Alternative pathway androgen biosynthesis and human fetal female virilization

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25 **Brief summary (25 words):**

26 An alternative androgen synthesis pathway is active during the major period of human fetal
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ABSTRACT

Androgen biosynthesis proceeds through the adrenal sex steroid precursor dehydroepiandrosterone, which is converted towards testosterone in the gonads, followed by further activation to 5α-dihydrotestosterone in genital skin, thereby facilitating male external genital differentiation. Congenital adrenal hyperplasia due to P450 oxidoreductase deficiency results in disrupted dehydroepiandrosterone biosynthesis, which explains undervirilization in affected boys. However, many affected girls are born severely virilized, despite low circulating androgens. We hypothesized that this is due to a prenatally active androgen pathway from 17α-hydroxyprogesterone to 5α-dihydrotestosterone, which bypasses dehydroepiandrosterone and testosterone, with increased activity in congenital adrenal hyperplasia. Here we employ explant cultures of human fetal organs (adrenal, gonads, genital skin) from the major period of sexual differentiation (6-10 weeks post-conception) and show that alternative pathway androgen biosynthesis is active in the fetus, and that female external genitalia respond sensitively to the androgens generated. Moreover, using steroid metabolite excretion analysis by mass spectrometry, we show that neonates with P450 oxidoreductase deficiency produce androgens through the alternative pathway during the first weeks of life. These results indicate a key role of alternative pathway androgen biosynthesis in the prenatal virilization of girls affected by congenital adrenal hyperplasia.

61 **INTRODUCTION**

62 Gonadal development depends on chromosomal sex, whereby the 46,XY or 46,XX
63 karyotype, established at fertilization, dictates subsequent development of either testis or ovary
64 (1-3). Gonadal hormones then direct differentiation of either male or female genitalia. In humans,
65 sexual differentiation is established at 7-12 weeks *post conception* (wpc) (4).
66 While secretion of testosterone by fetal testis Leydig cells is thought sufficient to drive
67 virilization of the internal genitalia in the male fetus (5), differentiation of the external genitalia
68 requires the action of 5α-dihydrotestosterone (DHT), which is generated locally from circulating
69 testosterone by the enzyme steroid 5α-reductase type 2 (SRD5A2) (6, 7). By contrast,
70 differentiation of human female genitalia has been regarded as the default of a low androgen
71 environment.

72 In humans, the regulation of sexual differentiation is intricately linked to early
73 development of the adrenal cortex (4, 8). Disorders affecting adrenal steroidogenesis commonly
74 affect sexual differentiation, as exemplified by the multiple variants of congenital adrenal
75 hyperplasia (CAH), which result either in inappropriate or disrupted androgen biosynthesis. This
76 consequently causes Disorder of Sex Development (DSD), which can manifest with external
77 genital virilization in newborn girls (46,XX DSD), or undermasculinization of external genitalia
78 in male neonates (46,XY DSD) (9). The most common variant of CAH, 21-hydroxylase
79 (CYP21A2) deficiency, manifests with 46,XX DSD, while 17α-hydroxylase/17,20-lyase
80 (CYP17A1) deficiency causes 46,XY DSD.

81 The congenital adrenal hyperplasia variant cytochrome P450 oxidoreductase (POR)
82 deficiency can manifest with both 46,XY and 46,XX DSD (10-12). POR plays a pivotal role as
83 the obligatory electron donor to all microsomal cytochrome P450 enzymes, including CYP21A2
84 and CYP17A1, the latter catalyzing the biosynthesis of dehydroepiandrosterone (DHEA), the
85 major precursor for testosterone biosynthesis. Consequently, POR deficiency (PORD) results in

86 low circulating androgen concentrations, which readily accounts for 46,XY DSD, but fails to
87 account for the severe virilization of external genitalia observed in affected 46,XX neonates.

