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

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Abstract (250 words max)

Context: Androgens are important modulators of immune cell function impacting proliferation, differentiation and cytokine production. The local generation of active androgens from circulating androgen precursors is an important mediator of androgen action in peripheral androgen target cells of tissue.

Objective: To characterize the activation of classic and 11-oxygenated androgens in human peripheral blood mononuclear cells (PBMCs).

Methods: PBMCs and natural killer cells were isolated from healthy male donors and incubated ex vivo with precursors and end products of the classic and 11-oxygenated androgen pathways. Steroid concentrations were quantified by liquid chromatography-tandem mass spectrometry. The expression of genes encoding steroid metabolizing enzymes was assessed by quantitative PCR.

Results: The enzyme AKR1C3 is the major reductive 17β-hydroxysteroid dehydrogenase in PBMCs and has higher activity for the generation of the active 11-oxygenated androgen 11-ketotestosterone than for the generation of the classic androgen testosterone from their respective precursors. Natural killer cells are the major site of AKR1C3 expression and activity within the PBMC compartment. Steroid 5α-reductase type 1 catalyzes the 5α-reduction of classic but not 11-oxygenated androgens in PBMCs. Lag time prior to the separation of cellular components from whole blood sample increases 11KT serum concentrations in a time-dependent fashion, with significant increases detected from two hours after blood collection.

Conclusions: 11-oxygenated androgens are the preferred substrates for androgen activation by AKR1C3 in PBMCs, primarily conveyed by natural killer cell AKR1C3 activity, yielding 11KT the major active androgen in PBMCs. Androgen metabolism by PBMCs can affect the levels of 11-oxygenated androgens measured in serum samples, if samples are not separated in a timely fashion.

45 **Introduction**

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

55 Recent *in-vitro* work has shown that AKR1C3 has an approximately 8-fold higher catalytic
56 efficiency for the generation of the 11-oxygenated androgen 11-ketotestosterone (11KT) from its precursor
57 11-ketoandrostenedione (11KA4) than for the generation of T from A4 (10). 11KT activates the androgen
58 receptor with potency and efficacy similar to T (11-13) and is the predominant circulating androgen in
59 androgen excess conditions including congenital adrenal hyperplasia, polycystic ovary syndrome and
60 premature adrenarche (12, 14, 15). 11KT is derived from the abundant adrenal precursor steroid 11β-
61 hydroxyandrostenedione (11OHA4) (13, 16), which is converted to 11KA4 by peripheral tissue expressing
62 11β-hydroxysteroid dehydrogenase type 2 (17, 18) prior to activation to 11KT by AKR1C3 (10, 16).

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

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™ (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™
84 Vacutainers™ (gold top). The tubes were left unseparated at room temperature in an air-conditioned room
85 and were separated by centrifugation (2000*g*, 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

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.

104 **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.

108 **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-

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 ΔCt. 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.

145 **Results**

146 **Androgen activation in PBMCs is primarily catalyzed by AKR1C3 and SRD5A1**

147 Using qPCR, we identified AKR1C3 as the major reductive, activating 17β-hydroxysteroid
148 dehydrogenase isoform in PBMCs, while HSD17B3, generally considered a testes-specific isoform, was
149 expressed at very low levels only (**Fig. 1A**). While we could not detect expression of steroid 5α-reductase
150 type 2 (SRD5A2) and 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) in any of the samples from
151 14 donors using our qPCR assay, we detected consistently high expression of steroid 5α-reductase type 1
152 (SRD5A1) in samples from all donors (n=14) and detected 17β-hydroxysteroid dehydrogenase type 4
153 (HSD17B4) expression in samples from 6 of 14 donors. This indicates that SRD5A1 is responsible for the
154 5α-reduction we observe in our PBMC incubations with androgen substrates while HSD17B4 catalyzes the
155 oxidative, inactivating 17β-hydroxysteroid dehydrogenase activity we observed (**Fig. 1A**), consistent with
156 previously published findings (6). HSD11B1 expression was detectable in samples from 13/14 donors,
157 while HSD11B2 mRNA was not detected. **Fig. 1B** schematically illustrates the enzymes identified by qPCR
158 as in PBMCs and their roles in androgen activation and inactivation in the classic and 11-oxygenated
159 androgen biosynthesis pathways.

