separation was performed on a Waters® Acquity™ UPLC HSS T3 1.8 μm column (diameter 1 mm, length 10 cm) and in-line filter frit 0.2 μm with acetonitrile/MeOH gradient. A Waters(XEVO-TQ-S system (Waters, Milford, MA, USA) was equipped with an ESI source operating in the electrospray positive mode except for DHEAS (negative ESI). Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers. The corresponding inter-assay coefficients of variation and lower limit of quantification (LLOQ) were androstenedione <6.5%; LLOQ 0.20 nmol/l; DHEAS <5.9%; LLOQ 0.25 μmol/l and testosterone <5%; LLOQ 0.07 nmol/l.

In women with PCOS or POI, serum was drawn at the outpatient clinic and insulin, glucose, SHBG and lipids were directly assessed. Insulin and SHBG were assessed with the Immulite 1000 assay (Diagnostics Products Corporation Breda, The Netherlands) until April 2007 and thereafter with the Roche Diagnostics Modular E170 (Roche Diagnostics, Almere, The Netherlands) [Conversion formula: Roche Modular E170 = 1.10 × (Immulite 1000) − 0.7]. Glucose and lipids were assessed with the VITROS Chemistry System (Ortho-Clinical Diagnostics, Strasbourg, France) until November 2006 and then with the Uncill DxC 800 assay (Beckman Coulter, Woerdern, The Netherlands). The corresponding intra- and inter-assay coefficient of variation with lower limits of detection (LLOD) of the last used assays were insulin <2 and <4%; SHBG <2 and <5%; LLOD: 0.35 nmol/l, glucose <4 and <4%; LLOD: 0.3 mmol/l, lipids <2 and <3%; LLOD: 0.1 mmol/l.

In natural post-menopausal women, serum was drawn during their evaluation for the Rotterdam Study, and, insulin, glucose, SHBG and lipid profiles were directly assessed. Insulin and SHBG were determined using Immulite 2000Xpi (Diagnostics Products Corporation Breda). Glucose and lipids were assessed using the COBAS 8000 system (Roche Diagnostics). The corresponding intra- and inter-assay coefficients of variation and the LLOD of the last used assays were insulin <6 and <8%; LLOD: 14 pmol/l, SHBG <4 and <5%; LLOD: 0.02 nmol/l, glucose <0.8 and <1.4%; LLOD: 0.11 mmol/l, lipids <1.1 and <2.1%; LLOD: 0.1 mmol/l.

There were no metabolic parameters available for the women of reproductive age with regular menstrual cycles. In these women, SHBG was assessed in serum samples that were previously stored at −20°C.

Hormones included in the statistical analyses were testosterone, DHEAS, androstenedione, SHBG and the calculated FAI. Cardiometabolic features included in the analyses were total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG), insulin and glucose. The calculated homeostatic model assessment-insulin resistance [HOMA-IR; (glucose × insulin)/22.5] and mean arterial pressure [MAP; (2 × diastolic + systolic)/3] were additionally included (Matthews et al., 1985; Domanski et al., 1999).

Statistical analyses

A power calculation was performed based on the groups in which we expected to detect the smallest difference in the concentration of total testosterone (i.e. women with POI). We expected a clinically significant lower testosterone level in the POI group of 1.12 nmol/l (SD ± 0.58) versus a testosterone of 1.36 nmol/l in women with regular menstrual cycles (RC) (Janse et al., 2012). To obtain a power of 0.9 with a significance level of 0.05/4 = 0.0125 (applying the Bonferroni correction for multiple testing), 167 patients needed to be included in the current study. Taking into account potential processing/measuring errors, a total of 170 patients were included in each group.

The primary research aim was to assess androgen levels and cardiometabolic characteristics in PCOS, POI, NM and RC women. All hormone levels and cardiometabolic features were log-transformed to obtain normally distributed variables. Crude and adjusted means were calculated and stratified per group using linear regression analyses. Due to the large age difference between the four groups, Model I was primarily adjusted for age. Model II was adjusted for age, ethnicity and smoking, and could be considered the fully adjusted model. Since BMI most likely an intermediate in the causal pathway between androgens and cardiometabolic characteristics, for instance, through direct inhibition of SHBG production and stimulation of insulin-like growth factor (IGF-I) production, adjusting for BMI could potentially result in an overadjustment (Utz et al., 2008). Nevertheless, a third model including BMI was made. Furthermore, androgen levels and cardiometabolic features between the four groups of women were compared. Due to large differences between the four groups of women, age differences in particular, we were not able to assign one reference group as this would result in unequal comparisons. Subsequently, we chose to make four specific one-to-one comparisons in order to obtain the most illustrative results. For androgen levels, these comparisons were: PCOS versus RC, POI versus RC, POI versus NM and NM versus RC. In the absence of values for the RC group, we compared cardiometabolic features between the following groups: PCOS versus POI and POI versus NM. t-tests were used to assess crude statistical differences and linear regression with a dichotomous class variable (e.g. PCOS 1 versus RC 0) for (multi)variable adjusted statistical differences.