88 An explanation for this striking and seemingly contradictory genital phenotype paradox
89 in PORD has been lacking. We hypothesized that this apparent paradox could be explained by the
90 existence of an alternative pathway to androgen production that generates DHT from 17α-
91 hydroxyprogesterone (17OHP) during human fetal sexual differentiation, thereby bypassing the
92 classic androgen biosynthesic pathway via DHEA and testosterone (11). Elements of this
93 pathway have been characterized in the fetal gonad of the tammar wallaby pouch young (13-15)
94 and fetal opossum urogenital tract (16, 17). 17OHP accumulates in PORD but also in the most
95 common CAH variant, 21-hydroxylase deficiency, and thus could feed into the proposed
96 alternative pathway, if present in the fetus. Indirect biochemical evidence has indicated that the
97 proposed alternative pathway is active postnatally in individuals with CAH due to 21-hydroxylase
98 deficiency (18, 19) and may explain maternal virlization observed in pregnancies affected by
99 PORD (20, 21). However, direct delineation of the putative alternative pathway during human
100 fetal development, and in particular during the major period of sexual differentiation, has been
101 lacking.

102 Here, we present conclusive evidence for the presence and activity of the alternative
103 pathway in the human fetus, producing potent androgens during the major period of sex
104 differentiation. We show that human fetal female external genitalia respond sensitively to
105 androgens during the same period. In concert, these findings define an alternative pathway for
106 androgen biosynthesis during the critical period of sexual differentiation in the human fetus that
107 represents a major mechanism to explain the prenatal virilization of female infants affected by
108 CAH.

109 **RESULTS AND DISCUSSION**

110 **Androgen biosynthesis in the human fetus during sexual differentiation**

111 To ascertain the presence and activity of the hypothesized alternative androgen pathway,
112 we performed incubations with male and female fetal adrenals, gonads and genital skin, which
113 were collected at 6-10 wpc during the major period of human sex differentiation as previously
114 described (8). We separately added deuterated steroid substrates for each step of the alternative
115 pathway and employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to
116 quantify the resulting products (**Supplemental Figure 1** and **Supplemental Tables 1 and 2**).
117 Experiments were conducted at least in triplicate for each organ of either sex (**Supplemental**
118 **Figures 2-3**).

119 Explant incubations with female tissue showed that some 17OHP entered the classic
120 androgen biosynthesis pathway yielding androstenedione in adrenal, ovary and genital skin.
121 However, testosterone was detected in only 2 of 9 female genital skin incubations, but not in
122 female adrenals and gonads (**Figure 1A**). By contrast, all three female tissues showed conversion
123 of 17OHP and subsequent intermediates through all steps of the proposed alternative androgen
124 pathway, with the end product, DHT, produced from 5α-androstanediol in genital skin (3/3),
125 ovaries (2/3) and adrenal (1/4) explant cultures (**Figure 1A**).

126 Male fetal tissues readily converted 17OHP along the classic androgen pathway to
127 testosterone and and, in the alternative pathway, to 5α-17-hydroxypregnanolone (5α-17HP). In
128 male genital skin, conversion from 17OHP proceeded until the generation of DHT (**Figure 1B)**,
129 feasibly arising from either route, as DHT represents the end product of both classic and
130 alternative androgen pathways. However, incubations with the intermediate substrates of the
131 alternative pathway demonstrated stepwise catalysis to DHT by male adrenal, testis and genital
132 skin (**Figure 1B**). Consistent with studies in non-human species (22, 23), we did not identify 5α-

133 pregnane-17α-ol-3,20-dione as a significant intermediate of the two-step conversion of 17OHP to
134 5α-17HP in tissues from either sex, likely due to its immediate onward conversion.

135 Taken together, these data show that adrenal, gonad and genital skin are capable of
136 androgen biosynthesis via both classic and alternative pathway in both sexes. Our data suggest
137 the presence of an adreno-genital steroidogenic unit that cooperates to produce DHT during the
138 period of sexual differentiation in both male and female fetuses.

139 **Steroidogenic enzyme expression during human fetal sexual differentiation**

140 The alternative pathway conversion of 17OHP to 5α-17HP requires sequential 5α-
141 reductase and 3α-hydroxysteroid dehydrogenase activities.

