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# Alternative pathway androgen biosynthesis and human fetal female virilization

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Androgen biosynthesis in the human fetus proceeds through the adrenal sex steroid precursor dehydroepiandrosterone, which is converted to testosterone in the gonads, followed by further activation to 5 $\alpha$ -dihydrotestosterone in genital skin, thereby facilitating male external genital differentiation. Congenital adrenal hyperplasia due to P450 oxidoreductase deficiency results in disrupted dehydroepiandrosterone biosynthesis, explaining undervirilization in affected boys. However, many affected girls are born virilized, despite low circulating androgens. We hypothesized that this is due to a prenatally active, alternative androgen biosynthesis pathway from 17 $\alpha$ -hydroxyprogesterone to 5 $\alpha$ -dihydrotestosterone, which bypasses dehydroepiandrosterone and testosterone, with increased activity in congenital adrenal hyperplasia variants associated with 17 $\alpha$ -hydroxyprogesterone accumulation. Here we employ explant cultures of human fetal organs (adrenals, gonads, genital skin) from the major period of sexual differentiation and show that alternative pathway androgen biosynthesis is active in the fetus, as assessed by liquid chromatography–tandem mass spectrometry. We found androgen receptor expression in male and female genital skin using immunohistochemistry and demonstrated that both 5 $\alpha$ -dihydrotestosterone and adrenal explant culture supernatant induce nuclear translocation of the androgen receptor in female genital skin primary cultures. Analyzing urinary steroid excretion by gas chromatography–mass spectrometry, we show that neonates with P450 oxidoreductase deficiency produce androgens through the alternative androgen pathway during the first weeks of life. We provide quantitative *in vitro* evidence that the corresponding P450 oxidoreductase mutations predominantly support alternative pathway androgen biosynthesis. These results indicate a key role of alternative pathway androgen biosynthesis in the prenatal virilization of girls affected by congenital adrenal hyperplasia due to P450 oxidoreductase deficiency.

fetal androgen biosynthesis | congenital adrenal hyperplasia | alternative androgen pathway | 5 $\alpha$ -dihydrotestosterone | human sexual differentiation

Gonadal development depends on chromosomal sex, whereby the 46,XY or 46,XX karyotype, established at fertilization, dictates subsequent development of either testis or ovary (1–3). Gonadal hormones then direct differentiation of either male or female genitalia. In humans, sexual differentiation is established at 7 to 12 wk post conception (wpc) (4).

While secretion of testosterone by fetal testis Leydig cells is thought sufficient to drive virilization of the internal genitalia in the male fetus (5), differentiation of the external genitalia requires the action of 5 $\alpha$ -dihydrotestosterone (DHT), which is generated locally from circulating testosterone by the enzyme steroid 5 $\alpha$ -reductase type 2 (SRD5A2) (6, 7). By contrast, differentiation of human female genitalia has been regarded as the default of a low-androgen environment.

In humans, the regulation of sexual differentiation is intricately linked to early development of the adrenal cortex (4, 8). Disorders affecting adrenal steroidogenesis commonly affect sexual differentiation, as exemplified by the multiple variants of congenital adrenal hyperplasia (CAH), which result either in inappropriate or disrupted androgen biosynthesis. This consequently causes disorders of sex development (DSDs), which can manifest with external genital virilization in newborn girls (46,XX DSD) or undermasculinization of external genitalia in male neonates (46,XY DSD) (9). The most common variant of CAH, 21-hydroxylase (CYP21A2) deficiency, manifests with 46,XX DSD, while 17 $\alpha$ -hydroxylase/17,20-lyase (CYP17A1) deficiency results in 46,XY DSD.

The congenital adrenal hyperplasia variant cytochrome P450 oxidoreductase (POR) deficiency can manifest with both 46,XY

## Significance

In the classic androgen biosynthesis pathway, testosterone is converted to 5 $\alpha$ -dihydrotestosterone, a step crucially required for normal male genital virilization. Congenital adrenal hyperplasia (CAH) due to P450 oxidoreductase deficiency (PORD) is an inborn disorder that disrupts classic androgen biosynthesis. However, some affected girls present with severe genital virilization at birth. We hypothesized that this is explained by a prenatally active, alternative biosynthesis pathway to 5 $\alpha$ -dihydrotestosterone. We show that adrenals and genital skin cooperate to produce androgens via the alternative pathway during the major period of human sexual differentiation and that neonates with PORD still produce alternative pathway androgens during the first weeks of life. This indicates that alternative pathway androgen biosynthesis drives prenatal virilization in CAH due to PORD.