160 **PBMCs preferentially activate 11-oxygenated androgens**

161 In order to unravel the pathways of androgen metabolism in PBMCs, we isolated PBMCs from
162 male healthy donors and performed ex vivo incubations, using as substrates several androgen precursors
163 and active androgens from both the classic (DHEA, A4 and T) and 11-oxygenated androgen pathways
164 (11OHA4, 11OHT, 11KA4, 11KT). Product formation was quantified by LC-MS/MS.

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

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).

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

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

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)

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.

Figure 1

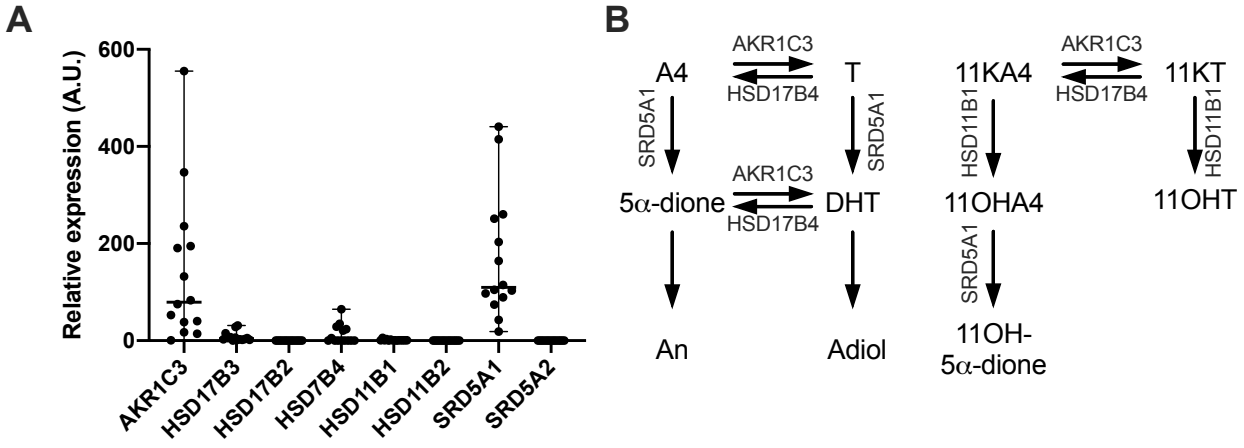

A
Relative expression (A.U.)
600
400
200
0
AKR1C3
HSD17B3
HSD17B2
HSD7B4
HSD11B1
HSD11B2
SRD5A1
SRD5A2
B
A4
AKR1C3
HSD17B4
T
SRD5A1
5α-dione
AKR1C3
HSD17B4
DHT
An
Adiol
11KA4
HSD11B1
11OHA4
SRD5A1
11OH-
5α-dione
AKR1C3
HSD17B4
11KT
HSD11B1
11OHT

