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Measurement of selected androgens using liquid chromatography-tandem mass spectrometry in reproductive-age women with Type 1 diabetes.

Running Title: Androgens measured by LC-MS/ MS in women with T1D

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Abstract

**Study question:** What information does androgen profiling using liquid chromatography tandem mass spectrometry (LC-MS/MS) provide in reproductive-age women with Type 1 diabetes (T1D)?

**Summary answer:** In T1D women, androstenedione proved most useful of the measured androgens in differentiating subgroups based on clinical phenotypes of hyperandrogenism (HA) and polycystic ovary syndrome (PCOS).

**What is known already:** The prevalence of HA and PCOS are increased in women with T1D. These observations are based on measurement of serum androgens using immunoassays, to-date no studies using LC-MS/MS have been reported in reproductive-age women with T1D.

**Study design, size, duration:** This was a cross-sectional study with recruitment of 3 groups of reproductive-age women: women with T1D (n=87), non-diabetic women with (N=97) and without PCOS (N=101).

**Participants/materials, setting, methods:** Using LC-MS/MS, we aimed to characterize androgen profiles and PCOS status in women with T1D, and interpret findings in relation to cohorts of non-diabetic women with and without PCOS.

**Main results and the role of chance:** Compared to non-diabetic women, dehydroepiandrosterone / dehydroepiandrosterone sulphate (DHEA/ DHEAS) ratio was lower (p<0.05) in women with T1D. Testosterone levels were greater in T1D women with clinical HA and anovulation compared to those without clinical HA and with regular cycles, while androstenedione levels were greater in T1D women with HA and anovulation compared to those with HA and regular cycles and also those without HA and with regular cycles (p<0.05 for all). Compared to T1D women without PCOS, the 18% of T1D women who had PCOS were younger with lower BMI, an older age of menarche, and were more likely to have a positive family history.
of PCOS (p<0.05 for all). Androgen levels did not differ between women with T1D and PCOS compared to BMI-matched non-diabetic women with PCOS, but androstenedione levels were greater in T1D women with PCOS compared to obese women with PCOS (p<0.05).

**Limitations, reasons for caution:** Relatively small subgroups of patients were studied, reducing the power to detect small differences. Free testosterone levels were not measured using equilibrium dialysis, and were not calculated – commonly used formulae have not been validated in T1D.

**Wider implications of the findings:** Androstenedione is a sensitive biochemical marker of clinical hyperandrogenism and PCOS in T1D. T1D women with PCOS are leaner than those without PCOS but are more likely to have a family history of PCOS. Women with T1D and PCOS have a similar biochemical phenotype to lean non-diabetic women with PCOS but differ from obese women with PCOS. The mechanisms underlying PCOS in T1D and its clinical significance require further investigation.

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**Key Words:** Hyperandrogenism, Polycystic Ovary Syndrome, Type 1 Diabetes, Liquid Chromatography-Mass Spectrometry, Androstenedione, Androgens, Biomarkers
Clinical hyperandrogenism (HA) and polycystic ovary syndrome (PCOS) commonly occur in reproductive-age women with Type 1 diabetes (T1D) (Codner, et al., 2007, Escobar-Morreale, et al., 2000, Miyoshi, et al., 2013, Zachurzok, et al., 2013). A recent meta-analysis has reported 24% prevalence of PCOS, 25% of HA, 25% of hirsutism, and 24% of menstrual dysfunction (Escobar-Morreale, et al., 2016) in such women. These high rates of androgen excess are probably due to increased systemic insulin levels, which result from subcutaneous injection in contrast to the physiologic situation in which insulin is released into the portal circulation. These observations are potentially important because of reproductive, gynecologic and cosmetic symptoms, and also because it has yet to be established whether HA or PCOS status influences insulin sensitivity and/or the significant burden of atherosclerotic disease in T1D.

To date measurement of androgens in all studies that have addressed HA and PCOS in T1D has been carried out using immunoassays. Direct immunoassays have been shown to overestimate testosterone concentrations, particularly in women, who have much lower androgen concentrations compared to men, and use of these assays has been demonstrated to result in frequent misclassification of HA and PCOS status in women without diabetes (Bhasin, et al., 2008, Rosner, et al., 2007, Tosi, et al., 2016). Although organic solvent extraction and/or gas chromatography steps prior to immunoassay analysis can improve their sensitivity and specificity, these assays still require proper validation. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a highly accurate method when properly validated and offers the advantage of measuring multiple steroids in a sample, and hence has been proposed as a “best prospect for gold standard” of testosterone measurement in the Endocrine Society Position Statement (Rosner, et al., 2007).