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DSD and 46,XX DSD (10–12). POR plays a pivotal role as the obligatory electron donor to all microsomal cytochrome P450 enzymes, including CYP21A2 and CYP17A1, the latter catalyzing the biosynthesis of dehydroepiandrosterone (DHEA), the major precursor for testosterone biosynthesis. Consequently, POR deficiency (PORD) results in low circulating androgen concentrations, which readily account for 46,XY DSD, but fails to account for the severe virilization of external genitalia regularly observed in affected 46,XX neonates.

An explanation for this striking and seemingly contradictory genital phenotype in PORD has been lacking. We hypothesized that this apparent paradox could be explained by the existence of an alternative pathway to androgen production that generates DHT from 17 $\alpha$ -hydroxyprogesterone (17OHP) during human fetal sexual differentiation, thereby bypassing the classic androgen biosynthesis pathway via DHEA and testosterone, as previously proposed by us (11) and others (13, 14). Elements of this pathway have been characterized in the fetal gonad of the tamar wallaby pouch young (15–17) and fetal opossum urogenital tract (18, 19). 17OHP accumulates in PORD but also in the most common CAH variant, 21-hydroxylase deficiency, and thus could feed into the proposed alternative pathway, if present in the fetus. Indirect biochemical evidence has indicated that the proposed alternative pathway is active postnatally in individuals with CAH due to 21-hydroxylase deficiency (20, 21) and may explain maternal virilization observed in pregnancies affected by PORD (22, 23). However, direct delineation of the putative alternative pathway during human fetal development, and in particular during the major period of sexual differentiation, has been lacking.

Here, we present conclusive evidence for the presence and activity of the alternative pathway in the human fetus, producing potent androgens during the major period of sexual differentiation, and show that human fetal female external genitalia respond

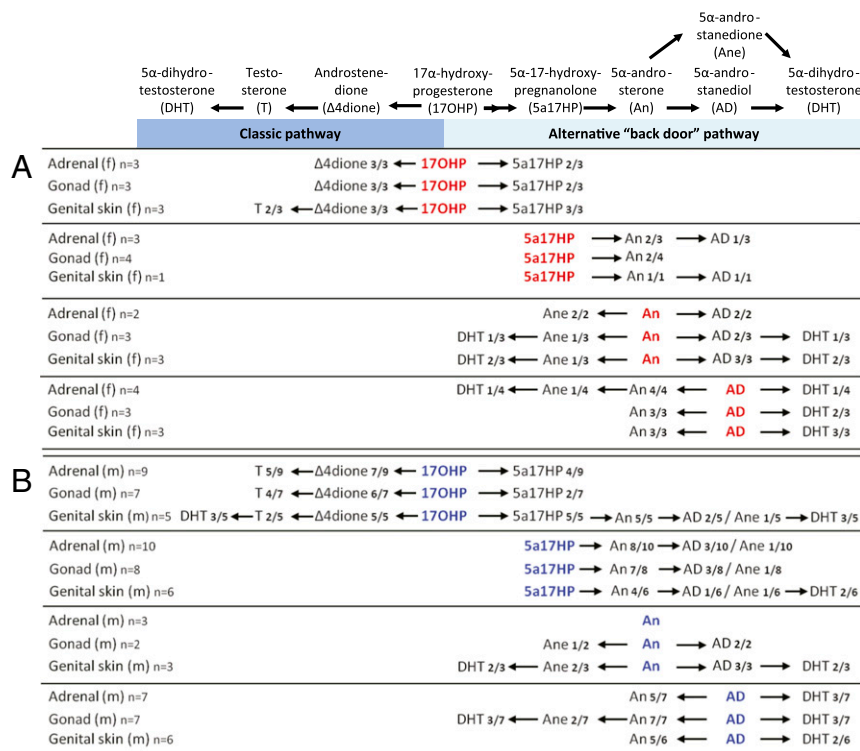
sensitively to androgens during the same period. In concert, these findings define an alternative pathway for androgen biosynthesis during the critical period of sexual differentiation in the human fetus that represents an important mechanism to explain the prenatal virilization of female infants affected by CAH.

