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

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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

39   of PCOS (p<0.05 for all). Androgen levels did not differ between women with T1D and PCOS

40   compared to BMI-matched non-diabetic women with PCOS, but androstenedione levels were

41   greater in T1D women with PCOS compared to obese women with PCOS (p<0.05).

42   **Limitations, reasons for caution**: Relatively small subgroups of patients were studied,

43   reducing the power to detect small differences. Free testosterone levels were not measured

44   using equilibrium dialysis, and were not calculated – commonly used formulae have not been

45   validated in T1D.

46   **Wider implications of the findings**: Androstenedione is a sensitive biochemical marker of

47   clinical hyperandrogenism and PCOS in T1D. T1D women with PCOS are leaner than those

48   without PCOS but are more likely to have a family history of PCOS. Women with T1D and

49   PCOS have a similar biochemical phenotype to lean non-diabetic women with PCOS but differ

50   from obese women with PCOS. The mechanisms underlying PCOS in T1D and its clinical

51   significance require further investigation.

52   **Study funding/competing interest(s):** The study was part-funded by the Meath Foundation.

53   The authors have no competing interests.

54

55   **Key Words:** Hyperandrogenism, Polycystic Ovary Syndrome, Type 1 Diabetes, Liquid

56   Chromatography-Mass Spectrometry, Androstenedione, Androgens, Biomarkers

57

58   **Introduction**

59   Clinical hyperandrogenism (HA) and polycystic ovary syndrome (PCOS) commonly occur in

60   reproductive-age women with Type 1 diabetes (T1D) (Codner, et al., 2007, Escobar-Morreale,

61   et al., 2000, Miyoshi, et al., 2013, Zachurzok, et al., 2013). A recent meta-analysis has reported

62   24% prevalence of PCOS, 25% of HA, 25% of hirsutism, and 24% of menstrual dysfunction

63   (Escobar-Morreale, et al., 2016) in such women. These high rates of androgen excess are

64   probably due to increased systemic insulin levels, which result from subcutaneous injection in

65   contrast to the physiologic situation in which insulin is released into the portal circulation. These

66   observations are potentially important because of reproductive, gynecologic and cosmetic

67   symptoms, and also because it has yet to be established whether HA or PCOS status

68   influences insulin sensitivity and/ or the significant burden of atherosclerotic disease in T1D.

69   To date measurement of androgens in all studies that have addressed HA and PCOS in T1D

70   has been carried out using immunoassays. Direct immunoassays have been shown to

71   overestimate testosterone concentrations, particularly in women, who have much lower

72   androgen concentrations compared to men, and use of these assays has been demonstrated to

73   result in frequent misclassification of HA and PCOS status in women without diabetes (Bhasin,

74   et al., 2008, Rosner, et al., 2007, Tosi, et al., 2016). Although organic solvent extraction and/or

75   gas chromatography steps prior to immunoassay analysis can improve their sensitivity and

76   specificity, these assays still require proper validation. Liquid chromatography-tandem mass

77   spectrometry (LC-MS/MS) is a highly accurate method when properly validated and offers the

78   advantage of measuring multiple steroids in a sample, and hence has been proposed as a "best

79   prospect for gold standard" of testosterone measurement in the Endocrine Society Position

80   Statement (Rosner, et al., 2007)

81   Using LC-MS/MS measurement of androgens, this study was designed to evaluate HA and

82   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

Discussion

224

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.

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294 **Authors' Roles**
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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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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.