1   **Predominant activation of 11-oxygenated androgens by peripheral blood mononuclear cells**

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9   **Short title**: Androgen activation in PBMCs

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24   **Abstract (250 words max)**

25   **Context**: Androgens are important modulators of immune cell function impacting proliferation,

26   differentiation and cytokine production. The local generation of active androgens from circulating androgen

27   precursors is an important mediator of androgen action in peripheral androgen target cells of tissue.

28   **Objective**: To characterize the activation of classic and 11-oxygenated androgens in human peripheral

29   blood mononuclear cells (PBMCs).

30   **Methods**: PBMCs and natural killer cells were isolated from healthy male donors and incubated ex vivo

31   with precursors and end products of the classic and 11-oxygenated androgen pathways. Steroid

32   concentrations were quantified by liquid chromatography-tandem mass spectrometry. The expression of

33   genes encoding steroid metabolizing enzymes was assessed by quantitative PCR.

34   **Results**: The enzyme AKR1C3 is the major reductive 17β-hydroxysteroid dehydrogenase in PBMCs and

35   has higher activity for the generation of the active 11-oxygenated androgen 11-ketotestosterone than for the

36   generation of the classic androgen testosterone from their respective precursors. Natural killer cells are the

37   major site of AKR1C3 expression and activity within the PBMC compartment. Steroid 5α-reductase type

38   1 catalyzes the 5α-reduction of classic but not 11-oxygenated androgens in PBMCs. Lag time prior to the

39   separation of cellular components from whole blood sample increases 11KT serum concentrations in a

40   time-dependent fashion, with significant increases detected from two hours after blood collection.

41   **Conclusions**: 11-oxygenated androgens are the preferred substrates for androgen activation by AKR1C3

42   in PBMCs, primarily conveyed by natural killer cell AKR1C3 activity, yielding 11KT the major active

43   androgen in PBMCs. Androgen metabolism by PBMCs can affect the levels of 11-oxygenated androgens

44   measured in serum samples, if samples are not separated in a timely fashion.

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Introduction

A critical step in androgen activation is the conversion of the androgen precursor androstenedione (A4) to the active androgen testosterone (T) (1-3), which occurs in the testes catalyzed by 17β-hydroxysteroid dehydrogenase type 3. However, in the majority of peripheral target tissues of androgen action, the intracrine activation of A4 to T is catalyzed by the enzyme AKR1C3 (aldo-keto reductase 1C3, also termed 17β-hydroxysteroid dehydrogenase type 5) (1-3), thereby representing a key regulator of local androgen exposure. Peripheral blood mononuclear cells (PBMCs) can activate A4 to T (4, 5) catalyzed by AKR1C3 (6). Androgens impact on a multitude of immune cell functions, including proliferation, differentiation, apoptosis, cytokine and immunoglobulin productions (7, 8) and also link immune and metabolic regulation (9).

Recent *in-vitro* work has shown that AKR1C3 has an approximately 8-fold higher catalytic efficiency for the generation of the 11-oxygenated androgen 11-ketotestosterone (11KT) from its precursor 11-ketoandrostenedione (11KA4) than for the generation of T from A4 (10). 11KT activates the androgen receptor with potency and efficacy similar to T (11-13) and is the predominant circulating androgen in androgen excess conditions including congenital adrenal hyperplasia, polycystic ovary syndrome and premature adrenarche (12, 14, 15). 11KT is derived from the abundant adrenal precursor steroid 11β-hydroxyandrostenedione (11OHA4) (13, 16), which is converted to 11KA4 by peripheral tissue expressing 11β-hydroxysteroid dehydrogenase type 2 (17, 18) prior to activation to 11KT by AKR1C3 (10, 16).

Of note, a number of studies have shown gradual increases in testosterone measured in serum, if the blood sample is left unseparated from cellular components for an increased period of time, using either immunoassays (19-21) or ultra-high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) for steroid quantification (22, 23). One of those studies additionally investigated the effect of time before separation on 11KT concentrations measured in serum and observed a much more pronounced increase in 11KT than T over time (23), which could have a differential impact on the measurement of circulating 11-oxygenated androgen concentrations.

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70   In this study, we explore this further and undertake a detailed characterization of androgen

71   activation via both classic and 11-oxygenated androgen biosynthesis pathways in the human peripheral

72   blood cell compartment and subpopulations, utilizing PBMCs isolated from healthy volunteers.

73   **Material and Methods**

74   **Blood collection**

75   The collection of blood for PBMC isolation and serum steroid analysis underwent ethical approval

76   by the Science, Technology, Engineering and Mathematics Ethical Review Committee of the University of

77   Birmingham (ERN_17-0494, ERN_17-0494A, and ERN_14-0446) and all participants gave informed,

78   written consent prior to study participation. Exclusion criteria for participation were any acute or chronic

79   disease affecting steroid biosynthesis or metabolism and intake of any medication known to interfere with

80   steroid biosynthesis or metabolism. All blood samples were collected between 09:00h and 11:00h; 50 mL

81   of blood were collected into K_2 EDTA Vacutainers^{TM} (purple top) for the immediate isolation of PBMCs.