## Results and Discussion

**Androgen Biosynthesis in the Human Fetus during Sexual Differentiation.** To ascertain the presence and activity of the hypothesized alternative androgen pathway, we performed incubations with male and female fetal adrenals, gonads, and genital skin, which were collected at 6 to 10 wpc as previously described (8). We separately added deuterated steroid substrates for each step of the alternative pathway and employed liquid chromatography–tandem mass spectrometry (LC-MS/MS) to identify the resulting products (*SI Appendix*, Fig. S1 and Tables S1 and S2). Experiments were conducted at least in triplicate for each organ of either sex (*SI Appendix*, Figs. S2 and S3).

Explant incubations with female tissue showed that some 17OHP entered the classic androgen biosynthesis pathway, yielding androstenedione in adrenal, ovary, and genital skin. However, testosterone was detected in only 2 of 9 female genital skin incubations, and not at all in female adrenals and gonads (Fig. 1A). By contrast, all 3 female tissues (genital skin, ovaries, and adrenals) showed conversion of 17OHP and subsequent intermediates through all steps of the proposed alternative androgen pathway, with the end product, DHT, produced from 5 $\alpha$ -androsterone, with either 5 $\alpha$ -androstanediol or 5 $\alpha$ -androstanedione as intermediates (Fig. 1A).

Male fetal tissues readily converted 17OHP along the classic androgen pathway to testosterone and, in the alternative pathway, to 5 $\alpha$ -17-hydroxypregnanolone (5 $\alpha$ -17HP). In male genital skin, conversion from 17OHP proceeded until the generation of DHT



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

(Fig. 1B), feasibly arising from either route, as DHT represents the end product of both the classic and alternative androgen pathways. However, incubations with the intermediate substrates of the alternative pathway demonstrated stepwise catalysis to DHT by male adrenal, testis, and genital skin (Fig. 1B). Consistent with studies in nonhuman species (24, 25), we did not identify 5 $\alpha$ -pregnane-17 $\alpha$ -ol-3,20-dione as a significant intermediate of the 2-step conversion of 17OHP to 5 $\alpha$ -17HP in tissues from either sex, likely due to its immediate forward conversion.

Taken together, these data show that adrenals, gonads, and genital skin are capable of androgen biosynthesis via both the classic and alternative pathways in both sexes. Our data suggest the presence of an integrated adrenogenital steroidogenic unit capable of producing DHT during the period of sexual differentiation in both male and female fetuses.

