

# Alternative (backdoor) androgen production and masculinization in the human fetus

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# Abstract

Masculinization of the external genitalia in humans is dependent on formation of 5 $\alpha$ -dihydrotestosterone (DHT) through both the canonical androgenic pathway and an alternative (backdoor) pathway. The fetal testes are essential for canonical androgen production but little is known about the synthesis of backdoor androgens despite their known critical role in masculinization. In this study, we have measured plasma and tissue levels of endogenous steroids in second trimester human male fetuses using multi-dimensional and high-resolution mass-spectrometry. Results show that androsterone is the principal backdoor androgen in the fetal circulation and that DHT is undetectable (<1ng/ml). Backdoor pathway intermediates are found primarily in the placenta and fetal liver with significant androsterone levels also in the fetal adrenal. Backdoor intermediates, including androsterone, are mostly undetectable in the fetal testes. This is consistent with transcript levels of enzymes involved in the backdoor pathway (SRD5A1, AKR1C2/4, CYP17A1), as measured by qPCR. These data identify androsterone as the predominant backdoor androgen in the human fetus and show that it is formed primarily in non-gonadal tissue with placental progesterone the likely substrate. Masculinization of the human fetus depends, therefore, on androgen synthesis by both the fetal testes and non-gonadal tissues leading to DHT formation at the genital tubercle. Our findings provide, for the first time, a solid basis to explain why placental insufficiency is associated with disorders of sex development in humans

# Introduction

The male external genitalia are the most common site of congenital abnormalities in the human, with up to 0.8% of male births affected (1;2). The most frequent of these abnormalities is hypospadias, which is characterized by abnormal opening of the urethra on the ventral side of the penis. Normal masculinization of the fetus is dependent upon androgen secretion by the testis and androgens act initially during a critical masculinization programming window to ensure normal male reproductive development (3). In humans, male-specific morphological differentiation and subsequent growth of the genital tubercle/penis begins around 10-12 weeks of gestation (4;5) and continues through the second and third trimesters (6;7). The etiology of hypospadias is probably multifactorial but it is likely that altered androgen exposure during the second trimester is a significant factor (8).

During masculinization testosterone acts directly to stabilize the mesonephric (Wolffian) ducts and to induce testis descent. However, it is conversion of testosterone to the more potent 5 $\alpha$ -dihydrotestosterone (DHT) at the target organ which leads to masculinization of the external genitalia (9). In humans, testosterone is synthesized in the testicular Leydig cells through the canonical  $\Delta^5$  pathway shown in Fig 1 (10;11). More recently, however, it has been reported that an alternative pathway to DHT formation exists which does not require testosterone as an intermediate. This alternative, “backdoor”, pathway (Fig 1) was first described in the testes of pouch young marsupials (12) and a similar pathway has since been reported in the prepubertal mouse testis (13) and the fetal human testis (14). The importance of the backdoor pathway to normal human development was initially unclear, but studies by Fluck *et al* (14) have shown that disordered sex development (DSD) will arise if the pathway is disrupted. In one individual with mutations in the aldo-keto reductase enzyme AKR1C2 and in another family with an added mutation in *AKR1C4*, there was failure of normal masculinization. Importantly, the consequences of these mutations in the backdoor pathway

are similar to those seen in individuals with mutations in the canonical pathway (15). These data demonstrate that both the canonical and backdoor pathways are essential for normal fetal masculinization.

Currently, the accepted model for masculinization is that circulating DHT, formed via the backdoor pathway in the fetal testis (14;15), is important for virilization alongside circulating testosterone. At present, however, our understanding of the regulation of human fetal masculinization is seriously hindered because we do not know which steroids are present in the male fetal circulation or fetal tissues, what the concentrations of these steroids are and which tissues are involved in their metabolism. This means that the circulating levels of DHT and potential substrates for DHT synthesis at the target organ, from either the canonical or backdoor pathways, remain unknown in the human fetus.