82   To assess the effect of the time period between blood collection and separation of the cellular components

83   by centrifugation on measured serum steroid concentrations, blood was collected into several SST^{TM}

84   Vacutainers^{TM} (gold top). The tubes were left unseparated at room temperature in an air-conditioned room

85   and were separated by centrifugation ($2000g$, 10 min) after defined time periods (0, 1, 2, 4, 6 and 24 hours).

86   The separated serum was stored at -80 °C.

87   **Ex vivo steroid metabolism assays in peripheral blood mononuclear cells**

88   PBMCs were isolated from blood by density gradient centrifugation using Ficoll Paque Plus (GE

89   Healthcare) following the manufacturer's instructions. Isolated PBMCs were washed in RPMI-1640

90   (Sigma-Aldrich) supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin, counted and

91   assessed for viability by trypan blue exclusion. A minimum of $3x10^6$ cells was frozen in 400 μL Trireagent

92   (Sigma Aldrich) and stored at -80 °C for RNA analysis at a later date. For steroid metabolism assays, $3x10^6$

93   cells were incubated with 100 nM of the respective steroid in a final volume of 500 μL RPMI-1640

94   supplemented with penicillin and streptomycin. Steroids were added from stock solutions in methanol and

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95   the final methanol concentration in the samples was 0.00304% (v/v). Technical replicates were prepared, if

96   sufficient cell numbers were available. During the incubation, the samples were constantly gently rotated

97   at 37 °C for 24 hours. For each experiment, incubations of cells with methanol only and cell-free incubations

98   for all steroids were prepared as controls as well as dilutions of each steroid at 100 nM in medium that were

99   frozen at -20 °C immediately after preparation. At the end of the incubation period samples centrifuged for

100   2 minutes at 10,000 rpm. The supernatant was stored at -20 °C for steroid analysis. The cell pellet was

101   washed in PBS, suspended in 50 µL lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5%

102   sodium deoxycholate, 1% Trition X-100, 0.1 mM DTE, 0.1 mM PMSF, 0.1 mM EDTA) and stored at -80

103   °C for protein quantification.

Natural killer cell ex vivo steroid metabolism assays

105   PBMCs were prepared as described above and NK cells were subsequently enriched using the

106   MACS human NK cell isolation kit (Miltenyi Biotec) as per the manufacturer's instructions. Cells were

107   checked for viability, counted and steroid conversion assays were set up as described above.

Steroid analysis by tandem mass spectrometry

109   The following steroids were quantified by LC-MS/MS in the medium supernatant of the PBMC

110   incubations and serum samples as previously described (15): 11β-hydroxy-5α-androstanedione (11OH-5α-

111   dione, 5α-androstane-11β-ol-3,17-dione), 11β-hydroxyandrostenedione (11OHA4, 4-androstene-11β-ol-

112   3,17-dione), 11β-hydroxytestosterone (11OHT, 4-androstene-11β,17β-diol-3-one), 11-ketoandrosterone

113   (11KA4, 4-androstene-3,11,17-trione), 11-ketotestosterone (11KT, 4-androstene-17β-diol-3,11-dione), 5α-

114   dihydrotestosterone (DHT, 5α-androstane-17β-ol-3-one), 5α-androstanediol (Adiol, 5α-androstane-3α,17β-

115   diol), 5α-androstanedione (5α-dione, 5α-androstane-3,17-dione), androstenedione (A4, 4-androstene-3,17-

116   dione), androsterone (An, 5α-androstane-3α-ol-17-one), dehydroepiandrosterone (DHEA, 5-androstene-

117   3β-ol-17-one), testosterone (T, 4-androstene-17β-ol-3-one). In addition, 11-keto-5α-dihydrotestosterone

118   (11KDHT, 5α-androstane-17β-ol-3,11-dione) and 11-keto-5α-androstanedione (11K-5α-dione, 5α-

119   androstane-3,11,17-dione) were analyzed by ultra-high performance supercritical fluid chromatography-

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120   tandem mass spectrometry (UHPSFC-MS/MS) as previously described (24). For *ex-vivo* cell incubations,

121   steroid concentrations or their ratios were normalized to the total protein content of the incubations as

122   determined in the supernatant after cell lysis using the DC Protein Assay (Bio-Rad).