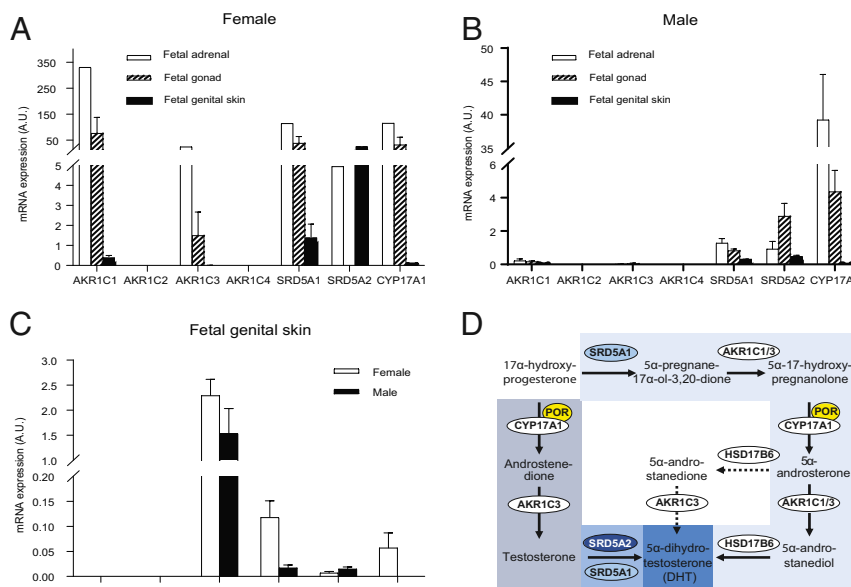
We did not study the recently described 11-oxygenated androgen biosynthesis pathway (26, 27), initiated by conversion of androstenedione to 11-hydroxyandrostenedione by CYP11B1 11 $\beta$ -hydroxylase activity, and eventually yielding 11-ketotestosterone, which activates the androgen receptor with similar potency to testosterone. Recent work indicated a significant role of 11-oxygenated androgens in CAH due to 21-hydroxylase deficiency (28) and polycystic ovary syndrome (29). We previously demonstrated the presence and activity of CYP11B1 in the human fetal adrenal during the period of sexual differentiation (8), and hence at least the initial step of the 11-oxygenated androgen pathway is likely to occur. However, urine steroid excretion analysis in infants with PORD (13) did not show increased excretion of 11-hydroxyandrostosterone, the major metabolite derived from 11-oxygenated androgens, indicating that this side arm of the classic androgen pathway is unlikely to play a major role in PORD.

**Steroidogenic Enzyme Expression during Human Fetal Sexual Differentiation.** The alternative pathway conversion of 17OHP to 5 $\alpha$ -17HP requires sequential 5 $\alpha$ -reductase and 3 $\alpha$ -hydroxysteroid dehydrogenase activities.

Of the 2 isoforms, the first step is expected to require catalysis by steroid 5 $\alpha$ -reductase type 1 (SRD5A1), since SRD5A2 does not convert 17OHP efficiently (30). A study of fetal tissues from 12 to 20 wpc did not detect SRD5A1 in adrenals, gonads, or genital skin (31); a recent study also using fetal tissues from the second trimester of pregnancy (11 to 21 wpc) described detection of SRD5A1 in liver, placenta, testis, and genital tubercle but not in the adrenal, while female gonads were not studied (32). However, studying tissue from the major period of human sexual differentiation, we found mRNA expression of SRD5A1 in adrenals, gonads, and genital skin of both sexes (Fig. 2A). The second step, the conversion of 5 $\alpha$ -pregnane-17 $\alpha$ -ol-3,20-dione to 5 $\alpha$ -17HP, requires 3 $\alpha$ -hydroxysteroid dehydrogenase activity, in keeping with the observed expression of *AKR1C1* and *AKR1C3*, both of which encode enzymes capable of catalyzing this reaction (Fig. 2A).

The next step in the alternative pathway requires CYP17A1 17,20-lyase activity; 5 $\alpha$ -17HP is the preferred substrate for this reaction and efficiently converted to 5 $\alpha$ -androsterone (33). We detected robust *CYP17A1* expression in human fetal adrenals from the major period of sexual differentiation, consistent with previous reports (8, 34) and also in the gonads of both sexes (Fig. 2B). The subsequent reduction of 5 $\alpha$ -androsterone to 5 $\alpha$ -androstenediol requires 17 $\beta$ -hydroxysteroid dehydrogenase activity, which can be provided by AKR1C3 or AKR1C1, expressed in adrenals, gonads, and genital skin (Fig. 2A).

The final step of the proposed pathway involves the conversion of 5 $\alpha$ -androstenediol to DHT, which requires 3 $\beta$ -epimerase (oxidative 3 $\alpha$ -HSD) activity. Several enzymes have been considered to catalyze this reaction (i.e., AKR1C2, RDH5, DHR59, HSD17B10, HSD17B6, and RDH16). However, only HSD17B6 and RDH16 are capable of efficient oxidation of 5 $\alpha$ -androstenediol to DHT, as previously demonstrated by transactivation of the androgen receptor following cell-based overexpression (24, 35). We found expression of both HSD17B6 and RDH16 in fetal genital skin of both sexes (Fig. 2C).