Figure 2

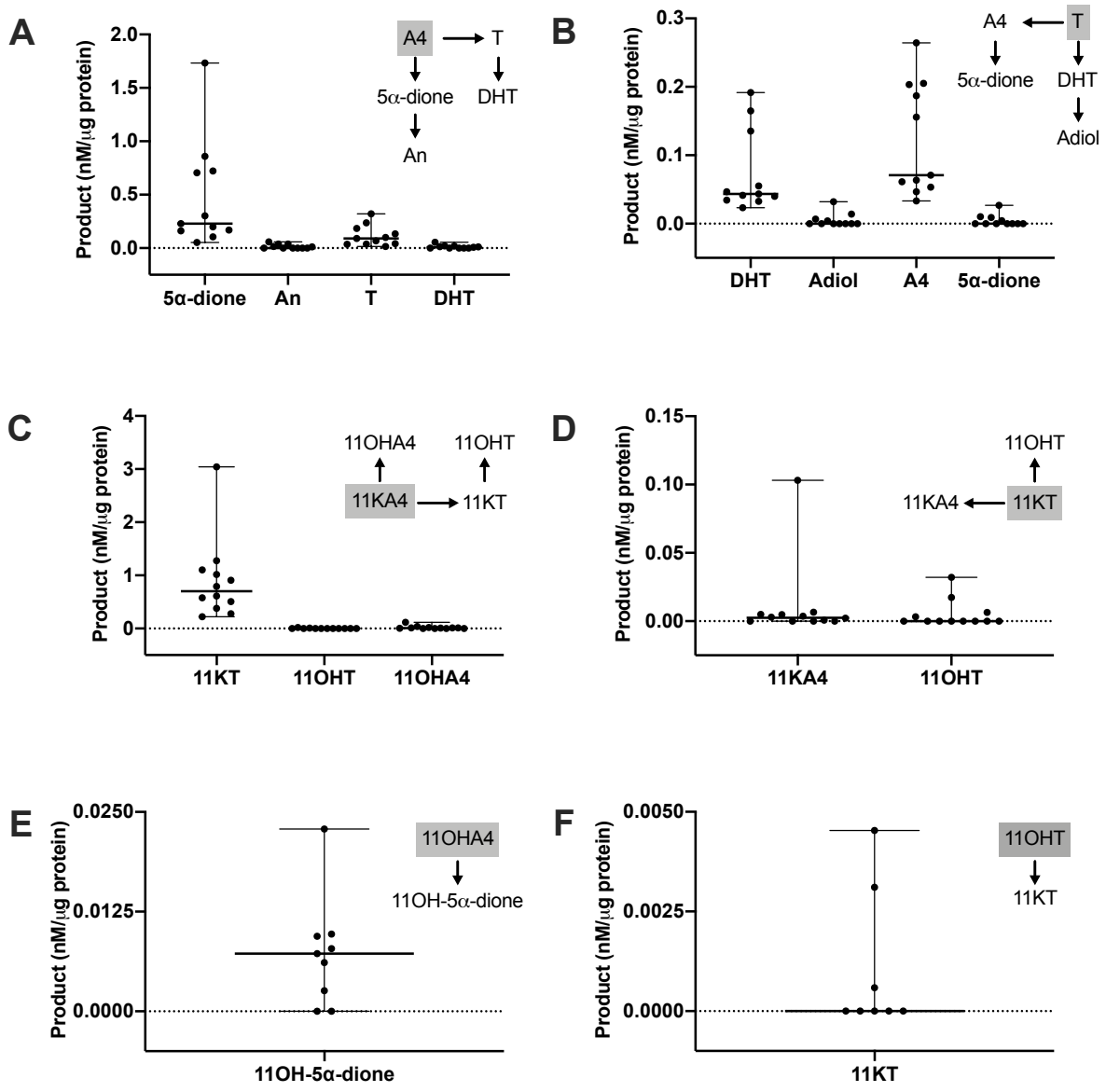

A
Product (nM/μg protein)
2.0
1.5
1.0
0.5
0.0
A4
T
5α-dione
DHT
An
5α-dione
An
T
DHT
B
Product (nM/μg protein)
0.3
0.2
0.1
0.0
A4
T
5α-dione
DHT
Adiol
DHT
Adiol
A4
5α-dione
C
Product (nM/μg protein)
4
3
2
1
0
11OHA4
11OHT
11KA4
11KT
11KT
11OHT
11OHA4
D
Product (nM/μg protein)
0.15
0.10
0.05
0.00
11OHT
11KA4
11KT
11KA4
11OHT
E
Product (nM/μg protein)
0.0250
0.0125
0.0000
11OHA4
11OH-5α-dione
11OH-5α-dione
F
Product (nM/μg protein)
0.0050
0.0025
0.0000
11OHT
11KT
11KT