Using LC-MS/MS measurement of androgens, this study was designed to evaluate HA and PCOS in reproductive-age women with T1D. The specific aims were to (1) compare androgen
profiles between women with and without T1D; (2) determine how androgen levels relate to clinical evidence of hyperandrogenism and PCOS in T1D; (3) further characterise PCOS in T1D with regard to clinical characteristics and androgen profile.
Materials and Methods

Study design and subjects

To address the research questions listed above, the following analyses were carried out:

1) A cross-sectional comparison of androgen levels in women with T1D compared to BMI- and age-matched healthy control women.

2) A comparison of androgen levels between T1D women with clinical hyperandrogenism and anovulatory cycles, those with clinical hyperandrogenism but regular menstrual cycles, and those without clinical hyperandrogenism.

3) Further characterization of PCOS in T1D with regard to clinical characteristics and androgen profile through:
   a. Determination of the prevalence of PCOS (NIH) criteria in T1D, and what characteristics distinguish them from T1D women without PCOS.
   b. Comparison of clinical and biochemical variables between women with T1D and PCOS, and non-diabetic women with PCOS.

Subjects

For each analysis, subjects were selected from three groups of reproductive-age women; women with T1D (n=87), non-diabetic women without PCOS (n=101) and non-diabetic women with PCOS (n=97).

Women with T1D were recruited from the Diabetes Database in Tallaght Hospital, Dublin, Ireland. Of a total patient population of 1109 with T1D, 354 were women between the ages of 18 and 45 years. There were no differences in age (28.7±6.1 vs. 28.0 ±6.6 years), BMI (25.4±4.4
vs. 25.8±4.6 kg/m²) or haemoglobin A1c (HbA1c) (8.7±1.5 vs. 8.4±1.8 %) between those who took part in the study and those who did not.

All eligible women were contacted either by phone or at the time of their scheduled clinic visit. Subjects were excluded if they were non-Caucasian, pregnant, or lactating; had a BMI less than 18 or greater than 55 kg/m²; had a recent illness or any chronic illness likely to influence results; or were taking any medications likely to influence the results including hormonal contraception, antihypertensive, lipid-lowering medications, antiplatelet agents, anti-inflammatory agents, or nonprescription agents. Those with normal menstrual cycles were studied in the follicular phase.

The control group was comprised of normal volunteers on no medications recruited from the general population. The recruitment of normal volunteers was done via advertisement in the study hospital, local schools and community centres. All normal subjects were eumenorrheic with testosterone levels within the normal female range and were studied in the follicular phase of the menstrual cycle.

Women with PCOS who met the eligibility criteria were recruited by the study physician from the Endocrinology outpatient clinics in the Adelaide and Meath Hospital, Tallaght, Dublin. PCOS was defined according to the NIH criteria as chronic oligomenorrhea (fewer than nine menstrual periods per year) and clinical and/ or biochemical evidence of hyperandrogenism, in the absence of other disorders causing the same phenotype (Zawadzki JK, 1992). However, for the purposes of the current study, we only included those who had clinical hyperandrogenism.

Clinical criteria included hirsutism with Ferriman-Gallwey score greater than 9, acne, or male pattern alopecia; biochemical criteria included total testosterone, androstenedione, or dehydroepiandrosterone sulphate (DHEAS) greater than the laboratory reference range. Serum thyroid-stimulating hormone (TSH), free thyroxine, prolactin, LH, FSH, estradiol and 17-hydroxyprogesterone were measured in all PCOS subjects to exclude other disorders.

To compare T1D women with non-diabetic women, subjects were pair-matched for age and for
BMI (Table I). The 87 women with T1D were then subdivided into 3 groups according to their clinical phenotypes: those with T1D and PCOS (clinical hyperandrogenism and anovulatory cycles) according to the NIH criteria (T1D-PCOS); those with clinical hyperandrogenism (hirsutism with a Ferriman-Gallwey score greater than 9, acne, or male pattern alopecia) but regular cycles (T1D-HA), and those with no clinical features (T1D-No CF). The 16 women with T1D-PCOS were also compared to 16 non-diabetic women with PCOS matched for age and BMI, and 16 obese non-diabetic women with PCOS matched for age.