In this study we have measured (i) concurrent levels of fetal plasma and tissue steroids by hyphenated mass spectrometric tools (ii) transcript levels of critical enzymes in the backdoor pathway in human fetal tissues and (iii) canonical and backdoor androgen synthesis by the human fetal testis *in vitro*. Our results show that high levels of intermediates in the backdoor pathway are present in the human fetal circulation and that androsterone is the major circulating backdoor androgen. Crucially, the results also show that the fetal testis contains only low levels of backdoor androgens and DHT and that androsterone is likely to be formed in non-gonadal tissues largely through metabolism of placental progesterone and adrenal dehydroepiandrosterone (DHEA).

## Results

### High levels of intermediates in the backdoor pathway are present in male fetal plasma but DHT is undetectable.

Overall levels of steroids involved in the synthesis of DHT in male fetal plasma (from cardiac puncture ex-vivo) are shown in Fig 2. As expected, all steroids in the canonical  $\Delta^4$  and  $\Delta^5$  pathways were present in the fetal circulation. Noticeably, the data also shows that intermediates in the backdoor pathway were present at levels comparable to the  $\Delta^5$  pathway, with the backdoor pathway clearly going from progesterone through 5 $\alpha$ -dihydroprogesterone (5 $\alpha$ DHP), allopregnanolone, 17 $\alpha$ -hydroxyallopregnanolone to androsterone (Fig 2). Circulating DHT, however, was not detectable in any of the 42 fetuses (<1ng/ml). The  $\Delta^5$  steroids pregnenolone, 17 $\alpha$ -hydroxypregnenolone and DHEA were present at the highest levels in the fetal circulation and these steroids probably come from the fetal adrenal gland (16;17). Levels of progesterone were also high and were likely to be derived principally from the placenta (18) (and see below). The key initial intermediates in the backdoor pathway, 5 $\alpha$ DHP and allopregnanolone, were present in the fetal circulation at similar concentrations to progesterone (means: progesterone 258ng/ml, 5 $\alpha$ DHP 135ng/ml, allopregnanolone 243ng/ml). The principal  $\Delta^4$  and backdoor androgens detectable in most samples were androstenedione, testosterone and androsterone, all potential substrates for DHT synthesis. Most forms of 5 $\alpha$ -androstanediol were undetectable in fetal plasma (including 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol), although 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (labelled androstanediol in Figs 1 and 2), which is a potential substrate for DHT synthesis, was detectable in 10/42 samples (Fig 2). Etiocholanolone, a metabolite of androstenedione, was also present in most samples (Supplementary Fig 1). Levels of most steroids did not change over the course of the second trimester, with the exceptions of testosterone which declined significantly ( $P<0.048$ ) and androstenediol which increased ( $P<0.004$ ) over the same period (Supplementary Fig 2).

Maternal smoking had no significant effect on fetal plasma steroid levels. A full list of steroids measured by GC-MS/MS in human male fetal plasma is shown in Supplementary Table 3.

**Backdoor intermediates are present primarily in the placenta and fetal liver with androsterone primarily in the fetal adrenal.**

Levels of major  $\Delta^4$ ,  $\Delta^5$  and backdoor androgens in placenta, fetal liver, fetal adrenal and fetal testis are shown in Fig 3. Note that 17 $\alpha$ -hydroxylated intermediates were not measured in this study while matrix effects meant that the 5 $\alpha$ -reduced androgen, androstenedione, was not detectable. The placenta contained high levels of progesterone with lower amounts of 5 $\alpha$ DHP and allopregnanolone. The backdoor androgens, androsterone and androstenediol were detectable in about half the placentas while the  $\Delta^4$  steroids androstenedione and testosterone were detectable in most placentas. DHT was also detectable in about half the placental samples (Fig 3). The major steroids detectable in the fetal liver were progesterone, allopregnanolone and DHEA. Low levels of androsterone and DHT were also detectable in most fetal livers while androstenediol was detectable in about half the samples (Fig 3). The fetal adrenals contained high levels of pregnenolone, progesterone and DHEA and androsterone was present in most adrenals. Testosterone was present in about half the adrenals but other steroids were not detectable (Fig 3). The fetal testes contained high levels of pregnenolone and testosterone with lower levels of progesterone and androstenedione. The backdoor intermediates 5 $\alpha$ DHP and allopregnanolone were detectable in 6 and 9 testes respectively (out of 25) but androsterone was not detectable and androstenediol was only detectable in one testis. Low levels of DHT were detectable in 5 testes. To confirm the low/undetectable levels of 5 $\alpha$ -reduced androgens in the fetal testis, testicular extracts from a further 6 fetuses were measured by GC-MS/MS (Table 2) to increase sensitivity (see