The secondary research aim was to assess potential associations between androgen levels and the cardiometabolic features for women with PCOS,
POI or NM. The FAI was used as a proxy for the androgen concentrations since it reflects the bioactive proportion of circulating testosterone levels. Associations were first depicted in scatterplots and subsequently assessed using linear regression analyses. Cardiometabolic features were used as the dependent and the FAI as independent variable, and adjusted for the same covariates as in Models I–III of the primary research aim. Furthermore, we assessed whether the association between FAI and cardiometabolic features was significantly different for POI versus NM and PCOS versus POI women (P-value for interaction).

SPSS version 21.0 was used for all analyses. Associations were considered statistically significant at a P-value of <0.05 after applying a Bonferroni correction for the number of performed comparisons.

**Results**

The baseline characteristics, median androgen concentrations and cardiometabolic characteristics of participating women with PCOS, POI, NM and RC are outlined in Table I. Women were predominantly from Northern European descent. The majority of women in the PCOS and NM group were overweight (64 and 62%, respectively). Figure 1 shows the multivariable-adjusted mean FAI of each group, which was used as a proxy for the androgen concentrations in further analyses. After adjustment for age, ethnicity, smoking and BMI, the FAI remained highest in women with PCOS, followed by the RC, NM and POI women (Fig. I).

The differences in androgen concentrations between the four study groups are shown in Table II. Women with PCOS exhibited a 3-fold increase in absolute FAI and a 2-fold increase in absolute testosterone levels compared with RC women, which remained significant after correction for age, ethnicity, smoking and BMI (both P < 0.001). Women with POI exhibited a 30% decrease in absolute FAI and 12% decrease in absolute testosterone levels compared with RC women, which remained significant after correction for age, ethnicity, smoking and BMI (P < 0.001 and =0.002, respectively). After adjustment for age, there were no significant differences in FAI, SHBG and androgen concentrations between POI versus NM and NM versus RC. Additional adjustment for other covariates did not alter these findings.

The differences in cardiometabolic features between PCOS versus POI and POI versus NM women are outlined in Table III. After correcting for age, ethnicity and smoking, we found significant differences in HDL cholesterol (P < 0.001), TG (P = 0.001), insulin (P < 0.001) and HOMA-IR (P < 0.001) between (hyperandrogenic) PCOS and (hypoandrogenic) POI women. After additional adjustment for BMI, only HDL cholesterol levels remained significantly decreased in PCOS compared

