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

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

3

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

5

6 A Gunness[1], A Pazderska [1], M Ahmed [1], A. McGowan[1], N Phelan[1], G Boran [2], A E Taylor[3], M W
7 O'Reilly[3], W Arlt[3], K Moore[1],LA Behan[1],M Sherlock[1] and J Gibney*[1]

8

9 [1]Department of Endocrinology and [2]Clinical Chemistry, Adelaide and Meath Hospital, Tallaght,
10 Dublin 24, Republic of Ireland .[3]Institute of Metabolism and Systems Research (IMSR),
11 University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

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13

14 **Correspondence address:** James.Gibney@amnch.ie

15 **Abstract**

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

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

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

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

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

31 **Main results and the role of chance**: Compared to non-diabetic women,
32 dehydroepiandrosterone / dehydroepiandrosterone sulphate (DHEA/ DHEAS) ratio was lower
33 ($p<0.05$) in women with T1D. Testosterone levels were greater in T1D women with clinical HA
34 and anovulation compared to those without clinical HA and with regular cycles, while
35 androstenedione levels were greater in T1D women with HA and anovulation compared to those
36 with HA and regular cycles and also those without HA and with regular cycles ($p<0.05$ for all).
37 Compared to T1D women without PCOS, the 18% of T1D women who had PCOS were younger
38 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

Introduction

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

83 profiles between women with and without T1D; (2) determine how androgen levels relate to
84 clinical evidence of hyperandrogenism and PCOS in T1D; (3) further characterise PCOS in T1D
85 with regard to clinical characteristics and androgen profile.
86

87 **Materials and Methods**

88 *Study design and subjects*

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

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

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

95 3) Further characterization of PCOS in T1D with regard to clinical characteristics and
96 androgen profile through:

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

99 b. Comparison of clinical and biochemical variables between women with T1D and
100 PCOS, and non-diabetic women with PCOS.

101

102 *Subjects*

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

106 Women with T1D were recruited from the Diabetes Database in Tallaght Hospital, Dublin,
107 Ireland. Of a total patient population of 1109 with T1D, 354 were women between the ages of 18
108 and 45 years. There were no differences in age (28.7±6.1 vs. 28.0 ±6.6 years), BMI (25.4±4.4

109 vs. 25.8±4.6 kg/m^2) or haemoglobin A1c (HbA1c) (8.7±1.5 vs. 8.4±1.8 %) between those who
110 took part in the study and those who did not.

111 All eligible women were contacted either by phone or at the time of their scheduled clinic visit.
112 Subjects were excluded if they were non-Caucasian, pregnant, or lactating; had a BMI less than
113 18 or greater than 55 kg/m^2; had a recent illness or any chronic illness likely to influence results;
114 or were taking any medications likely to influence the results including hormonal contraception,
115 antihypertensive, lipid-lowering medications, antiplatelet agents, anti-inflammatory agents, or
116 nonprescription agents. Those with normal menstrual cycles were studied in the follicular phase.

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

122 Women with PCOS who met the eligibility criteria were recruited by the study physician from the
123 Endocrinology outpatient clinics in the Adelaide and Meath Hospital, Tallaght, Dublin. PCOS
124 was defined according to the NIH criteria as chronic oligomenorrhea (fewer than nine menstrual
125 periods per year) and clinical and/ or biochemical evidence of hyperandrogenism, in the
126 absence of other disorders causing the same phenotype (Zawadzki JK, 1992). However, for the
127 purposes of the current study, we only included those who had clinical hyperandrogenism.
128 Clinical criteria included hirsutism with Ferriman-Gallwey score greater than 9, acne, or male
129 pattern alopecia; biochemical criteria included total testosterone, androstenedione,
130 or dehydroepiandrosterone sulphate (DHEAS) greater than the laboratory reference
131 range. Serum thyroid-stimulating hormone (TSH), free thyroxine, prolactin, LH, FSH, estradiol
132 and 17-hydroxyprogesterone were measured in all PCOS subjects to exclude other disorders.