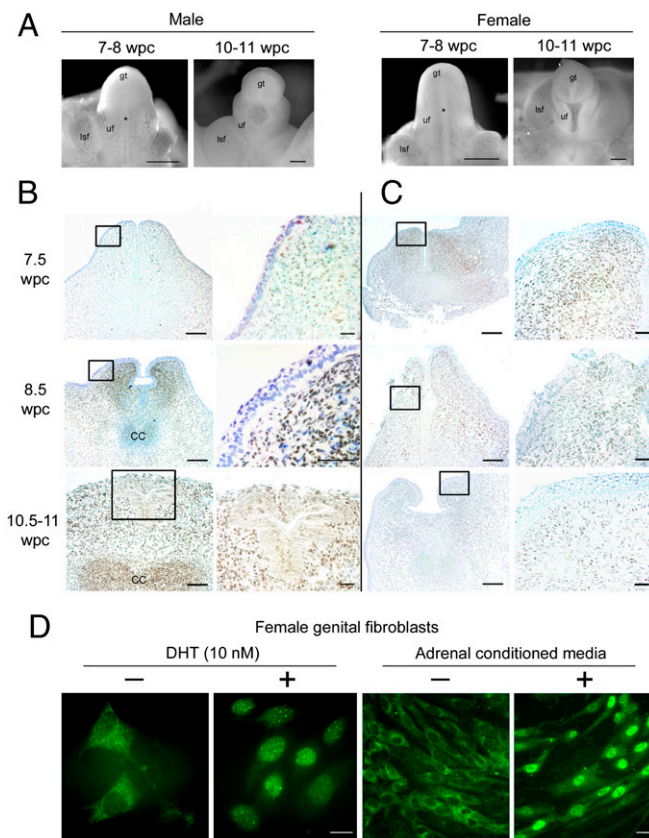


**Fig. 2.** Steroidogenic enzyme expression in human fetal tissue from the major period of sexual differentiation and their proposed role(s) in alternative pathway synthesis. (A and B) mRNA expression (mean  $\pm$  SEM) in human fetal tissues collected at 6 to 10 wpc as measured by qPCR in female adrenals ( $n = 1$ ), gonads ( $n = 2$ ), genital skin ( $n = 2$ ), and corresponding male tissues ( $\geq 3$  biological replicates for adrenals, gonads, and genital skin). (C) Fetal genital skin mRNA expression of all enzymes potentially capable of converting 5 $\alpha$ -androstenediol to 5 $\alpha$ -dihydrotestosterone. Expression data were normalized to ribosomal 18S. (D) 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 5 $\alpha$ -dihydrotestosterone. Arrows indicate observed conversions in the fetal organ explant cultures; dotted arrows represent reactions only rarely observed.

In summary, we detected the transcripts encoding all enzymes required to catalyze the alternative androgen pathway (Fig. 2D). Taken together with the steroid conversion studies, these data comprehensively demonstrate that the normal adrenal, gonad, and genital skin are capable of androgen biosynthesis via both the classic and alternative pathways in both sexes. Our data point to an adrenogenital steroidogenic unit that can cooperate to produce DHT via the alternative pathway during the major period of human sexual differentiation.

**Androgen Receptor in Female Genitalia from the Start of Sexual Differentiation.** Having demonstrated the capacity for DHT production from steroidogenic precursors in female fetuses, we corroborated its ability to function by examining the presence of the androgen receptor (AR) in female external genitalia from the start of sexual differentiation. Previously, AR expression was documented in 4 female human fetuses from 9 to 18 wpc (36). In our study, we readily detected AR protein in stromal cells in the urethral folds of the external genitalia in both male and female fetuses at the onset of sexual differentiation (Fig. 3 A–C). Its nuclear localization in fixed tissues in both sexes implied AR was ligand-bound. To explore this further, we studied the intracellular localization of AR by immunofluorescence in female external genital fibroblasts taken into primary culture from the same stage of development. In steroid-free media, the external genital cells demonstrated cytoplasmic AR localization. As expected, the addition of 1 nM DHT induced nuclear translocation of AR. Strikingly, the same translocation was observed when using medium conditioned overnight from the corresponding female adrenal gland (Fig. 3D). In combination, these data show AR from the start of sexual differentiation in both male and female external genitalia and demonstrate a functional adrenogenital steroidogenic unit capable of causing AR nuclear translocation.