123   **RNA extraction and semi-quantitative PCR**

124   Samples stored in TRI reagent were defrosted and the RNA in the aqueous phase of the phenol-

125   chloroform extraction was purified using the RNeasy Mini Kit (Qiagen). RNA concentrations were

126   determined from the absorbance of the sample at 260 nm using a Nanodrop spectrophotometer and reverse

127   transcription was performed using Applied Biosystems™ TaqMan™ Reverse Transcription Reagents

128   following the manufacturer's protocol. Quantitative PCR was performed on an ABI 7900HT sequence

129   detection system (Perkin Elmer, Applied Biosystems) using TaqMan™ Gene Expression Assays (FAM-

130   labelled) and the SensiFASTTM Probe Hi-ROX kit (Bioline). ΔCt was calculated as Ct [Target]-Geometric

131   mean (Ct [HPRT1], Ct [GAPDH]). Gene expression in arbitrary units (A.U.) was calculated as $1000*2^{-}$

132    $^{\Delta Ct}$. For targets not reproducibly detected in duplicate reactions, relative gene expression is shown as 0.

133   **Analysis of published RNAseq data from PBMC subpopulations**

134   We accessed dice-database.org (25) to investigate *AKR1C3* expression determined by RNAseq in

135   FACS-sorted PBMC subpopulations. The database contains expression data from 54 male and 37 female

136   healthy donors (age range 18-61 years). *AKR1C3* expression data in transcript per million (TPM) were

137   downloaded and plotted in GraphPad Prism.

138   **Statistical analysis**

139   Changes in serum steroid concentrations were analyzed in GraphPad Prism 8 using ANOVA

140   followed by a Dunnett multiple comparison test to compare each timepoint against the sample processed

141   immediately after collection (0h). Statistical analysis of differences in steroid concentrations in the

142   supernatants from the PBMC *ex-vivo* incubations was performed by Wilcoxon matched-pairs signed rank

143   test. Statistical analysis of RNAseq was performed by one-way ANOVA followed by Tukey's multiple

144   comparisons test.

Results

Androgen activation in PBMCs is primarily catalyzed by AKR1C3 and SRD5A1

Using qPCR, we identified AKR1C3 as the major reductive, activating 17β-hydroxysteroid dehydrogenase isoform in PBMCs, while HSD17B3, generally considered a testes-specific isoform, was expressed at very low levels only (**Fig. 1A**). While we could not detect expression of steroid 5α-reductase type 2 (SRD5A2) and 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) in any of the samples from 14 donors using our qPCR assay, we detected consistently high expression of steroid 5α-reductase type 1 (SRD5A1) in samples from all donors (n=14) and detected 17β-hydroxysteroid dehydrogenase type 4 (HSD17B4) expression in samples from 6 of 14 donors. This indicates that SRD5A1 is responsible for the 5α-reduction we observe in our PBMC incubations with androgen substrates while HSD17B4 catalyzes the oxidative, inactivating 17β-hydroxysteroid dehydrogenase activity we observed (**Fig. 1A**), consistent with previously published findings (6). HSD11B1 expression was detectable in samples from 13/14 donors, while HSD11B2 mRNA was not detected. **Fig. 1B** schematically illustrates the enzymes identified by qPCR as in PBMCs and their roles in androgen activation and inactivation in the classic and 11-oxygenated androgen biosynthesis pathways.

PBMCs preferentially activate 11-oxygenated androgens

In order to unravel the pathways of androgen metabolism in PBMCs, we isolated PBMCs from male healthy donors and performed ex vivo incubations, using as substrates several androgen precursors and active androgens from both the classic (DHEA, A4 and T) and 11-oxygenated androgen pathways (11OHA4, 11OHT, 11KA4, 11KT). Product formation was quantified by LC-MS/MS.

Initial time course experiments with PBMCs isolated from three male donors (aged 22, 23 and 28 years) identified 24 hours as a suitable incubation time allowing for robust product quantification within the linear range of product formation over time (data not shown). These initial experiments additionally revealed very low 3β-hydroxysteroid dehydrogenase activity in PBMCs and thus excluded DHEA as a relevant substrate for metabolism in PBMCs (data not shown).

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170   All further experiments were performed with PBMCs isolated from men aged 18-30 years (n=4-5;

171   age 22-30 years; BMI 20.2-29.1 kg/m^2) and men aged >50 years (n=4-7; age 53-72 years; BMI 21.2-30.4).

172   Since we did not observe any significant differences in androgen metabolism and expression of genes

173   encoding key androgen-metabolizing enzymes between the two age groups, we present and discuss the

174   combined data for the entire cohort below (n=8-12; age 22-72; BMI 20.2-30.4 kg/m^2).

175   Incubations with A4 and T yielded their respective 5α-reduced products, 5α-androstanedione (5α-

176   dione) and 5α-dihydrotestosterone (DHT), and revealed efficient interconversion of A4 and T by reductive

177   and oxidative 17β-hydroxysteroid dehydrogenases (**Fig. 2A+B**). 5α-dione and DHT were further converted

178   to their 3α-hydroxy metabolites androsterone (An) and 5α-androstanediol (Adiol).