### Table I  Characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>PCOS (n = 170)</th>
<th>POI (n = 170)</th>
<th>NM (n = 170)</th>
<th>RC (n = 170)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.0 (23.0–31.0)</td>
<td>37.0 (34.0–40.0)</td>
<td>55.8 (53.2–58.3)</td>
<td>33.0 (29.0–36.0)</td>
</tr>
<tr>
<td>Age at menopause (years)</td>
<td>NA</td>
<td>35.0 (29.3–37.0)</td>
<td>52.0 (50.0–54.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Time since menopause (years)</td>
<td>NA</td>
<td>1.0 (0.0–3.0)</td>
<td>3.5 (2.3–4.9)</td>
<td>NA</td>
</tr>
<tr>
<td>Northern European descent (yes)</td>
<td>122 (72%)</td>
<td>134 (79%)</td>
<td>157 (92%)</td>
<td>145 (85%)</td>
</tr>
<tr>
<td>Current smoking (yes)</td>
<td>37 (22%)</td>
<td>45 (27%)</td>
<td>40 (24%)</td>
<td>24 (14%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 (23.4–32.5)</td>
<td>23.0 (21.4–26.6)</td>
<td>26.3 (24.0–29.1)</td>
<td>23.4 (21.7–25.9)</td>
</tr>
<tr>
<td>Overweight (BMI ≥ 25)</td>
<td>108 (64%)</td>
<td>62 (37%)</td>
<td>106 (62%)</td>
<td>52 (31%)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>91.0 (78.0–104.0)</td>
<td>80.0 (75.0–90.0)</td>
<td>85.4 (80.3–93.6)</td>
<td>–</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>122.0 (116.0–133.0)</td>
<td>122.0 (113.0–135.0)</td>
<td>127.0 (116.0–140.0)</td>
<td>–</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79.0 (73.0–85.0)</td>
<td>80.0 (73.0–88.0)</td>
<td>81.0 (75.0–89.3)</td>
<td>–</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>93.3 (87.7–101.0)</td>
<td>95.0 (86.2–102.2)</td>
<td>97.3 (89.3–106.8)</td>
<td>–</td>
</tr>
<tr>
<td>Hypertension (≥ 140 systolic and/ or 90 diastolic mmHg)</td>
<td>36 (21%)</td>
<td>44 (26%)</td>
<td>67 (39%)</td>
<td></td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.6 (1.3–2.01)</td>
<td>0.7 (0.5–0.9)</td>
<td>0.8 (0.6–1.0)</td>
<td>0.8 (0.6–1.0)</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>7.6 (6.3–9.5)</td>
<td>2.4 (1.9–3.2)</td>
<td>2.4 (1.9–3.4)</td>
<td>3.5 (2.7–4.5)</td>
</tr>
<tr>
<td>DHEAS (μmol/l)</td>
<td>5.2 (3.7–6.7)</td>
<td>3.5 (2.6–4.8)</td>
<td>2.5 (1.6–3.5)</td>
<td>4.0 (2.9–5.8)</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>32.0 (22.9–44.0)</td>
<td>54.0 (38.9–75.8)</td>
<td>56.3 (41.2–77.7)</td>
<td>48.5 (33.8–62.2)</td>
</tr>
<tr>
<td>FAI</td>
<td>4.9 (3.6–7.4)</td>
<td>1.2 (0.8–1.7)</td>
<td>1.3 (1.0–2.0)</td>
<td>1.7 (1.1–2.8)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.6 (4.1–5.2)</td>
<td>5.0 (4.5–5.7)</td>
<td>5.8 (5.2–6.4)</td>
<td>–</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (1.0–1.6)</td>
<td>1.7 (1.4–1.9)</td>
<td>1.6 (1.3–1.9)</td>
<td>–</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.8 (2.4–3.3)</td>
<td>3.0 (2.5–3.4)</td>
<td>3.6 (3.1–4.2)</td>
<td>–</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8 (0.5–1.2)</td>
<td>0.8 (0.5–1.0)</td>
<td>1.1 (0.8–1.5)</td>
<td>–</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.1 (4.9–5.4)</td>
<td>5.1 (4.8–5.4)</td>
<td>5.1 (4.9–5.4)</td>
<td>–</td>
</tr>
<tr>
<td>Insulin (mIU/l)</td>
<td>10.4 (6.5–18.1)</td>
<td>6.5 (3.9–10.4)</td>
<td>8.4 (6.5–13.4)</td>
<td>–</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.3 (1.5–4.1)</td>
<td>1.6 (0.8–2.3)</td>
<td>2.0 (1.4–3.1)</td>
<td>–</td>
</tr>
</tbody>
</table>

Continuous parameters are presented as medians with inter-quartile ranges, categorical variables as absolute numbers with percentages. BMI, body mass index; MAP, mean arterial pressure; DHEAS, dehydroepiandrosterone sulfate; FAI, free androgen index; HOMA-IR, homeostasis model assessment-insulin resistance; PCOS, polycystic ovary syndrome; POI, premature ovarian insufficiency; NM, natural menopausal women; RC, women with regular menstrual cycles; NA, not applicable.
with POI women (1.2 versus 1.7 mmol/l, respectively; \(P < 0.001\)). When comparing POI and NM women, significant differences were found for MAP and insulin levels in models adjusted for age, ethnicity and smoking (\(P < 0.001\) and \(=0.013\), respectively). After additional adjustment for BMI, only MAP remained significantly different between POI and NM women (Table III).