**Ethical Approval**

All study subjects gave their written signed consent to the study, which was approved by the Research Ethics Committee of the Adelaide and Meath Hospital and St. James’ Hospital (Dublin, Ireland).

**Measurement of anthropological data and baseline characteristics**

All subjects were studied after a 12-h fast and having avoided excessive exercise and alcohol for the previous 24 hours. They underwent estimation of body composition using auxological methods. Height (measured with a Harpenden stadiometer) and weight were measured in a hospital gown. Waist circumference (WC) and hip circumference were measured with a non-distensible flexible tape measure at the waist and hip. Each participant completed a health and lifestyle questionnaire, which included reproductive history, smoking history, and alcohol consumption.

**Laboratory methods**

Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module (Roche, Stockholm, Sweden); insulin was measured by electrochemiluminescence immunoassay on the Roche E Module; eGDR, (estimation of the glucose disposal rate, a validated measure of insulin sensitivity in T1D was calculated as previously described (Williams, et al., 2000) in T1D women.
only. Sex-hormone binding globulin (SHBG), estradiol, thyroid stimulating hormone (TSH), free thyroxine (FT4), prolactin, and cortisol were measured by standard chemiluminescence immunoassays (CVs <5% for all).

Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and plasma and serum was stored at −80°C until the end of the study. They were then transported on dry ice by courier delivery to the Institute of Metabolism and Systems Research (IMSR), University of Birmingham, Edgbaston, Birmingham. Using previously described techniques (O'Reilly, et al., 2014), serum testosterone, androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) were measured using a Waters Xevo mass spectrometer with Acquity uPLC system (Waters Ltd, Elstree, UK). LC/MS-MS conditions were an electrospray ionization source with capillary voltage 4.0 kV, a source temperature of 150°C, and a desolvation temperature of 500°C. Serum steroid oxime analysis (Kushnir, et al., 2010) was used for the measurement of testosterone, androstenedione, and DHEA and carried out in positive mode, whereas the measurement of serum DHEAS was performed in negative mode. Testosterone, androstenedione, and DHEA were extracted from 200 μL serum via liquid-liquid extraction using 1 mL tert-butyl-methyl-ether followed by derivatization into steroid oximes using 100 μL derivatization mixture (0.16 g hydroxylamine in 8 mL pyridine). For protein precipitation and extraction of DHEAS, 20 μL ZnSO₄, 0.1 mM, and 100 μL acetonitrile were added to 20 μL serum before evaporation under constant nitrogen flow (Chadwick, et al., 2005). All steroids were separated using an optimized gradient system consisting of methanol with 0.1% formic acid and quantified referring to a linear calibration series with appropriate internal standards. Each steroid was identified by matching retention times and two mass transitions in comparison with a deuterated reference compound. The information regarding the precision for each androgen assay has been previously described (Buttler, et al., 2015, O'Reilly, et al., 2017) and is shown in detail in Supplementary Table S1.
Statistical analysis was performed using Graph Pad Prism version 7.00, GraphPad Software, La Jolla California USA, www.graphpad.com. Results are presented as median and interquartile range (IQR) unless otherwise specified. Statistical analysis of clinical characteristics was made using Student’s t-test or Mann–Whitney U test for independent samples. One-Way ANOVA was used to analyze between group differences when more than 2 groups were reviewed, and the Kruskal-Wallis test was used for post hoc analysis. A p value <0.05 was considered significant. Graphical representation of data excluded any results 2 standard deviation above the mean, but all results were used in the analysis.
Results

Comparison of androgen levels in women with T1D compared to BMI and age-matched healthy control women

32 women with T1D were compared to 32 BMI- and age-matched healthy control women. Baseline characteristics are shown in Table I and androgen levels in Figure 1. There were no significant differences in waist-to-hip ratio (WHR) between the two groups (Table I). There were no significant differences in testosterone, androstenedione, SHBG, DHEAS or DHEA between the two groups. Compared to non-diabetic women, DHEA/ DHEAS ratio was significantly lower (p <0.05) in women with T1D (Figure 1).