Materials and Methods). These fetuses were aged 15-19 weeks and the levels of pregnenolone, DHEA, progesterone, androstenedione and testosterone were generally within the range shown in Fig 3 (all data in Fig 3 is from fetuses aged 13 or 14 weeks). In those cases measured by GC-MS/MS the backdoor steroids allopregnanolone, androstanedione, androsterone and androstanediol were detectable at very low levels in all 6 testes. Low levels of DHT were also detected in all testes (Table 2).

To determine whether human fetal testes produce backdoor steroids under hormonal stimulation, dispersed fetal testicular cells were incubated with or without hCG for 24 h and the steroids produced were measured by GC-MS/MS. In the canonical pathway  $17\alpha$ -hydroxyprogesterone, DHEA and androstenedione were detectable in most samples as was pregnenolone at low levels (Supplementary Fig 3). The presence of hCG had a stimulatory effect on DHEA levels. DHT was detected in one control culture. No backdoor androgens, or intermediates in their synthesis, were detectable in any testicular cell cultures.

### **Enzymes associated with the backdoor pathway are predominantly expressed in non-gonadal tissues.**

The critical entry-point to the backdoor pathway is through  $5\alpha$ -reduction of progesterone or  $17\alpha$ -hydroxyprogesterone by *SRD5A1*. The highest levels of *SRD5A1* expression in the second trimester fetus were in the liver, with significant, but lower expression in the placenta, testis and genital tubercle (Fig 4). *SRD5A2* expression was only consistently detectable in the genital tubercle. The placenta and fetal liver are considerably larger than the other organs measured in this study (Table 1) and, in terms of *total* fetal transcript levels, therefore, these tissues have about 1,000 times greater *SRD5A1* expression than the testis. *AKR1C2* is specific to the backdoor pathway and is critical for human fetal masculinization (14). Mean *AKR1C2* transcript levels were highest in the fetal liver and fetal testis (Fig 4) although,

taking tissue mass into account, liver and placenta each have ~200 times more total *AKR1C2* transcript than the fetal testes. There was also significant *AKR1C2* expression in the genital tubercle which is likely to be important for local DHT synthesis from androsterone (Fig 1). *AKR1C4* is the other backdoor enzyme which may be required for masculinization (14) and transcripts were only consistently detected in the fetal liver (Fig 4). Expression of *HSD17B6* was highest in the testis with transcripts also consistently detected in the adrenal and placenta and lower expression in the genital tubercle (Fig 4). *HSD17B3* was expressed at similar levels in the testis and liver, with very low or undetectable expression in the placenta, adrenal and genital tubercle (Fig 4). *AKR1C3* showed the highest level of expression in the liver with low but detectable expression in other tissues. The cytochrome P450 enzyme CYP17A1 is essential in both canonical and backdoor pathways of androgen synthesis (Fig 1). Predictably, expression was high in both fetal adrenal and fetal testis, with the mean adrenal level about 6 times that of the testis (Fig 4). Expression was very low or undetectable in the genital tubercle, liver and placenta. The HSD3B2 enzyme is essential for *de novo* androgen synthesis and highest levels of *HSD3B* were in the placenta with lower expression in the testis and adrenal (Fig 4).

## Discussion

Masculinization of the fetus is dependent on the action of testosterone at the Wolffian ducts and on the action of DHT at the external genitalia (19). The process of masculinization at the external genitalia starts in the early second trimester and the most intense phase of penile growth also occurs later in the second trimester (20). This is a critical period for normal masculinization, therefore, and it was assumed, until recently, that growth of the external genitalia was solely dependent on DHT formed in the target organ through 5 $\alpha$ -reduction of testis-derived testosterone. However, the recent demonstration that the alternative, backdoor



pathway to DHT synthesis is also required for normal human fetal male development (14) has shown that the process is a complex interaction between different steroidogenic pathways. In this study we now show for the first time that androsterone is the major circulating backdoor androgen in the human male fetus and that most circulating androsterone comes from non-gonadal tissues that use placental progesterone (or its metabolites) as substrate. Masculinization depends, therefore, not only on the fetal testes, but also on other, non-gonadal tissues.