142 Of the two isoforms, the first step is expected to require catalysis by steroid 5α-reductase
143 type 1 (SRD5A1), since SRD5A2 does not to convert 17OHP efficiently (24). While a study of
144 fetal tissues from 12-20 wpc did not detect SRD5A1 in adrenals, gonads or genital skin (25), we
145 found mRNA expression of SRD5A1 in all tissues during the major period of human sexual
146 differentiation (6-10 wpc) (**Figure 2A**). The second step, the conversion of 5α-pregnane-17α-ol-
147 3,20-dione to 5α-17HP requires 3α-hydroxysteroid dehydrogenase activity, in keeping with the
148 observed expression of *AKR1C1* and *AKR1C3*, both of which encode enzymes capable of
149 catalyzing this reaction (**Figure 2A**).

150 The next step in the alternative pathway requires CYP17A1 17,20 lyase activity; 5α-
151 17HP is the preferred substrate for this reaction and efficiently converted to 5α-androsterone (26).
152 *CYP17A1* was robustly detected in human fetal adrenals, consistent with our previous work (8),
153 and also in the gonads of both sexes (**Figure 2B**). The subsequent reduction of 5α-androsterone to
154 5α-androstanediol requires 17β-hydroxysteroid dehydrogenase activity, which can be provided by
155 AKR1C3, which was expressed in all tissues investigated (**Figure 2A**).

156 The final step of the proposed pathway involves the conversion of 5α-androstanediol to
157 DHT, which requires 3β-epimerase (oxidative 3α-HSD) activity. Several enzymes have been

158 considered to catalyze this reaction (i.e. AKR1C2, RDH5, DHRS9, HSD17B10, HSD17B6 and
159 RDH16). However, only HSD17B6 and RDH16 are capable of efficient oxidation of 5α-
160 androstanediol to DHT, as previously demonstrated by transactivation of the androgen receptor
161 following cell-based overexpression (22, 27). We found expression of both HSD17B6 and
162 RDH16 in fetal genital skin of both sexes (**Figure 2C**).

163 In summary, we detected expression of all enzymes required to catalyze the alternative
164 androgen pathway (**Figure 2D**). Taken together with the steroid conversion studies, these data
165 comprehensively demonstrate that the normal adrenal, gonad and genital skin are capable of
166 androgen biosynthesis via both classic and alternative pathway in both sexes. Our data point to an
167 adreno-genital steroidogenic unit that can cooperates to produce DHT via the alternative pathway
168 during the major period of human sexual differentiation.

169 **Androgen receptor in female genitalia from the start of sex differentiation**

170 Having demonstrated the capacity for DHT production from steroidogenic precursors in
171 female fetuses, we corroborated its ability to function by examining the presence of the androgen
172 receptor (AR) in female external genitalia from the start of sex differentiation. Previously, AR
173 expression has been documented in four female human fetuses from 9-18 wpc (28). AR protein
174 was readily detected in stromal cells in the urethral folds of the external genitalia in both male
175 and female fetuses at the onset of sexual differentiation (**Figure 3A-C**). Its nuclear localization in
176 fixed tissues in both sexes implied AR was ligand-bound. To explore this further we studied the
177 intracellular localization of AR by immunofluorescence in female external genitalia fibroblasts
178 taken into primary culture from the same stage of development. In steroid-free media, the external
179 genitalia cells demonstrated cytoplasmic AR localization. As expected, the addition of 1 nM DHT
180 induced nuclear translocation of AR. Strikingly, the same translocation was observed when using
181 medium conditioned overnight from the corresponding female adrenal gland (**Figure 3D**). In
182 combination, these data show AR from the start of sex differentiation in both male and female

183 external genitalia and demonstrate a functional adreno-genital steroidogenic unit capable of
184 causing AR nuclear translocation.