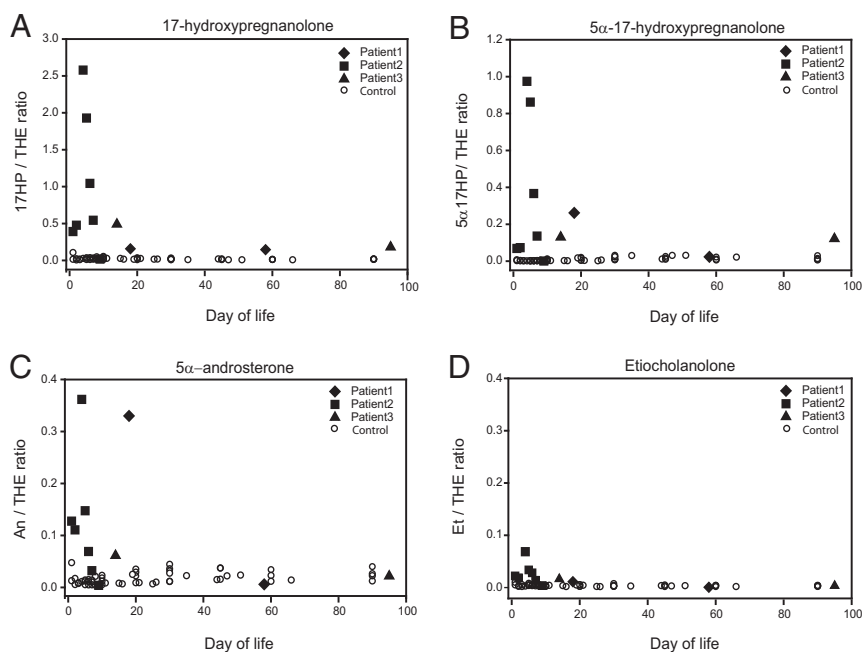
**Androgen Biosynthesis in Neonates with Congenital Adrenal Hyperplasia Due to P450 Oxidoreductase Deficiency.** Having shown evidence of the alternative pathway during human sexual differentiation, we next investigated whether we could demonstrate equivalent activity *in vivo*. To address our hypothesis that excess alternative pathway androgen biosynthesis in fetal life explains female virilization (46,XX DSD) in CAH due to PORD (12, 37), in whom the classic androgen pathway is disrupted, we identified 3 patients with a mutation known to cause 46,XX DSD in affected girls and facilitate normal male appearance of external genitalia in affected boys (POR A287P) (11, 12). All 3 individuals had a 46,XY karyotype; patients 1 and 2 harbored homozygous A287P mutations while patient 3 was compound-heterozygous for POR A287P/G188\_V191dup. While postnatal circulating androgens in PORD are low in infancy and beyond (12, 37), we hypothesized that affected individuals would still show evidence of excess alternative pathway androgen biosynthesis in the immediate neonatal period. We analyzed urinary steroid metabolite excretion by gas chromatography–mass spectrometry and detected significant alternative pathway activity in the neonatal period, consistent with previous preliminary findings that argued for the adrenal gland as a major site for alternative pathway activity (13). During the first 3 wk after birth, we documented increased excretion of the 17OHP metabolite 17-HP compared with healthy controls (Fig. 4A), consistent with accumulation of 17OHP, the initial substrate of the alternative androgen pathway. We found significantly increased excretion of 5 $\alpha$ -17HP (Fig. 4B), a key intermediate of the alternative pathway; and, finally, there was significantly increased excretion of the major DHT metabolite 5 $\alpha$ -androsterone (Fig. 4C). By contrast, etiocholanolone excretion, derived from classic pathway androgen biosynthesis, did not differ between the affected and healthy control groups (Fig. 4D). This result corroborates a previous report on urine steroid metabolite excretion in a 46,XY infant affected by PORD, which also described increased