Comparison of androgen levels between sub-groups of women with T1D

Baseline characteristics are shown in Table II and androgen levels in Figure 2. Testosterone levels were greater (p <0.05) in T1D-PCOS compared to T1D-No CF. Androstenedione levels were greater (p <0.005) in T1D-PCOS compared to T1D-HA and T1D-no CF. No between-group changes were observed in SHBG, DHEAS, DHEA or DHEA/ DHEAS ratio.

Characterisation of PCOS in T1D with regard to clinical characteristics and androgen profile.

a) Determination of the prevalence of PCOS (NIH) criteria in T1D, and what characteristics distinguish them from T1D women without PCOS

The prevalence of PCOS in women with T1D was 18% (16/ 87). T1D-PCOS women compared to those without PCOS were younger (26.5 vs. 29.0 years) and had lower BMI (23.4 vs. 25.3 kg/m2). T1D-PCOS women had an older age of menarche (13.0 vs. 12.5 years, p=0.024), and were more likely (12.5% vs. 2.8 %) to have a positive family history of PCOS.

b) Comparison of androgens between women T1D- PCOS women, BMI-matched non-diabetic women with PCOS and obese non-diabetic women with PCOS
Demographic variables are shown in Table III and androgen levels in Figure 3. Compared to obese women with PCOS, androstenedione levels were greater ($p < 0.05$) in T1D-PCOS women. SHBG levels were greater in T1D-PCOS women compared to obese non-diabetic women with PCOS. No other differences were observed between groups.
Discussion

To our knowledge this is the first study using LC-MS/MS to report androgen levels in reproductive-age women with T1D. Previous studies (Codner, et al., 2007, Escobar-Morreale, et al., 2000, Escobar-Morreale, et al., 2016, Miyoshi, et al., 2013, Zachurzok, et al., 2013) have used immunoassay techniques which can lack sensitivity and specificity, particularly at the relatively low concentrations of androgens observed in women (Tosi, et al., 2016). Among women with T1D, only testosterone and androstenedione helped discriminate between T1D-PCOS and those with T1D-No CF, androstenedione proving more sensitive as it also discriminated between those with T1D-HA and those with T1D-PCOS. Women with T1D-PCOS were younger with lower BMI, had an older age of menarche, and were more likely to have a family history of PCOS than those without PCOS. Androgen levels did not differ between women with T1D-PCOS compared to BMI-matched non-diabetic women with PCOS, but androstenedione levels were greater in T1D-PCOS compared to obese non-diabetic women with PCOS.

Clinical HA was observed in 49% of women with T1D, consistent with most previous studies (Escobar-Morreale, et al., 2016), while anovulatory cycles were observed in 30%. Eighteen percent had both and would thus meet NIH criteria for diagnosis of PCOS. Previous studies in non-diabetic women with PCOS demonstrated androstenedione (O'Reilly, et al., 2014) total testosterone/ dihydrotestosterone ratio (Munzker, et al., 2015), estrone (Stener-Victorin, et al., 2010) and 11-oxygenated androgens (O'Reilly, et al., 2017) as the most sensitive markers of androgen excess. In the current study, among the variables studied, androstenedione proved to be the most sensitive biochemical marker of PCOS diagnosis, levels being greater in T1D-PCOS compared to those with T1D-HA or T1D-No CF. Androstenedione levels also differentiated between T1D-PCOS women, and obese non-diabetic women with PCOS suggesting that it is potentially a useful clinical androgen measurement in T1D. We did not
measure dihydrotestosterone, estrone or 11-oxygenated androgens and therefore cannot compare the usefulness of these variables with androstenedione.

Although absolute levels of DHEA-OX and DHEAS did not differ between women with T1D and healthy controls, a lower DHEA/ DHEAS ratio was observed among T1D subjects implying increased inactivation of DHEA by sulfation in the adrenal cortex and in the liver. This effect is potentially explained by systemic hyperinsulinemia, which is characteristic of T1D. Systemic hyperinsulinemia is generally considered to play a role in increasing active androgen burden in the circulation through direct effects on the ovary and adipose tissue (O'Reilly, et al., 2017, O'Reilly, et al., 2014); it could therefore be hypothesized that enhanced inactivation of DHEA to DHEAS, resulting in a lower DHEA/ DHEAS ratio, is a compensatory mechanism to remove active androgens from the circulation in patients with T1D.