185 **Androgen biosynthesis in neonates with congenital adrenal hyperplasia**

186 Having shown evidence of the alternative pathway during human sexual differentiation,
187 we next investigated whether we could demonstrate equivalent activity *in vivo*. To address our
188 hypothesis that excess alternative pathway androgen biosynthesis in fetal life explains female
189 virilization (46,XX DSD) in CAH due to PORD (12, 29), in whom the classic androgen pathway
190 is disrupted, we identified three patients with a mutation known to cause 46,XX DSD in affected
191 girls with normal male genitalia in affected boys (POR A287P) (11, 12). While postnatal
192 circulating androgens in PORD are low in infancy and beyond (12, 29), we hypothesized that
193 affected individuals would still show evidence of excess alternative pathway androgen
194 biosynthesis in the immediate neonatal period. We analysed urinary steroid metabolite excretion
195 by gas chromatography-mass spectrometry and detected significant alternative pathway activity
196 in the neonatal period, consistent with previous preliminary findings that argued for the adrenal
197 gland as a major site for alternative pathway activity (30). During the first three weeks after birth
198 we documented increased excretion of the 17OHP metabolite 17-HP compared to healthy
199 controls (**Figure 4A**), consistent with accumulation of 17OHP, the initial substrate of the
200 alternative androgen pathway. We found significantly increased excretion of 5α-17HP **(Figure**
201 **4B**), a key intermediate of the alternative pathway; and finally, there was significantly increased
202 excretion of the major DHT metabolite 5α-androsterone (**Figure 4C**). By contrast,
203 etiocholanolone excretion, derived from classic pathway androgen biosynthesis, did not differ
204 between the affected and healthy control groups (**Figure 4D**). These findings indicate increased
205 *in vivo* alternative pathway androgen biosynthesis in neonates with PORD, which ceases shortly
206 after birth.

207 **Conclusions**

208 We have provided ex vivo and in vivo evidence for the existence and activity of an
209 alternative pathway for the synthesis of the most potent androgen, DHT, during early human
210 development. Our data demonstrate that, through cooperation of an adreno-genital steroidogenic
211 unit, the alternative androgen pathway yields active androgen synthesis in the female fetus, with
212 excess activity driving female virilization, 46,XX DSD, in CAH.

213

214 **METHODS**

215 Detailed descriptions of experimental procedures are reported in the Supplemental Methods.

216 **AUTHOR CONTRIBUTIONS**

217 WA and NAH conceived and designed experiments; NR, AET, EFN, DJA, and CHLS
218 contributed to the study design. NR, EFN, DJA, VD and AB performed and analyzed the explant
219 culture experiments, supervised by WA and NAH. AET and NK performed and analyzed mass
220 spectrometry experiments, supervised by WA and CHLS. RJA provided reagents. NR wrote the
221 manuscript jointly with WA and NAH; all authors contributed to the discussion and
222 reviewed/edited the manuscript.