179   The quantitatively dominant formation observed among all substrates tested was the generation of

180   11KT from 11KA4, which was significantly higher than the generation of T from A4 (p=0.001; n=11; **Fig.**

181   **2C**). After an incubation period of 24 hours, PBMCs generated approximately 8 times more 11KT than T

182   from their respective precursors 11KA4 and A4 (**Fig. 2A+C**). However, while T was converted back to A4

183   in large quantities, incubation with 11KT led to only minor generation of 11KA4 (quantifiable in 8/12

184   incubations, **Fig. 2D**), further contributing to preferential 11KT activation by the PBMCs.

185   Expressing the observed steady state between activation and inactivation as product/substrate ratios

186   made this difference even more obvious, clearly indicating that the generation of active 11-oxygenated

187   androgens is favored in PBMCs. Product/substrate-ratios for different substrates of 5α-reductase reflect the

188   established substrate preference for SRD5A1 with A4 resulting in the highest activity followed by T and

189   with only minor activity for 11OHA4 (**Fig. 3B**).

190   The generation of 11OHA4 from 11KA4 (quantifiable in 8/12 incubations) and of 11OHT from

191   11KT (quantifiable in 4/12 incubations) indicated 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1)

192   activity in PBMCs, however, at negligible levels compared to the 17β-hydroxysteroid dehydrogenase and

193   5α-reductase activities observed (**Fig. 2C+D**). We did not detect any 5α-reduced products of 11KA4 and

194   11KT (11-keto-5α-androstanedione and 11-keto-5α-dihydrotestosterone) (n=3; data not shown).

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195   Incubation with the 11-oxygenated androgen precursors 11OHA4 and 11OHT led to only

196   negligible product formation compared to the other substrates tested. After 24 hours the generation of the

197   5α-reduced product 11β-hydroxy-5α-androstanedione (11OH-5α-dione) from 11OHA4 could be

198   quantified, as well as the generation of 11KT from 11OHT (**Fig. 1E+F**).

199   **AKR1C3 expression and activity in PBMCs is primarily driven by natural killer cells**

200   In order to identify the subpopulation(s) within the PBMC compartment that are responsible for the

201   AKR1C3-catalysed androgen activation observed in our PBMC incubations, we used publicly available

202   RNAseq-based gene expression data from 15 FACS-sorted PBMCs subpopulations including B-cells,

203   different T-cell populations, monocytes and natural killer (NK) cells (dice-database.org (25)). This revealed

204   significantly higher AKR1C3 expression in NK cells ($p<0.0001$) than in any other PBMC subpopulation

205   (**Fig. 4A**). There was no difference between AKR1C3 expression in NK cells from male and female donors

206   (**Fig. 4B**). To confirm NK cells as the major site of AKR1C3 activity, we isolated PBMCs from a leukocyte

207   cone of an anonymous donor and subsequently enriched NK cells in a fraction of the PBMCs. Ex vivo

208   incubations of the matched crude PBMC isolations and the enriched NK cell fraction with the AKR1C3

209   substrates A4 and 11KA4 confirmed higher AKR1C3 activity in the NK cell enriched incubation compared

210   to the incubation with crude PBMC isolates (**Fig. 4C**).

211   **Lag time prior to the separation of cellular components increases 11-ketotestosterone**

212   We collected blood samples from six healthy volunteers (3m, 3 f; age range 28-50 years) to assess

213   the effect of an extended incubation of their blood samples unseparated from cellular components on the

214   quantification of serum steroids by LC-MS/MS (**Fig. 5**). We observed a time-dependent increase in the

215   serum concentrations of the AKR1C3 product 11KT, reaching a median relative increase of 44% after 24

216   hours, with significant increases in 11KT observed after 2 hours of leaving the full blood samples

217   unseparated (**Fig. 5I**). Additionally, we observed decreases in the concentrations of the AKR1C3 substrates

218   A4 and 11KA4, with median relative decreases of 19% and 34%, respectively, at 24 hours. The observed

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219   decreases in serum A4 concentrations were statistically significant for the majority of time points assessed

220   (p<0.05), while statistical significance was not reached for the decrease in 11KA4 (**Fig. 5C+H**).

221   Among the other analytes investigated (DHEA, T, DHT, An, 11OHA4), only 11OHA4 exhibited

222   significant changes over time (p<0.05), with a median relative decrease of 38% occurring within one hour

223   after blood collection (**Fig. 5G**).