The multivariable adjusted associations between FAI and cardiometabolic features, stratified for PCOS, POI and NM women, are depicted in Figure 1. Square, Model I is adjusted for age. Triangle, Model II is adjusted for age, ethnicity and smoking. The upper bounds of the confidence intervals of Models I and II in the PCOS group have been truncated (10.87 and 12.83, respectively). PCOS, polycystic ovary syndrome; POI, premature ovarian insufficiency; NM, women who experienced natural menopause; RC, women with regular menstrual cycles.

**Figure 1** Adjusted backtransformed means and confidence interval of the FAI. Square, Model I is adjusted for age. Triangle, Model II is adjusted for age, ethnicity and smoking. Circle, Model III is adjusted for age, ethnicity, smoking and BMI. The upper bounds of the confidence interval of Models I and II in the PCOS group have been truncated (10.87 and 12.83, respectively). PCOS, polycystic ovary syndrome; POI, premature ovarian insufficiency; NM, women who experienced natural menopause; RC, women with regular menstrual cycles.

**Table II** \(P\)-values for differences in androgens, SHBG and FAI between groups.

<table>
<thead>
<tr>
<th></th>
<th>PCOS versus RC</th>
<th>POI versus RC</th>
<th>POI versus NM</th>
<th>RC versus NM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model I</td>
<td>(&lt;0.001)</td>
<td>0.002</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>Model II</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.24</td>
<td>0.35</td>
</tr>
<tr>
<td>Model III</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.31</td>
<td>0.47</td>
</tr>
<tr>
<td>Androstenedione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model I</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>Model II</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>Model III</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>DHEAS</td>
<td>0.43</td>
<td>0.12</td>
<td>0.96</td>
<td>0.73</td>
</tr>
<tr>
<td>Model II</td>
<td>0.49</td>
<td>0.06</td>
<td>0.91</td>
<td>0.51</td>
</tr>
<tr>
<td>Model III</td>
<td>0.37</td>
<td>0.07</td>
<td>0.85</td>
<td>0.43</td>
</tr>
<tr>
<td>SHBG</td>
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</tr>
<tr>
<td>Model I</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.60</td>
<td>0.35</td>
</tr>
<tr>
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<td>(&lt;0.001)</td>
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<td>0.31</td>
</tr>
<tr>
<td>Model III</td>
<td>0.16</td>
<td>(&lt;0.001)</td>
<td>0.83</td>
<td>0.18</td>
</tr>
<tr>
<td>FAI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model I</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.12</td>
<td>0.99</td>
</tr>
<tr>
<td>Model II</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.17</td>
<td>0.85</td>
</tr>
<tr>
<td>Model III</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>0.51</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Model I is adjusted for age. Model II is adjusted for age, ethnicity and smoking. Model III is adjusted for age, ethnicity, smoking and BMI (conservative model).

**Table S1** \(P\)-values in bold are significant at \(P = 0.05\) after the Bonferroni correction for four comparisons (i.e. \(P < 0.0125\)).

DHEAS, dehydroepiandrosterone sulfate; FAI, free androgen index; PCOS, polycystic ovary syndrome; POI, premature ovarian insufficiency; NM, natural menopausal women.
Discussion

The primary aim of the current study was to compare androgen levels assessed by LC-MS/MS and to explore its potential association with various cardiometabolic features in women under different clinical conditions (i.e., PCOS, POI, NM, and RC). As expected, we found women with PCOS to be hyperandrogenic, and women with POI to be hypoandrogenic, compared with RC women. Differences in androgens between NM versus POI and NM versus RC were no longer significant after adjusting for age.

The second research aim of this study was to assess potential associations between androgen levels and cardiometabolic features in these women. We found that a higher FAI was associated with increased cardiovascular risk factors, i.e., elevated TG, insulin, HOMA-IR, and MAP in all women. A high FAI was also associated with increased glucose and decreased HDL levels in women with PCOS and NM, and with increased LDL in POI women. Adjustment for BMI substantially attenuated these associations. The strongest associations were observed in women with PCOS, even after accounting for age and following the menopausal transition. Previous cross-sectional studies have described lower androgen levels in post-menopausal women compared with pre-menopausal women, suggesting an association between androgen concentrations and menopausal status (Rozenberg et al., 1998; Bancroft and Cawood, 1996). However, most longitudinal studies demonstrated a continuous decline in total testosterone, DHEAS, and androstenedione levels with age, with no or very little variation occurring in relation to the menopausal status (Overlie et al., 1999; Davison et al., 2005; Rannevik et al., 2008). Our finding that androgen levels between POI versus NM and NM versus RC did not differ significantly after adjustment for age further supports the results from these longitudinal studies.