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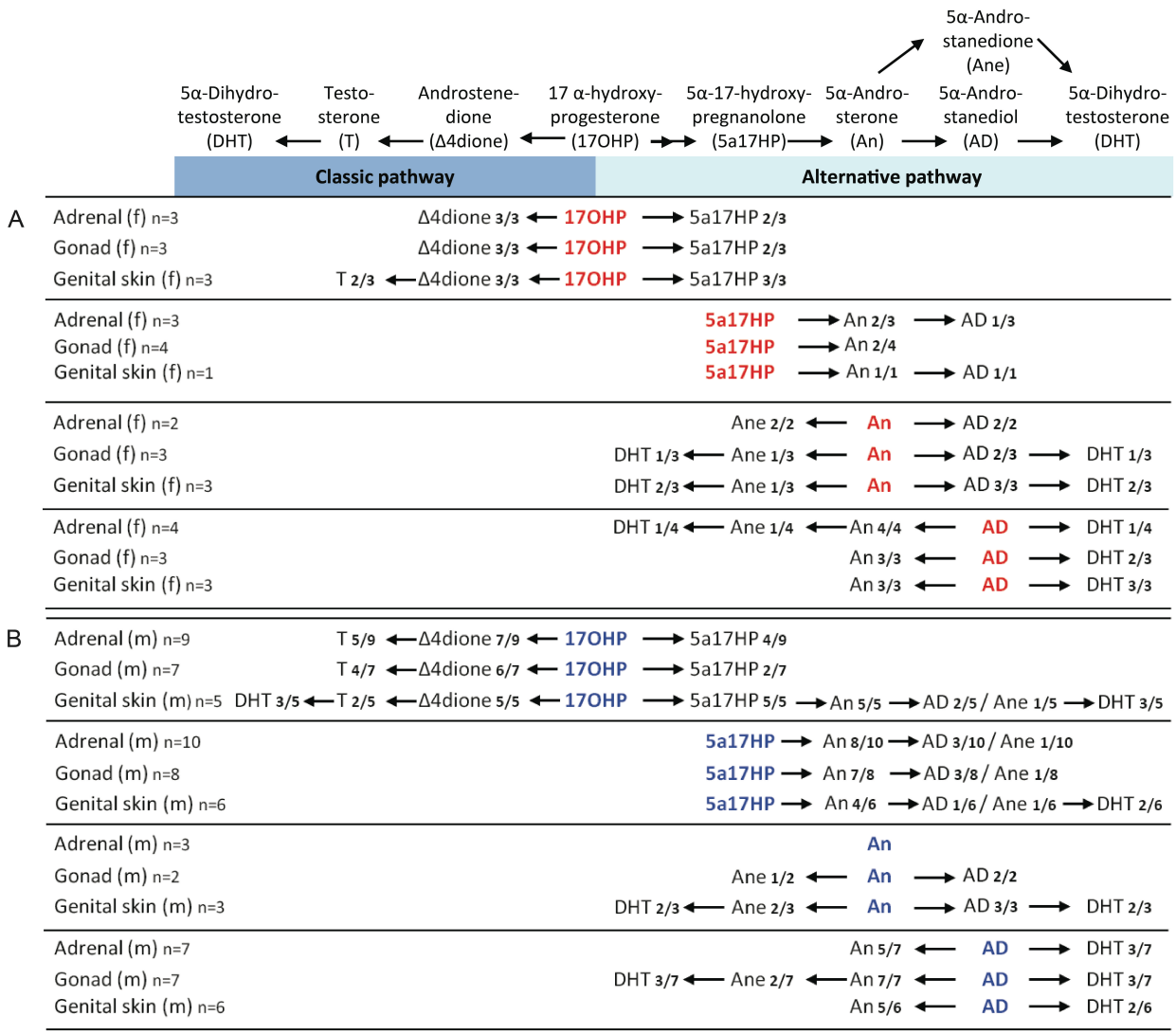

Figure 1: Androgen biosynthesis via classic and alternative androgen pathways as observed in human fetal organ explant cultures from the major period of sexual differentiation (6-10 wpc). The steroid substrates added to the explant cultures derived from fetal adrenals, gonads and genital skin are shown in red and blue for fetal female (A) and male (B) tissues, respectively. Conversion products detected and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) are given in black. f, female; m, male; n numbers indicate the number of biological replicates per tissue and fetal sex and how many of the cultures showed detectable synthesis of the indicated products.