224   **Discussion**

225   Androgen signaling is vital for immune cell function by regulating proliferation, differentiation,

226   cytokine production and other pathways (7, 8). Intracrine androgen activation from adrenal androgen

227   precursors contributes a large proportion of androgen receptor activation in peripheral target cells and

228   tissues of androgen action (17, 26). While we have previously shown that PBMCs can activate the classic

229   androgen precursor A4 to T via AKR1C3 and further convert T to the most potent human androgen DHT

230   (6), we show for the first time in this study that PBMCs preferentially activate the 11-oxygenated androgen

231   precursor 11KA4 to its active counterpart 11KT. We show that PBMCs generate approximately 8-fold more

232   11KT than T from their respective precursors, revealing 11KT as the predominant active androgen within

233   the human PBMC compartment. Using in vitro systems overexpressing AKR1C3, Barnard et al. (10)

234   previously demonstrated that AKR1C3 has a significantly higher catalytic efficiency for the activation of

235   11-oxygenated androgens compared to classic androgens, which we have shown here, for the first time, ex

236   vivo in human cells.

237   In addition, we show that the inactivating conversion of the active androgens T and 11KT to their

238   respective precursors A4 and 11KA4 by oxidative 17β-hydroxysteroid dehydrogenase activity is relevant

239   only for T, while inactivation of 11KT to 11KA4 occurs only in negligible amounts. Taken together, this

240   demonstrates that PBMCs preferentially activate 11-oxygenated androgens. This is in agreement with the

241   study by Barnard et al. (10) who showed that while 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2)

242   catalyzes the inactivation of T and 11KT with similar efficiencies, increased ratios of AKR1C3 to

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243   HSD17B2 favor the activation of 11-oxygenated androgens due to the catalytic preference of AKR1C3 for

244   11KA4 over A4.

245   Our results confirm previous findings that steroid 5α-reduction contributes to the activation of

246   classic androgens in PBMCs by generating the most potent human androgen DHT from T (6). The observed

247   substrate preference of 5α-reduction for A4 over T is consistent with the established substrate preference

248   of steroid 5α-reductase type 1 (SRD5A1) (27), which is the major steroid 5α-reductase in PBMCs (6). We

249   did not observe relevant 5α-reduction of 11-oxygenated androgens. Using in vitro promoter reporter assays

250   11KDHT has been shown to activate the AR with potency and efficacy comparable to DHT. However it is

251   not clear if the generation of 11KDHT by the 5α-reduction of 11KT is relevant under physiological

252   conditions. Recently we showed that 11KT metabolism primarily proceeds via the AKR1D1 mediated 5β-

253   reduction of the steroid A-ring and that SRD5A2, but not SRD5A1 can efficiently catalyze the 5α-reduction

254   of 11KT (28). We now confirm that 11KT is not 5α-reduced by human cells with SRD5A1 expressed at

255   levels that efficiently convert T to DHT, confirming that the 5α-reduction of 11KT would require the

256   expression of SRD5A2.

257   In this study, we did not observe any significant effect of age on androgen activation in PBMCs

258   with only a trend for increased median AKR1C3 and SRD5A1 activity in contrast to a published study by

259   our group that described significantly increased AKR1C3 and SRD5A1 activity in men aged over 50

260   compared to men aged 18-30 years (6). The small sample numbers of both studies and differences in the

261   assays used for steroid quantification (LC-MS/MS in our assay vs. thin layer chromatography) are likely to

262   have caused the discrepancy. However, when assessing the effect of age on androgen activation in

263   peripheral tissues, age-related changes in the supply of androgen precursors from circulation need to be

264   considered, in addition to age-dependent changes in the expression of androgen-activating and -inactivating

265   enzymes in the peripheral target tissues of androgen action. While circulating levels of classic androgens

266   significantly decline with age, levels of 11-oxygenated androgens remain constant across adulthood (29,

267   30). Hence, the peripheral activation of 11-oxygenated androgens is favored over the activation of classic

268   androgens not only by the substrate preference of the key androgen activating enzyme AKR1C3, but also

269   by constant high substrate availability across the life span.

270   Primary adrenal insufficiency is associated with an increased risk of infections compared to the

271   general population (31). In addition, patients with primary adrenal insufficiency show significantly reduced

272   NK cell cytotoxicity (32). Interestingly, this finding not affected by DHEA replacement therapy, excluding

273   the deficiency of the classic androgen pathway precursor DHEA as a cause of the reduced NK cell

274   cytotoxicity. Our study identifies adrenal 11-oxygenated androgen precursors rather than classic androgen

275   precursors as predominant androgens activated in the PBMC compartment and particularly in NK cells,

276   potentially linking the lack of adrenal 11-oxygenated androgen precursors in primary adrenal insufficiency

277   to the observed decrease in NK cell cytotoxicity.

278   The preference of PBMCs to generate 11KT from 11KA4 via AKR1C3 activity is further reflected

279   in the significant increases in 11KT serum concentrations, if cellular components were not removed from

280   the full blood samples in a timely fashion, confirming previous preliminary observations (23). We found

281   that these changes became significant after two hours, suggesting that blood samples for the measurement

282   of 11-oxygenated androgens should be processed within two hours of collection. 11KT is the dominant

283   circulating active androgen in polycystic ovary syndrome (15) and CAH (14, 33) and a useful marker for

284   the diagnosis of androgen excess. The limited stability of 11KT levels in unseparated full blood samples

285   suggests that saliva, a cell-free biofluid, could be a superior matrix for the measurement 11KT (33, 34).