Figure 3

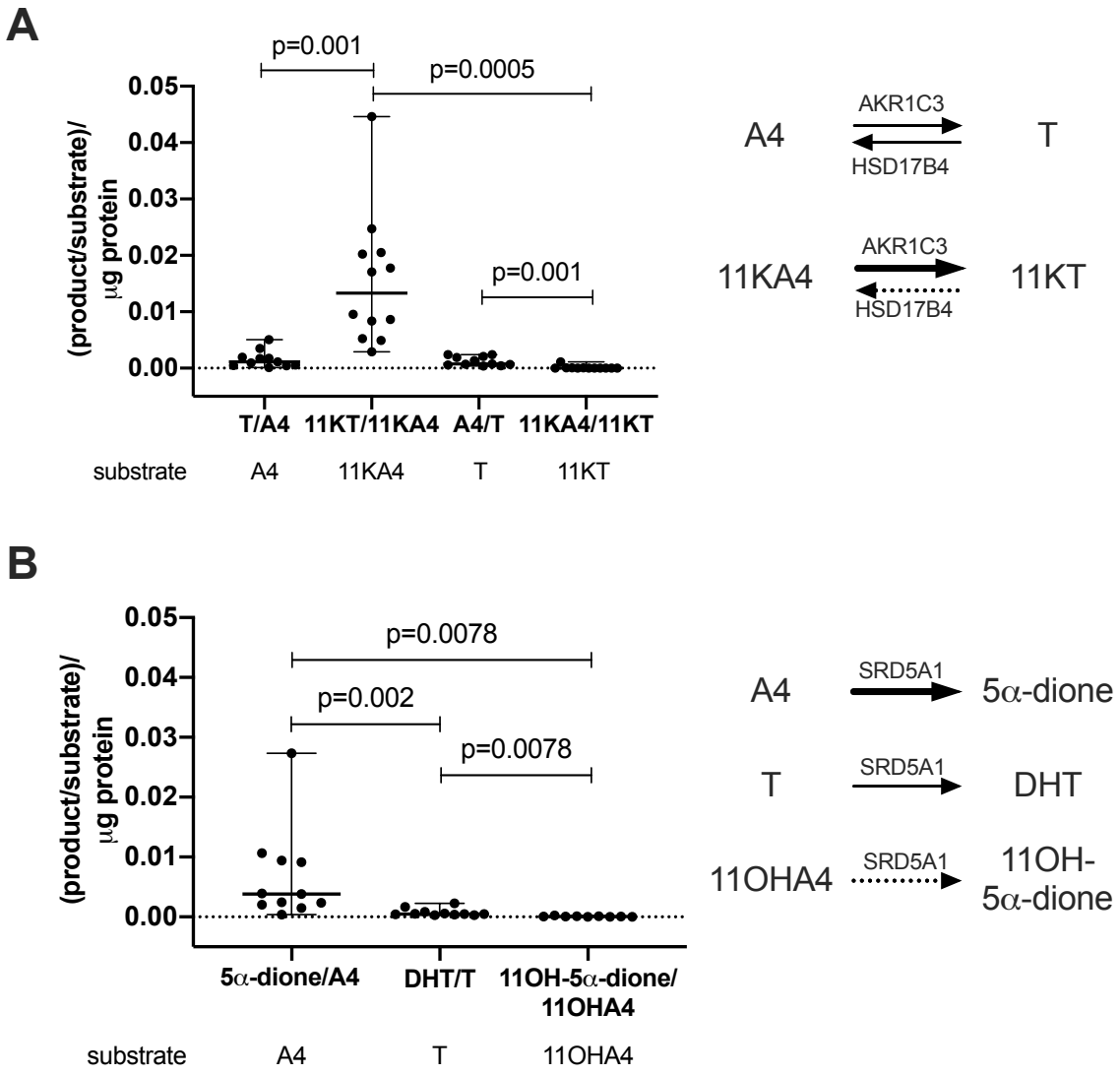

A
p=0.001
p=0.0005
p=0.001
(product/substrate)/ µg protein
0.05
0.04
0.03
0.02
0.01
0.00
T/A4
11KT/11KA4
A4/T
11KA4/11KT
substrate
A4
11KA4
T
11KT
A4
AKR1C3
HSD17B4
T
11KA4
AKR1C3
HSD17B4
11KT
B
p=0.0078
p=0.002
p=0.0078
(product/substrate)/ µg protein
0.05
0.04
0.03
0.02
0.01
0.00
5α-dione/A4
DHT/T
11OH-5α-dione/ 11OHA4
substrate
A4
T
11OHA4
A4
SRD5A1
5α-dione
T
SRD5A1
DHT
11OHA4
SRD5A1
11OH-5α-dione