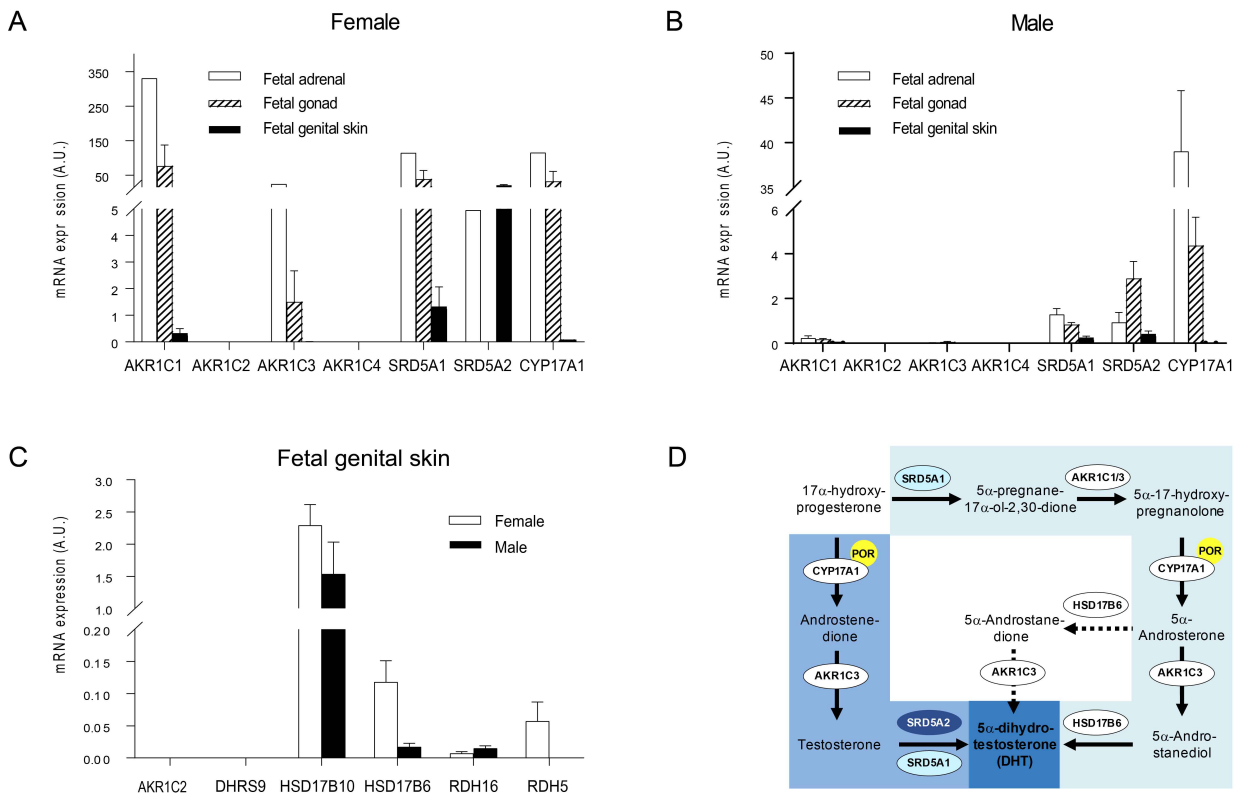

Figure 2: **Steroidogenic enzyme expression in human fetal tissue from the major period of sexual differentiation (6-10 wpc) and their proposed role(s) in alternative pathway synthesis.**

(A+B) mRNA expression (mean+SEM) in human fetal tissues from the major period of sexual differentiation (6-10 wpc) as measured in by qPCR in female adrenal (n=1), gonad (n=2), genital skin (n=2) and corresponding male tissues (≥3 biological replicates for adrenal, gonads, and genital skin). Expression data were normalized to ribosomal 18S.

(C) Schematic summary of the proposed distinct roles of the identified enzymes in the classic androgen pathway (dark blue) and the alternative androgen synthesis pathway (light blue) both resulting in the synthesis of potent 5α-dihydrotestosterone. Arrows indicate observed conversions in the fetal organ explant cultures; dotted arrows represent reactions only rarely observed.