286   In conclusion, we show that 11-oxygenated androgen precursors are the preferred substrates for

287   androgen activation by AKR1C3 in PBMCs, yielding 11KT as the major active androgen in the PBMC

288   compartment. Importantly, AKR1C3 is predominantly expressed in NK cells, potentially linking adrenal

289   11-oxgenated androgen deficiency to the reduced NK cell cytotoxicity in primary adrenal insufficiency.

290   Androgen metabolism by PBMCs can affect the concentrations of 11-ketotestosterone measured in serum

291   samples, if samples are not processed in a timely, standardized way.

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388

389   **Figure Legends**

390   **Fig. 1:** Expression of genes encoding key androgen-metabolizing enzymes in PBMCs isolated from healthy

391   men (A, n=14; age range 22-72 years; BMI range 20.2-30.4 kg/m^2). Gene expression was assessed by semi-

392   quantitative PCR, normalized to HPRT1 and GAPDH and arbitrary units (A.U.) were calculates as

393   1000*2^-ΔCt. (B) Schematic representation of the expressed steroid-metabolizing enzymes and their roles

394   in activation and inactivation of androgens in classic and 11-oxygenated androgen pathways.

395   **Fig. 2:** Ex vivo metabolism of classic (A+B) and 11-oxygenated androgens (C-F) by PBMCs isolated from

396   healthy men (n=8-12; age range 22-72 years; BMI range 20.2-30.4 kg/m^2). PBMCs were incubated with

397   100 nM substrate for 24 hours. The respective substrate is shown in a grey box for each graph. Product

398   formation was quantified by LC-MS/MS and normalized to the total protein content of the incubation.

399   Median and range are indicated. Product concentrations below the LLOQ are shown as 0.

400   **Fig. 3:** Product/substrate ratios in ex vivo incubations of PBMCs isolated from healthy men (n=8-12; age

401   range 22-72 years; BMI range 20.2-30.4 kg/m^2) after the addition of different steroid substrates. (A) Steady-

402   state ratios of 17β-hydroxysteroid dehydrogenase products and substrates, reflecting the dominant

403   contribution of the reductive, activating 17β-hydroxysteroid dehydrogenase AKR1C3 to 11-oxygenated

404   androgen metabolism. (B) 5α-reductase activity by SRD5A1. Product and substrate were quantified by LC-

405   MS/MS after a 24-hour incubation. Ratios were normalized to the total protein content of the incubation.

406   Statistical analysis was performed using Wilcoxon matched-pairs signed rank test.

407   **Fig. 4:** Natural killer cells are the major peripheral blood mononuclear cell population with *AKR1C3*

408   expression (A and B) and activity (C). RNAseq analysis of FACS-sorted PBMC subpopulations (dice-

409   database.org; (25)) revealed natural killer cells (NK cells) as the cell type with the highest *AKR1C3*

410   expression (A) with comparable expression in women (n=37) and men (n=54) (B). NK cell-enriched

411   incubations with AKR1C3 substrates (n=4 technical replicates) showed higher AKR1C3 activity (shown

412   as product/substrate ratio) compared to incubations of crude PBMC preparations from the same donor (n=1)

17

413   (C). NK cells enriched using MACS negative selection (Miltenyi Biotec) were incubatedand product

414   formation after 24 hours analyzed by LC-MS/MS. Statistical analysis of AKR1C3 expression in the PBMC

415   subpopulation was performed by one-way ANOVA followed by Tukey's multiple comparisons test.

416   AKR1C3 expression in male and female NK cells was compared by Mann-Whitney test. TPM, transcripts

417   per million.

418   **Fig. 5:** Relative changes in androgen concentrations compared to baseline, measured in serum after

419   incubation of unseparated whole blood samples. Baseline samples were separated by centrifugation within

420   1h after collection. The remaining samples were incubated as whole blood at room temperature for the

421   times indicated prior to separation of serum and cellular content (sample numbers: n=6 for DHEA (**B**), n=6

422   for A4 (**C**), n=5 for T (**D**), n=3 for DHT (**E**), n=6 for An (**F**), n=6 for 11OHA4 (**G**), 11KA4 (**H**) and 11KT

423   (**I**)). Individual data points are shown with range and median. Statistical analysis was performed on the