T1D women with PCOS were younger and of lower BMI than those without PCOS. Indices of insulin resistance including daily insulin dose and eGDR, a validated index of insulin sensitivity in T1D (Williams, et al., 2000) did not differ between those with and without PCOS. A family history of PCOS was predictive of PCOS in T1D suggesting contribution of genes associated with PCOS to the development of the phenotype in T1D. Other than a marginally later age of menarche in those with PCOS, we did not find any other differences compared to those without PCOS. The androgen profile of women with T1D and PCOS did not differ from BMI-matched non-diabetic women with PCOS, although androstenedione levels were greater in the diabetic PCOS compared to obese non-diabetic women with PCOS. It appears therefore that PCOS in T1D is a similar condition to lean PCOS, but potentially differs from the more common phenotype of PCOS in obese women.

Testosterone circulates bound to SHBG and albumins and only free, unbound testosterone exerts biological effect. A limitation of this study is the absence of data for free testosterone levels. This is important as substantially higher SHBG levels in women with T1D (probably due
to low insulin levels in the portal circulation (Yki-Jarvinen, et al., 1995) compared to non-diabetic women potentially result in lower free testosterone levels. To confidently understand this effect, however, it would be necessary to measure free testosterone using the gold standard technique of equilibrium dialysis (Vermeulen, et al., 1999). While free testosterone is often calculated, there are conflicting reports as to how well this correlates with measured free testosterone, (Zakharov, et al., 2015) (Hackbarth, et al., 2011, Ly, et al., 2010, Salameh, et al., 2010) and importantly there is no data to support the validity of this estimation in T1D, where particularly high levels of SHBG would potentially influence calculated values.

In summary, our findings have helped further characterize HA and PCOS in women with T1D. Androstenedione appears to be the most discriminatory biochemical marker as it differs between T1D women with and without HA, and between lean (PCOS and non-diabetic) and obese women with PCOS. The biochemical phenotype of PCOS in T1D is similar to that in lean non-diabetic women with PCOS, and is more likely to occur when there is a family history of PCOS. The clinical relevance of PCOS and HA in T1D is not known but future studies aimed at determining whether they contribute cardio-metabolic abnormalities are now warranted.
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Authors’ Roles
A.G.: study design, data entry and collection, data analysis and interpretation, manuscript writing; A.P.: data analysis and interpretation, manuscript writing; M.A.: data entry and collection; A.McG.: study design, critical reading of manuscript; N.P.: study design, data entry and collection, data analysis and interpretation; G.B.: study design, critical reading of manuscript; A.E.T.: data analysis and interpretation, manuscript writing, critical reading of manuscript; M.W.O.R.: data analysis and interpretation, manuscript writing, critical reading of manuscript; W.A.: data analysis and interpretation, critical reading of manuscript; K.M.: study design, critical reading of manuscript; L.A.B.: data analysis and interpretation, critical reading of manuscript; M.S.: data analysis and interpretation, manuscript writing, critical reading of manuscript; J.G.: study design, data analysis and interpretation, manuscript writing and critical reading of manuscript.

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Conflict of interest
The authors report no financial or other conflict of interest relevant to the subject of this article.
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Figure Legends

Figure 1. Comparison of androgen levels in women with Type 1 diabetes (T1D) and non-diabetic women (* p< 0.05). Horizontal lines represent median with interquartile range.

Figure 2. Comparison of androgen levels between T1D women with clinical hyperandrogenism and anovulatory cycles (T1D-PCOS), those with clinical hyperandrogenism but regular cycles (T1D-HA) and those without clinical features (T1D-No CF) (* p< 0.05 ** p<0.005 *** p<0.0005). Horizontal lines represent median with interquartile range.

Figure 3. Comparison of androgen levels in three different groups of women with PCOS: T1D and PCOS (T1D-P), BMI-matched women with PCOS (Lean-P) and overweight women with PCOS (Obese-P) (* p<0.05, ** p<0.005, *** p<0.0005). Horizontal lines represent median with interquartile range.