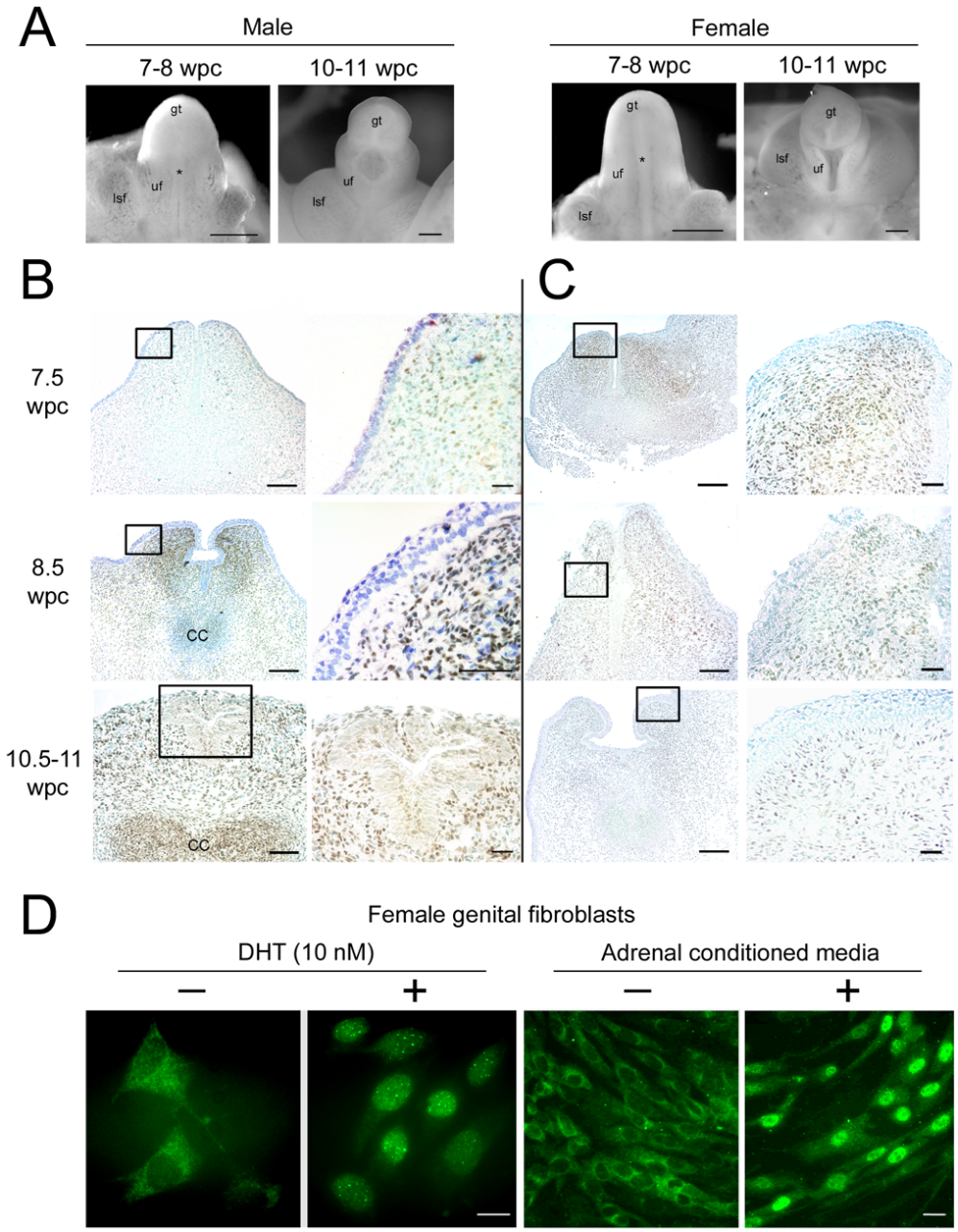

Figure 3: The androgen receptor is present in both male and female genitalia from the onset of sex differentiation. (A) Morphology of male and female external genitalia at the onset of sex differentiation. gt, genital tubercle; uf, urethral fold; lsf, labioscrotal fold; *, patency between urethral folds at 7-8 wpc that is partially sealed in males by 10-11 wpc. (B-C) Immunohistochemistry in trasverse sections through the phallus for AR in male (B) and female (C) external genitalia from the start of human sexual differentiation at 7-8 wpc counter-stained with toluidine blue. Boxes in the left-hand panel are shown at higher magnification to the right. (**D**) Immunofluorescence for AR in female external genitalia fibroblasts in the presence (+) or absence (-) of 10nM DHT (left) or medium conditioned by overnight incubation with an adrenal gland from the same female fetus (right). Size bars: (A) 500µm; (B)+(C) 100µm (low) and 20µm (high magnification); (D) 25µm.