The backdoor pathway of androgen synthesis depends, initially, on progesterone formed from pregnenolone and in the fetus the major *de novo* sources of pregnenolone are the adrenal and the testis. In both tissues, however, pregnenolone is metabolized predominantly through the  $\Delta^5$  pathway to DHEA because pregnenolone is bound with a significantly higher affinity by human CYP17A1 than by HSD3B2 (21). In addition, HSD3B activity is likely to be relatively low compared to CYP17A1 in both tissues, based on transcript levels (this study and (22;23)). Both tissues contain progesterone, at levels similar to those reported previously (24), but the tissue concentrations, particularly in the testis, are not markedly greater than those in the liver which lacks significant *HSD3B* expression and does not have the capacity to synthesize progesterone *de novo* (25). This means that most progesterone in fetal human tissues and circulation is likely to come from the placenta which has been shown previously to secrete progesterone directly into the fetal circulation at high levels (26;27), similar to those reported here.

Metabolism of progesterone to 5 $\alpha$ DHP depends on SRD5A1 and transcript levels of this enzyme are highest in the liver, with lower levels also present in the placenta and testis. This is consistent with earlier *in vivo* studies showing high relative 5 $\alpha$ -reductase activity in the fetal liver (28). Tissue levels of 5 $\alpha$ DHP are highest in the placenta, however, which probably reflects the high concentration of progesterone substrate in this tissue. Within the

backdoor pathway, the conversion of 5 $\alpha$ DHP to allopregnanolone by AKR1C2 and/or AKR1C4 has been shown to be critical for masculinization (14) and the highest consistent tissue concentrations of allopregnanolone are found in the placenta and fetal liver (Fig 3). High concentrations in the fetal liver are likely to be a reflection of high AKR1C2 and AKR1C4 and, as the fetal liver lacks *CYP17A1* expression, allopregnanolone will not be further metabolized. High expression of *AKR1C2* and *AKR1C4* in the fetal liver is likely to be another reason why 5 $\alpha$ DHP levels are low in this tissue. The placenta does not contain high levels of *AKR1C2*, and *AKR1C4* is absent, but substrate levels for the enzymes are high and, like the liver, there will be little further metabolism of allopregnanolone via *CYP17A1*. In the fetal testis 5 $\alpha$ DHP and allopregnanolone levels were undetectable in about two thirds of samples but were at a significant level in the remaining samples and might be expected to contribute to circulating steroid concentrations in these fetuses. The fetal adrenals do not express *SRD5A1/2* and would not be expected to contribute to fetal 5 $\alpha$ DHP or allopregnanolone production. Taking tissue mass into account, the liver and placenta are likely to be the major sites of 5 $\alpha$ DHP and allopregnanolone production in the second trimester fetus. Placental allopregnanolone production would also be consistent with the increasing plasma levels in pregnant women during gestation (29). Finally, it should be noted that 50% or more of any steroids secreted into the fetal circulation by the placenta will go directly to the fetal liver through the umbilical vein and so most fetal 5 $\alpha$ -reduction or 3 $\alpha$ -hydroxylation of placental progesterone is likely to take place in the fetal liver.

Once formed, there is a high affinity between allopregnanolone and human *CYP17A1* (30) but the only tissues which express the enzyme are the fetal testes and adrenals. The high circulating levels of allopregnanolone in the fetus, and the relatively low 17 $\alpha$ -hydroxyallopregnanolone and androsterone levels would suggest that conversion by *CYP17A1* is limiting in the fetus. Tissue androsterone levels were highest in the adrenals