Figure 4

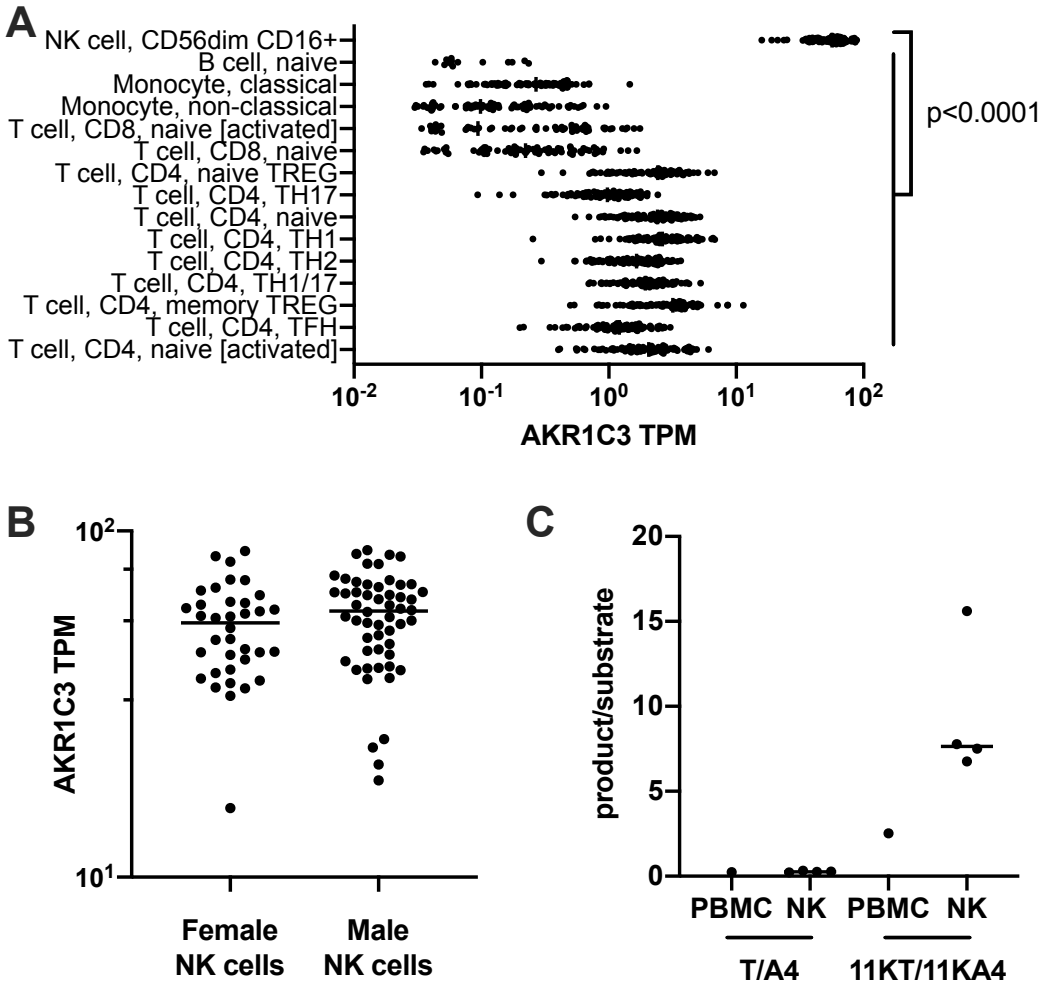
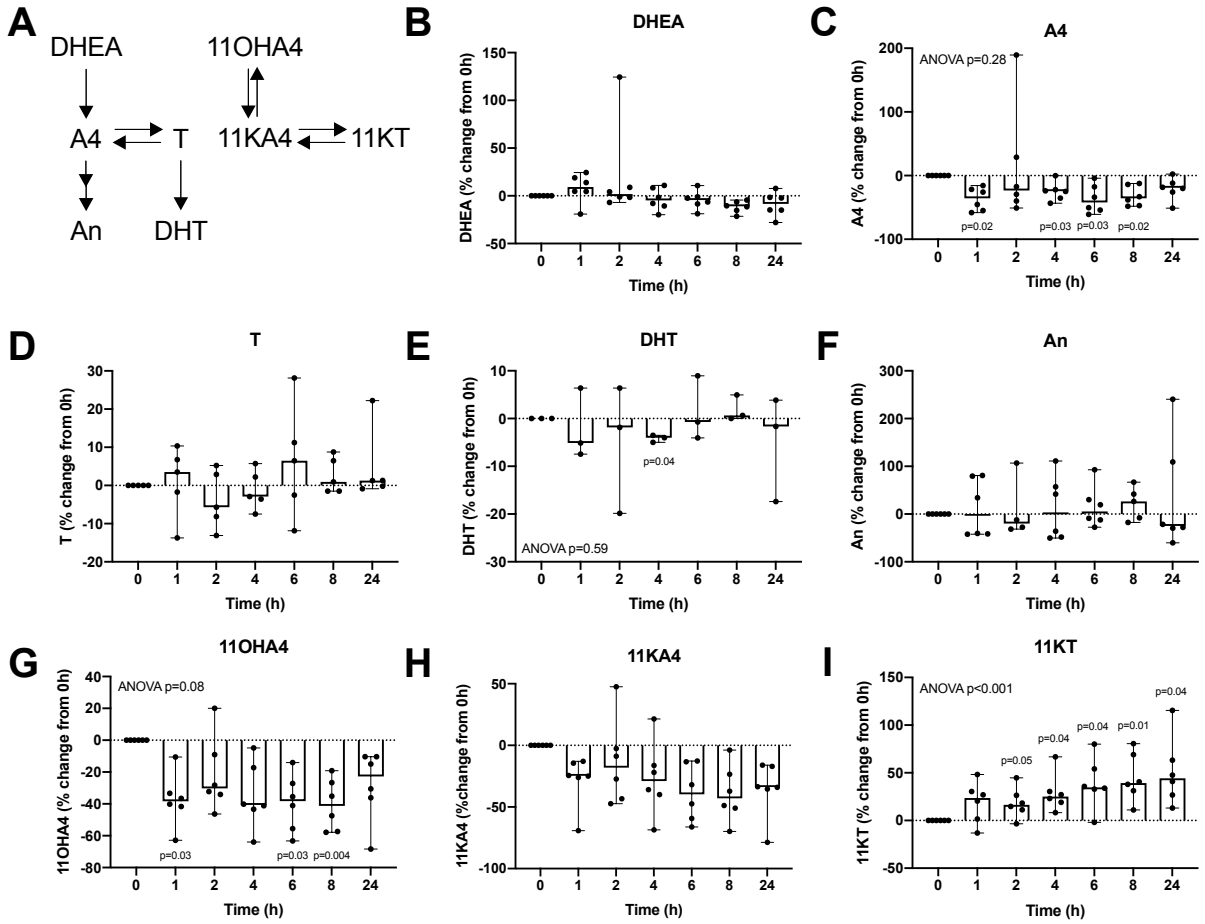