424   concentrations in nM using ANOVA, followed by Dunnett multiple comparison test to compare each time
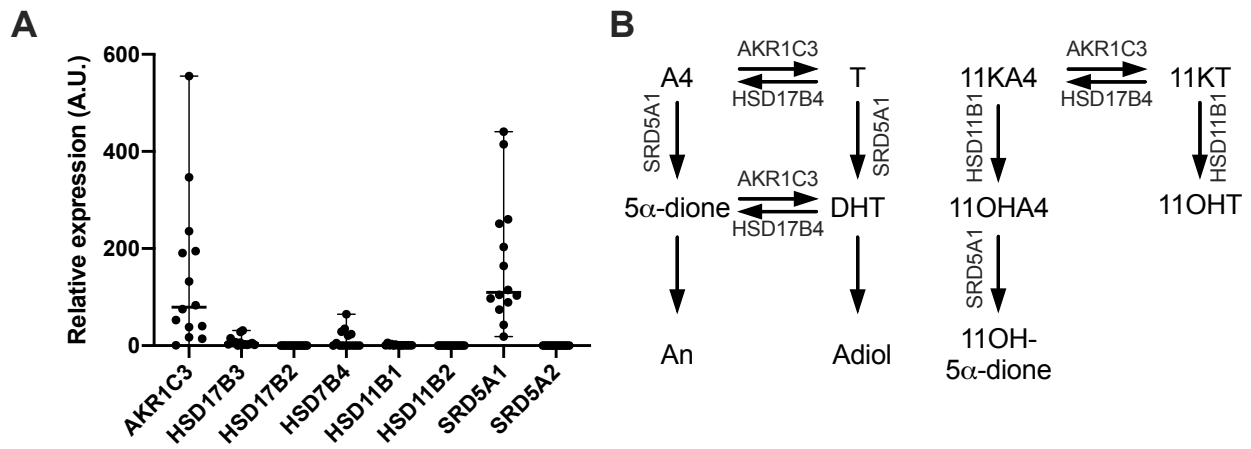
425   point against baseline.