which is consistent with high *CYP17A1* transcript levels and, since the adrenals lack *SRD5A1* and appear unable to make 5 $\alpha$ -reduced steroids, the substrate for this reaction must come from circulating allopregnanolone. It should be noted that this androsterone was mostly the sulfated form which is a reflection of the high *SULT2A1* levels in the human fetal adrenal (17). The presence of androsterone in the fetal adrenals at the end of the first trimester has also been reported previously (22). In contrast to the adrenals, androsterone levels in the fetal testis were either undetectable or extremely low. Differences between the fetal testis and adrenal are likely to be a reflection of the lower *CYP17A1* in the testis, much greater vascularization in the fetal adrenal for allopregnanolone substrate provision (31) and, possibly, greater competition from other substrates in the testis. Interestingly, the placenta also contained detectable androsterone in most samples. There is some debate about whether the human placenta expresses significant *CYP17A1* and associated 17 $\alpha$ -hydroxylase and C<sub>17</sub>-<sub>20</sub> lyase activity (32;33) and our data shows that transcripts are either absent or are present only at very low levels. This suggests that most placental androsterone must be derived from adrenal DHEA although the possibility exists for some *de novo* synthesis. Given the relative sizes of the fetal organs involved and the tissue concentrations, the placenta and the fetal adrenals are likely to be, by far, the major sources of backdoor androsterone production in the fetus.

When the backdoor pathway was shown to be essential for human fetal masculinization, it was suggested that DHT is synthesized via this pathway in the fetal testes and released into the circulation (14;15). This now appears very unlikely because DHT is undetectable, or present at very low levels, in testes from most fetuses and because circulating DHT levels are undetectable (<1ng/ml). These results are consistent with earlier studies which either failed to detect DHT in the human fetal testis (34;35) or found very low levels (36). DHT can be metabolized to androstenediol by all AKR1 enzymes, and AKR1C2

in particular (11;37), and given the high levels of AKR1 enzyme transcripts in the fetal liver, it is likely that any DHT released into the fetal circulation is rapidly metabolized. Our results show, therefore, that the major circulating backdoor androgen in the fetus is androsterone which is present at similar levels to testosterone. It remains to be shown whether the genital tubercle can convert androsterone to DHT but the tissue expresses high levels of *AKR1C2*, as well as detectable *AKR1C3*, and AKR1C2 can catalyze both oxidation and reduction steps required for androsterone conversion to DHT (Figs 1 and 5). [ A schematic diagram of the proposed pathways involved in backdoor androgen synthesis in the human fetal male is shown in Fig 5.

In the DSD cases described by Flück et al (14) the 46,XY patients carried hypomorphic mutations in *AKR1C2* and *AKR1C4* or *AKR1C2* alone. From data reported here, reduced fetal AKR1C2 activity would be expected to affect backdoor pathways in the liver and testis reducing production of allopregnanolone. In addition, loss of enzyme activity in the genital tubercle may also affect production of DHT at the target organ. The potential involvement of placental AKR1C2 in the reported 46,XY DSD patients is also of interest. The placenta develops from both maternal and fetal cells and it is not known whether placental AKR1C2 is of fetal or maternal origin. It is of note, however, that two 46,XY individuals who are known heterozygotes for a mutation in *AKR1C2* show divergent phenotypes with one showing a normal male phenotype and the other DSD (14). The mother of the affected individual is also heterozygous for a mutation in *AKR1C2* and if placental AKR1C2 is of maternal origin then this would be expected to affect backdoor steroid production in the placenta. Unfortunately the genotype of the mother of the unaffected heterozygous individual is not available.

Further strong evidence for a non-testicular pathway of backdoor androgen synthesis comes from patients with P450 oxidoreductase (POR) or 21-hydroxylase deficiency who

show 46,XX virilization. It is likely that virilization in these individuals *in utero* is due to excessive backdoor androgen synthesis which is occurring in the absence of testes (38-40). It has been postulated that this increase in fetal backdoor androgen production comes from the fetal adrenals (41;42). The lack of *SRD5A1* and intermediates in the backdoor pathway in normal fetal adrenals make this unlikely, however, unless the condition itself increases adrenal *SRD5A1* activity (e.g. through increased adrenal stimulation by ACTH). Our data would suggest that the increased fetal adrenal 17 $\alpha$ -hydroxyprogesterone seen in these conditions acts initially as substrate for backdoor androgen production through 5 $\alpha$ -reduction in other tissues, probably the fetal liver.