**Fig. 3.** 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; lsf, labioscrotal fold; uf, urethral fold. Asterisks indicate patency between urethral folds at 7 to 8 wpc that is partially sealed in males by 10 to 11 wpc. (B and C) Immunohistochemistry in transverse sections through the phallus for AR in male (B) and female (C) external genitalia from the start of human sexual differentiation at 7 to 8 wpc counterstained with toluidine blue. Boxes (Left) are shown at higher magnification (Right). (D) Immunofluorescence for AR in female external genital fibroblasts in the presence (+) or absence (–) of 10 nM DHT (Left) or medium conditioned by overnight incubation with an adrenal gland from the same female fetus (Right). [Scale bars, 500  $\mu$ m (A); 100  $\mu$ m (low) and 20  $\mu$ m (high magnification) (B and C); and 25  $\mu$ m (D).]

5 $\alpha$ -androsterone but unremarkable etiocholanolone excretion (38). Of note, the POR A287P-homozygous patients in our study had higher excretion of 5 $\alpha$ -17HP and 5 $\alpha$ -androsterone than the compound-heterozygous patient, who can be expected to have less alternative pathway activity due to the greater impairment of residual POR function. Taken together, these findings indicate increased *in vivo* alternative pathway androgen biosynthesis in neonates with PORD, which ceases shortly after birth.

These findings were further supported by *in vitro* 17,20-lyase activity assays employing yeast microsomes cotransformed with human CYP17A1 and either wild-type or mutant POR. The results demonstrated that the residual activity of POR A287P was higher in the alternative androgen pathway than in the classic pathway. POR A287P demonstrated significantly higher activity than POR mutant H628P, which in the homozygous state is associated with severe male undervirilization (46,XY DSD) and normal female genital phenotype (SI Appendix, Fig. S1 A–C). Additional experiments with yeast microsomes cotransformed with CYP19A1 and wild-type or mutant POR demonstrated that neither mutant affected aromatase activity (SI Appendix, Fig. S1D), thus excluding impaired aromatization of classic pathway androgens as a driver of prenatal virilization.



**Fig. 4.** Urinary steroid excretion in 3 46,XY neonates with POR deficiency (closed symbols) in comparison with 9 sex- and age-matched healthy controls (open symbols). POR-deficient neonates harbored the A287P mutation, which in the homozygous state is associated with normal male genitalia in boys and genital virilization (46,XX DSD). We included data from 3 46,XY neonates with PORD; 2 harbored homozygous POR mutations (A287P/A287P), and 1 harbored compound-heterozygous mutations (A287P/G188\_V191dup). Longitudinal urine collections were carried out during the first 3 mo of life and analyzed by gas chromatography–mass spectrometry. Depicted are the urinary excretion of (A) the 17OHP metabolite 17-hydroxypregnanolone (17HP) and the 2 alternative pathway intermediates (B) 5 $\alpha$ -17-hydroxypregnanolone (5 $\alpha$ -17HP) and (C) 5 $\alpha$ -androsterone (An), in comparison with (D) etiocholanolone (Et), which is only generated via the classic androgen pathway. All steroids are shown relative to tetrahydrocortisone (THE), an abundant adrenal-derived steroid metabolite, as the denominator.

In conclusion, we have provided *in vitro*, *ex vivo*, and *in vivo* evidence for the existence and activity of an alternative pathway for the synthesis of the most potent androgen, DHT, during early human development. Our data demonstrate that, through cooperation of an adrenogenital steroidogenic unit, the alternative androgen pathway yields active androgen synthesis in the female fetus, with excess activity driving female virilization, 46,XX DSD, in CAH due to P450 oxidoreductase deficiency. Given that the alternative pathway substrate 17OHP also accumulates in 21-hydroxylase deficiency, it is conceivable that alternative pathway androgens contribute to prenatal virilization in this most common CAH variant.