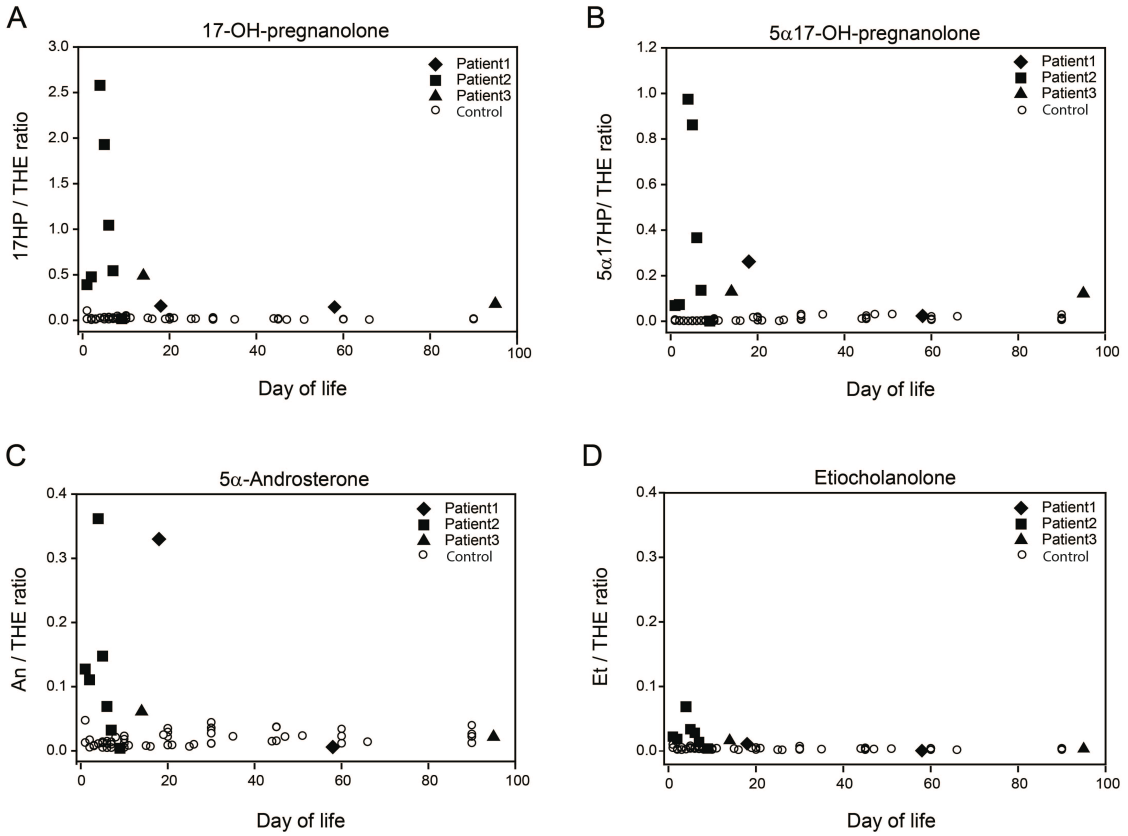

Figure 4: Urinary steroid excretion in three male neonates with POR deficiency (closed symbols) in comparison to nine sex- and age-matched healthy controls (open symbols). POR deficient neonates harbored the A287P mutation (2 homozygous, 1 heterozygous) associated with normal male genitalia in boys and genital virilization (46,XX DSD) in girls. Longitudinal urine collections were carried out during the first three months of life and analyzed by gas chromatography-mass spectrometry. Depicted are the urinary excretion of (A) the 17OHP metabolite 17-hydroxypregnanolone (17HP) and the two alternative pathway intermediates (B) 5α-17-hydroxypregnanolone (5α-17HP) and (C) 5α-androsterone (An), in comparison to (D) etiocholanolone (Et), which is only generated via the classic androgen pathway. All steroids are shown relative to tetrahydrocortisone (THE) as the denominator; THE represents an abundant adrenal-derived steroid metabolite.