In the current study, we observed that an increase in FAI is associated with various cardiometabolic derangements in all women, irrespective of their clinical condition. Previous large studies in pre-, peri, and post-menopausal women also reported a positive correlation between circulating androgen levels and CVD risk (Rexrode et al., 2003; Sutton-Tyrrell et al., 2005; Mongraw-Chaffin et al., 2015). We observed a linear association between FAI and cardiometabolic features in all study groups. A linear association would indicate that women with the lowest androgen concentrations exhibit the most favorable cardiometabolic profile. However, in the current study, POI women exhibited the lowest androgen concentrations compared with other study groups, and POI has been repeatedly associated with an increased CVD risk (Knauff et al., 2008; Roeters van Lennep et al., 2014). These findings would correspond more with a u-shaped association between androgens and CVD with increased risk at both ends, as previously proposed (Laughlin et al., 2010; Soisson et al., 2013). This apparent discrepancy may be explained by variations in other biological factors contributing to CVD risk (e.g., circulating estrogen levels) or differences in study type, design, and sample size. Moreover, it is noteworthy that the cardiometabolic characteristics of women with POI and PCOS already approximate those of NM women, although these women are nearly 20–30 years older. This emphasizes the importance of performing a cardiometabolic evaluation in women diagnosed with PCOS as well as POI (Fauzer et al., 2012; Faubion et al., 2015).

Aside from testosterone, endogenous estrogen levels have also been extensively studied as a potential predictor of CVD risk. Circulating estradiol and testosterone are both bound to SHBG, although the binding affinity of SHBG for testosterone is higher than that for estradiol (Rosner, 1991). Increasing SHBG levels with steady estradiol/testosterone levels, therefore result in a relative increase in the bioactive fraction of estradiol compared with testosterone (Burke and Anderson, 1972). Bearing this interactive relation in mind, we performed a post hoc analysis in which we additionally adjusted the associations between FAI and cardiometabolic abnormalities for endogenous estradiol levels. We found that the observed associations between FAI and lipid metabolism were slightly attenuated. However, in the BMI-adjusted models, we did not find any significant changes in the associations between FAI and cardiometabolic features after additional adjustment for estradiol levels (data not shown). These results are in line with previous reported effects of estrogen on lipid metabolism and body fat distribution in women. Estradiol is known to influence the size and number of subcutaneous adipocytes and attenuates lipolysis, which may cause post-menopausal women to gain body fat after menopause (Ferrara et al., 2002; Kolovou et al., 2014).

BMI exerted a distinct effect on the observed associations between FAI and cardiometabolic features in our study. After adjustment for BMI, FAI remained significantly associated with most cardiometabolic features.
features in PCOS women. However, in NM women, only glucose metabolism parameters remained associated with FAI after adjustment for BMI, whereas in women with POI, there we no longer any significant associations. It has been proposed that the association between androgens and BMI is modulated through obesity-related changes in circulating levels of insulin and IGF-1 (Lukanova et al., 2004; Bezemer et al., 2005). Increasing BMI results in a concomitant rise in insulin levels, which inhibits the hepatic production of SHBG and therefore leads to higher levels of bioactive testosterone (Calle and Kaaks, 2004). Furthermore, insulin and IGF-1 directly stimulate the ovarian synthesis of androgens (Lukanova et al., 2004). Adipose tissue is also able to actively produce androgens through activity of 17β-hydroxysteroid dehydrogenase (Quinkler et al., 2004). Increased enzyme activity occurring with obesity might further contribute to androgen excess.

One of the strengths of this study is that we measured androgen levels with LC-MS/MS, which is currently considered the gold standard for androgen assessment in women (Rosner et al., 2010; Handelsman and Wartofsky, 2013). Furthermore, by selecting four distinct groups of women, we were able to study associations between androgen concentrations and cardiometabolic features in women with contrasting endocrine profiles, which, as such, has not been previously performed. Since this study provided abundant data, we presented selected data based on clinical/scientific relevance in order to restrict the number of performed comparisons.