## Materials and Methods

**Collection of Human Embryonic and Fetal Material.** Ethical approval for these studies was granted by the North West Haydock Research Ethics Committee of the UK Health Research Authority (approval no. 18/NW/0096). The collection and staging of human embryonic and fetal material were carried out with informed consent, as described previously (8, 39), using Carnegie classification and fetal foot length to provide a direct assessment of developmental age as days or weeks post conception (dpc or wpc), respectively, and male fetal material was identified by SRY expression, as previously described (1). We analyzed organs and tissue from 30 fetuses: 25 male and 5 female; median age 55 dpc (range 44 to 84 dpc).

**RNA Extraction, Reverse Transcription, and Quantitative PCR.** Total RNA was extracted from whole organs using the TRI Reagent system (Sigma-Aldrich). RNA integrity and concentrations were assessed using a NanoDrop spectrophotometer. Reverse transcription was carried out employing a standard protocol. mRNA expression levels were quantified using an ABI 7500 sequence detection system (PerkinElmer Applied Biosystems), employing the Applied Biosystems “assay on demand” probe and primers for specific amplification of SRD5A1, SRD5A2, CYP17A1, AKR1C1, AKR1C2, AKR1C3, AKR1C4, HADH2/HSD17B10, HSD17B6, RDH5, DHRS9, and RDH16 (for further details, see *SI Appendix*).

**Tissue Explant Culture and Steroid Identification by Tandem Mass Spectrometry.** Whole-organ tissue explants (fetal adrenals, gonads, and genital skin) were cultured in DMEM/F12 (PAA Laboratories) supplemented with 2% Ultraser SF (i.e., steroid-free; BioSeptra) and 1% ITS+ (BD) at 37 °C in humidified 5% CO<sub>2</sub> and 95% air for 64 h. Genital skin was cultured as monolayers and used for experiments at passage 4.

Identification of steroid products from the explant cultures was achieved using LC-MS/MS. Steroids were positively identified by comparison of retention time and MS/MS mass transitions with authentic steroid standards (*SI Appendix, Table S1*). Two mass transitions were used to positively identify each steroid, referred to as quantifier and qualifier ions, respectively; the resolution of a series of authentic steroid standards is shown in *SI Appendix, Fig. S2*, alongside further method details.

For steroid conversion assays, tissue explants were incubated with precursor steroids purchased from Steraloids and Sigma-Aldrich. For explant cultures assessing the conversion of 17-hydroxyprogesterone, 5 $\alpha$ -androsterone, and 5 $\alpha$ -androstenediol, we used deuterated steroids (for details, see *SI Appendix, Table S2*). Representative results of steroid detection following explant culture incubations are shown in *SI Appendix, Figs. S3 and S4* for female and male tissues.

**Immunohistochemistry.** Immunohistochemistry, immunoblotting, and immunofluorescence were carried out as reported previously (40), using monoclonal mouse anti-AR (1:100; LabVision).

**Urine Steroid Metabolite Excretion Analysis.** Urine samples were collected longitudinally from birth until 90 d of life in 3 neonates affected with PORD (2 homozygous for POR A287P, and the other 1 compound-heterozygous for POR A287P/G188\_V191dup) and compared with those collected from 9 healthy controls. These were matched for sex, age, and gestational age at birth and collected during the same time window. The parents of PORD patients and healthy controls provided written informed consent prior to urine collection. The study protocol was reviewed and approved by the Research Ethics Committee of the University College London Institute of Child Health/Great Ormond Street Hospital NHS Trust (REC reference 05/Q0508/24).

Urinary steroid hormone profiles were determined by gas chromatography–mass spectrometry analysis as described previously (41). The final analytical samples are the methoxime-trimethylsilyl derivatives of steroids

enzymatically released from sulfate and glucuronide conjugation. Analytes quantified by selected ion monitoring were normalized to tetrahydrocortisone, the most abundant steroid metabolite consistently excreted throughout life, with no significant difference in urinary tetrahydrocortisone concentrations identified between PORD patients ( $n = 3$ ; 13 urine samples; median 285  $\mu\text{g/L}$ , range 56 to 1,256  $\mu\text{g/L}$ ) and healthy controls ( $n = 9$ ; 48 urine samples; median 306  $\mu\text{g/L}$ , range 59 to 1,663  $\mu\text{g/L}$ ).

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