18

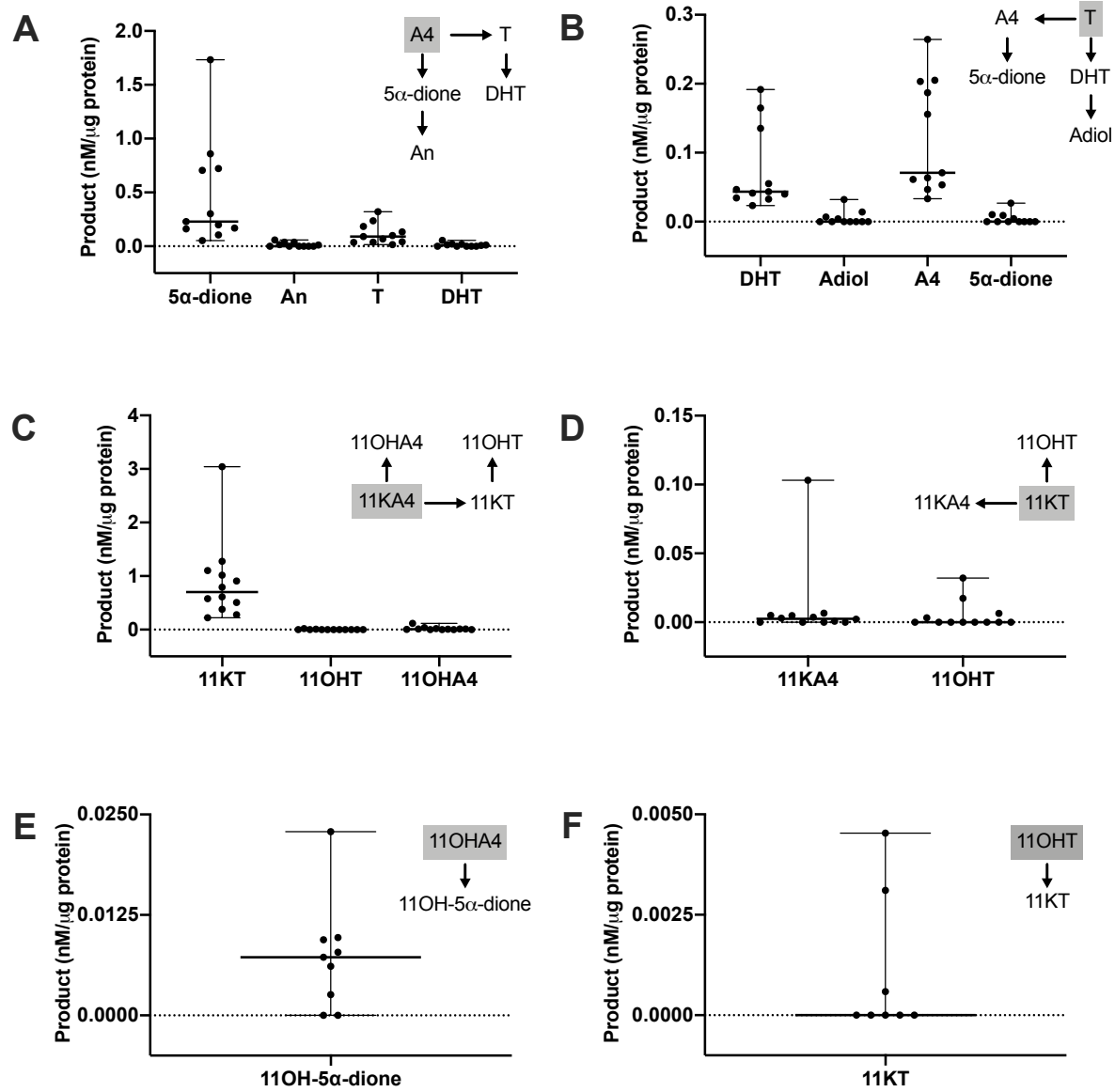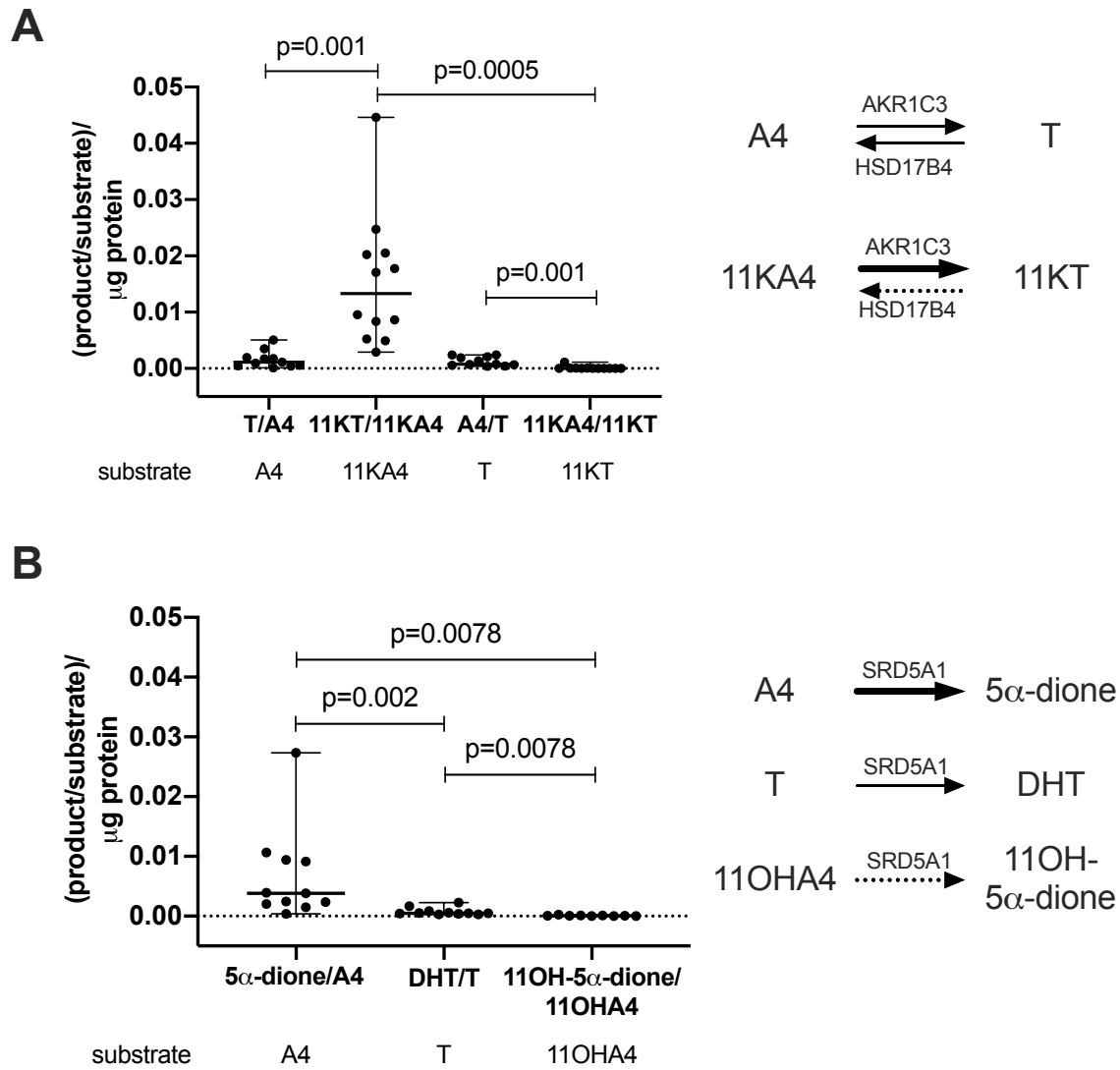**Figure 1**

Figure 2

Figure 3

Figure 4

Figure 5