133 To compare T1D women with non-diabetic women, subjects were pair-matched for age and for

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

141 *Ethical Approval*

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

145 *Measurement of anthropological data and baseline characteristics*

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

153 *Laboratory methods*

154 Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module (Roche,
155 Stockholm, Sweden); insulin was measured by electrochemiluminescense immunoassay on the
156 Roche E Module; eGDR, (estimation of the glucose disposal rate, a validated measure of insulin
157 sensitivity in T1D was calculated as previously described (Williams, et al., 2000) in T1D women

158 only. Sex-hormone binding globulin (SHBG), estradiol, thyroid stimulating hormone (TSH), free
159 thyroxine (FT4), prolactin, and cortisol were measured by standard chemiluminescence
160 immunoassays (CVs <5% for all).
161 Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and plasma and serum was
162 stored at −80°C until the end of the study. They were then transported on dry ice by courier
163 delivery to the Institute of Metabolism and Systems Research (IMSR), University of Birmingham,
164 Edgbaston, Birmingham. Using previously described techniques (O'Reilly, et al., 2014), serum
165 testosterone, androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone
166 sulfate (DHEAS) were measured using a Waters Xevo mass spectrometer with Acquity uPLC
167 system (Waters Ltd, Elstree, UK). LC/MS-MS conditions were an electrospray ionization source
168 with capillary voltage 4.0 kV, a source temperature of 150°C, and a desolvation temperature of
169 500°C. Serum steroid oxime analysis (Kushnir, et al., 2010) was used for the measurement of
170 testosterone, androtenedione, and DHEA and carried out in positive mode, whereas the
171 measurement of serum DHEAS was performed in negative mode. Testosterone,
172 androstenedione, and DHEA were extracted from 200 µL serum via liquid-liquid extraction using
173 1 mL tert-butyl-methyl-ether followed by derivatization into steroid oximes using 100 µL
174 derivatization mixture (0.16 g hydroxylamine in 8 mL pyridine). For protein precipitation and
175 extraction of DHEAS, 20 µL $ZnSO_4$, 0.1 mM, and 100 µL acetonitrile were added to 20 µL
176 serum before evaporation under constant nitrogen flow (Chadwick, et al., 2005). All steroids
177 were separated using an optimized gradient system consisting of methanol with 0.1% formic
178 acid and quantified referring to a linear calibration series with appropriate internal standards.
179 Each steroid was identified by matching retention times and two mass transitions in comparison
180 with a deuterated reference compound. The information regarding the precision for each
181 androgen assay has been previously described (Buttler, et al., 2015, O'Reilly, et al., 2017) and
182 is shown in detail in Supplementary Table S1.
183

184 *Statistical analysis*

185 Statistical analysis was performed using Graph Pad Prism version 7.00, GraphPad Software, La
186 Jolla California USA, www.graphpad.com
187 Results are presented as median and interquartile range (IQR) unless otherwise specified.
188 Statistical analysis of clinical characteristics was made using Student's t-test or Mann–Whitney
189 U test for independent samples. One-Way ANOVA was used to analyze between group
190 differences when more than 2 groups were reviewed, and the Kruskal-Wallis test was used for
191 post hoc analysis. A p value <0.05 was considered significant. Graphical representation of data
192 excluded any results 2 standard deviation above the mean, but all results were used in the
193 analysis.
194

195 **Results**

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

198 32 women with T1D were compared to 32 BMI- and age-matched healthy control women.
199 Baseline characteristics are shown in Table I and androgen levels in Figure 1. There were no
200 significant differences in waist-to-hip ratio (WHR) between the two groups (Table I). There were
201 no significant differences in testosterone, androstenedione, SHBG, DHEAS or DHEA between
202 the two groups. Compared to non-diabetic women, DHEA/ DHEAS ratio was significantly lower
203 ($p < 0.05$) in women with T1D (Figure 1).

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

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

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

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

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

216 b) Comparison of androgens between women T1D- PCOS women, BMI-matched non-diabetic
217 women with PCOS and obese non-diabetic women with PCOS

218 Demographic variables are shown in Table III and androgen levels in Figure 3. Compared to
219 obese women with PCOS, androstenedione levels were greater ($p < 0.05$) in T1D-PCOS
220 women. SHBG levels were greater in T1D-PCOS women compared to obese non-diabetic
221 women with PCOS. No other differences were observed between groups.