A
NK cell, CD56dim CD16+
B cell, naive
Monocyte, classical
Monocyte, non-classical
T cell, CD8, naive [activated]
T cell, CD8, naive
T cell, CD4, naive TREG
T cell, CD4, TH17
T cell, CD4, naive
T cell, CD4, TH1
T cell, CD4, TH2
T cell, CD4, TH1/17
T cell, CD4, memory TREG
T cell, CD4, TFH
T cell, CD4, naive [activated]
p<0.0001
AKR1C3 TPM
B
AKR1C3 TPM
Female NK cells
Male NK cells
C
product/substrate
PBMC
NK
PBMC
NK
T/A4
11KT/11KA4

Figure 5


A
DHEA
11OHA4
A4
T
11KA4
11KT
An
DHT
B
DHEA
DHEA (% change from 0h)
Time (h)
C
A4
ANOVA p=0.28
A4 (% change from 0h)
p=0.02
p=0.03
p=0.03
p=0.02
Time (h)
D
T
T (% change from 0h)
Time (h)
E
DHT
DHT (% change from 0h)
p=0.04
ANOVA p=0.59
Time (h)
F
An
An (% change from 0h)
Time (h)
G
11OHA4
ANOVA p=0.08
11OHA4 (% change from 0h)
p=0.03
p=0.03
p=0.004
Time (h)
H
11KA4
11KA4 (%change from 0h)
Time (h)
I
11KT
ANOVA p<0.001
11KT (% change from 0h)
p=0.05
p=0.04
p=0.04
p=0.01
p=0.04
Time (h)