A limitation of the current study is the lack of cardiometabolic features of RC women. Therefore, we were unable to directly compare associations between androgens and cardiometabolic features between these women and the other study groups. Another potential limitation is that although the use of FAI to study the unbound fraction of testosterone in women has been validated, it might be less precise than the direct measurement of the unbound fraction of testosterone in serum (Handelsman and Wartofsky, 2013). However, we solely used the FAI to study associations between androgens and cardiometabolic features, and did not attempt to establish absolute normative values of free androgens.
circulating androgen concentrations in different groups of women for clinical usage outside the current study.

The association between sex hormone levels, cardiometabolic abnormalities and the development of actual cardiovascular events in women has not been clearly established yet. Results from the few available long-term follow-up studies in the general female population report either no independent relationship between endogenous sex hormone levels and CVD events or only suggest a potential role for testosterone (Barrett-Connor and Goodman-Gruen, 1995; Laughlin et al., 2010; Chen et al., 2011). The potential association between ovarian dysfunction and future CVD events also remains partially unsettled. In a recent meta-analysis, POI was found to be an independent modest risk factor for ischemic heart disease and overall CVD, but not for stroke (Roeters van Lennep et al., 2014). The association between PCOS and cardiometabolic abnormalities (e.g. obesity, dyslipidemia, insulin resistance) has indeed been clearly established. However, previous reports on the actual development of CVD events in PCOS women have been inconsistent (Shaw et al., 2008; Iftikhar et al., 2012; Morgan et al., 2012; Hart and Doherty, 2015). Many of these studies suffer from several limitations, such as retrospective design, unclear phenotyping, limited follow-up, all of which hinder the interpretation of reported results. Unfortunately, due to the cross-sectional design of our study, we were not able to assess the potential relation between androgen levels and the development of actual cardiovascular events.

In summary, this study demonstrates that androgens intrinsically affect the cardiometabolic features of women with and without various forms of ovarian dysfunction. Increased androgen levels were strongly associated with impaired cardiometabolic features in all women participating in the current study. Differences in androgen levels between pre- and post-menopausal women were no longer significant after correcting for age, which indicates that predominantly chronological aging, rather than ovarian aging, influences variations in circulating androgen levels. Furthermore, we observed a substantial effect of BMI on circulating androgen levels. This study affirms the potent effect of androgens on

Figure 3  Associations between log FAI and cardiometabolic features for NM, POI and PCOS women, adjusted for age, smoking, ethnicity and BMI. Positive associations are depicted on the right side of the null line; negative associations are depicted on the left side of the null line. Associations are significant when the confidence interval (visualized as the horizontal line) does not reach the vertical null line. \( P\)-int: \( P\)-value for interaction, PCOS, polycystic ovary syndrome; POI, premature ovarian insufficiency; NM, natural menopausal women; HOMA-IR, homeostasis model assessment-insulin resistance; MAP, mean arterial pressure.
cardiometabolic features, implicating that androgens should indeed be regarded as important denominators of women’s health. Future research, regarding the role of androgens in the development of CVD and the potential modulatory effects of BMI, is required.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles
N.M.P.D. and L.J. contributed to the design of the study, the acquisition, analysis and interpretation of data, and the drafting and revising of the manuscript and gave final approval of the version to be published. M.P.H.K. and Y.B.R. contributed to the design of the study, the acquisition of data and the drafting and revising of the manuscript and gave final approval of the version to be published. O.H.F. and M.K. contributed to the analysis and interpretation of data, and the drafting and revising of the manuscript and gave final approval of the version to be published. J.S.E.L. and F.J.M.B. contributed to the design of the study, the interpretation of data and the drafting and revising of the manuscript and gave final approval of the version to be published. B.C.J.M.F. contributed to the conception and the design of this study, the interpretation of data and the drafting and revising of the manuscript and gave final approval of the version to be published.

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Conflict of interest
J.S.E.L. has received fees and grant support from the following companies (in alphabetical order): Ferring, Merck-Serono, Merck Sharpe & Dome, Organon, Shering Plough and Serono. In the last 5 years, B.C.J.M.F. has received fees and grant support from the following companies (in alphabetical order): Actavis, COGI, Euroscreen, Ferring, Finox, Genovum, Gedeon-Richter, Merck-Serono, OvaScience, Pantharei Bioscience, Preglem, Roche, Uteron and Watson laboratories. With regard to potential conflicts of interest, there is nothing further to disclose.

References
Androgens and cardiometabolic features in women


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