222

223

224 **Discussion**

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

238 Clinical HA was observed in 49% of women with T1D, consistent with most previous studies
239 (Escobar-Morreale, et al., 2016), while anovulatory cycles were observed in 30%. Eighteen
240 percent had both and would thus meet NIH criteria for diagnosis of PCOS. Previous studies in
241 non-diabetic women with PCOS demonstrated androstenedione (O'Reilly, et al., 2014) total
242 testosterone/ dihydrotestosterone ratio (Munzker, et al., 2015), estrone (Stener-Victorin, et al.,
243 2010) and 11-oxygenated androgens (O'Reilly, et al., 2017) as the most sensitive markers of
244 androgen excess. In the current study, among the variables studied, androstenedione proved to
245 be the most sensitive biochemical marker of PCOS diagnosis, levels being greater in T1D-
246 PCOS compared to those with T1D-HA or T1D-No CF. Androstenedione levels also
247 differentiated between T1D-PCOS women, and obese non-diabetic women with PCOS
248 suggesting that it is potentially a useful clinical androgen measurement in T1D. We did not

249 measure dihydrotestosterone, estrone or 11-oxygenated androgens and therefore cannot
250 compare the usefulness of these variables with androstenedione.

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

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

271 Testosterone circulates bound to SHBG and albumins and only free, unbound testosterone
272 exerts biological effect. A limitation of this study is the absence of data for free testosterone
273 levels. This is important as substantially higher SHBG levels in women with T1D (probably due

274 to low insulin levels in the portal circulation (Yki-Jarvinen, et al., 1995) compared to non-diabetic
275 women potentially result in lower free testosterone levels. To confidently understand this effect,
276 however, it would be necessary to measure free testosterone using the gold standard technique
277 of equilibrium dialysis (Vermeulen, et al., 1999). While free testosterone is often calculated,
278 there are conflicting reports as to how well this correlates with measured free testosterone,
279 (Zakharov, et al., 2015) (Hackbarth, et al., 2011, Ly, et al., 2010, Salameh, et al., 2010) and
280 importantly there is no data to support the validity of this estimation in T1D, where particularly
281 high levels of SHBG would potentially influence calculated values.

282 In summary, our findings have helped further characterize HA and PCOS in women with T1D.
283 Androstenedione appears to be the most discriminatory biochemical marker as it differs
284 between T1D women with and without HA, and between lean (PCOS and non-diabetic) and
285 obese women with PCOS. The biochemical phenotype of PCOS in T1D is similar to that in lean
286 non-diabetic women with PCOS, and is more likely to occur when there is a family history of
287 PCOS. The clinical relevance of PCOS and HA in T1D is not known but future studies aimed at
288 determining whether they contribute cardio-metabolic abnormalities are now warranted.

289

290

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293
294 **Authors' Roles**
295
296 A.G.: study design, data entry and collection, data analysis and interpretation,
297 manuscript writing; A.P.: data analysis and interpretation, manuscript writing; M.A.: data
298 entry and collection; A.McG.: study design, critical reading of manuscript; N.P.: study
299 design, data entry and collection, data analysis and interpretation; G.B.: study design,
300 critical reading of manuscript; A.E.T.: data analysis and interpretation, manuscript
301 writing, critical reading of manuscript; M.W.O.R.: data analysis and interpretation,
302 manuscript writing, critical reading of manuscript; W.A.: data analysis and interpretation,
303 critical reading of manuscript; K.M.: study design, critical reading of manuscript; L.A.B.:
304 data analysis and interpretation ,critical reading of manuscript; M.S.: data analysis and
305 interpretation, manuscript writing, critical reading of manuscript; J.G.: study design, data
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318 The authors report no financial or other conflict of interest relevant to the subject of this
319 article.
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323

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325

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397 **Figure Legends**

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

400

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

405

406 **Figure 3.** Comparison of androgen levels in three different groups of women with PCOS : T1DM
407 and PCOS (T1D-P), BMI-matched women with PCOS (Lean-P) and overweight women with
408 PCOS (Obese-P) (* p<0.05, ** p<0.005, *** p<0.0005). Horizontal lines represent median with
409 interquartile range.