If the placenta is a critical component of the fetal backdoor androgen pathway, as suggested by this data, then it has implications for our understanding of the regulation of masculinization and DSD. It is now established that placental insufficiency, associated with intrauterine growth restriction (IUGR), is associated with abnormalities in development of the male external genitalia, and hypospadias in particular (43;44). Severe forms of placenta-mediated IUGR start during the first trimester (43) and could interfere, therefore, with all aspects of fetal masculinization. It has been suggested that DSD associated with placental dysfunction could be due to reduced placental hCG production (44) but other studies have shown that maternal hCG levels tend to be increased in placental insufficiency (43). In contrast, maternal progesterone levels are reported to be reduced during IUGR (45), suggesting placental steroidogenesis is affected. If the placenta is central to fetal backdoor androgen production, as we suggest, then altered placental steroidogenesis may lead directly to abnormalities in masculinization.

Studies in the 1950s/60s by Jost and others showed that androgen is required for masculinization of the external genitalia (reviewed in (46)). Later studies showed that testicular testosterone must be converted to DHT at the target organ to induce

masculinization (47). Most recently, it has been shown that the normal process of masculinization in the human depends on two separate pathways of androgen synthesis, the canonical and backdoor pathways (14). We now report that the backdoor pathway in the human fetus is driven by placental progesterone production which acts as substrate for androsterone synthesis, primarily in non-gonadal tissues. This means that our current understanding of the endocrine control of masculinization in the human fetus is that it is mediated through circulating testosterone and androsterone and is dependent on a complex interaction and exchange between the testes and non-gonadal tissues, particularly the placenta.

## Methods

### **Sample collection.**

Three sources of human fetal tissues were used in this study: 1) In Aberdeen, human fetuses between 11 and 21 weeks of gestation and classified as normal at scan were collected from women over 16 years of age undergoing elective termination (17) Information about maternal smoking during pregnancy was available for most fetuses. Fetuses were transported to the laboratory within 30 minutes of delivery, weighed, crown-rump length recorded, and sexed. Blood samples from a total of 42 male fetuses were collected by cardiac puncture *ex vivo* and plasma was stored at -80°C. Tissues were snap-frozen in liquid N<sub>2</sub> and then stored at -80°C. 2) In Stockholm, human fetal testes were obtained for *in vitro* incubation studies after elective termination of pregnancy at 10–12 weeks of gestation. 3) Additional fetal livers and placentas were provided by the MRC/Wellcome-Trust funded Human Developmental Biology Resource (HBDR, <http://www.hdb.org>). Available fetal and maternal characteristics relevant to samples used in different parts of this study are shown in Supplementary Table 1.

Collections of fetal material in Aberdeen, Stockholm and by HBDR were respectively approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21), the Regional Ethics Committee of Stockholm (EPN dnr 2014/1022-32) and the relevant Research Ethics Committees, in accordance with the relevant UK Human Tissue Authority (HTA; [www.hta.gov.uk](http://www.hta.gov.uk)) Codes of Practice. Written, informed, maternal consent was received from participants prior to inclusion in the study.

### **RNA Extraction, Reverse Transcription and Real-Time PCR**

Total RNA was extracted from frozen fetal tissues either using TRIzol (Life Technologies, Paisley, UK) (48) or using Qiagen AllPrep kits (Qiagen, Manchester, UK). Reverse transcription, primer design and real-time PCR were carried out as previously described (49;50) and the primers used are shown in Supplementary Table 2. RNA which is free of genomic DNA contamination is required to amplify *SRD5A1* because of the presence of a processed pseudogene in the genome and this was carried out using RNAeasy Plus Micro-columns (Qiagen Ltd, Manchester, UK) followed by DNase treatment (DNA-free, Life Technologies, Paisley, UK). To normalize data, Normfinder was used to identify the most stable housekeeping genes in each tissue using the housekeeping genes and primers described earlier (51). The best combinations of housekeeping genes varied between tissues and so, to allow comparison of transcript expression between tissues, *TBP* (TATA box binding protein) was used as housekeeping gene for all samples since it was the most consistently stable transcript across all tissues (51;52). Some steroid enzyme transcript data (*SRD5A1*, *SRD5A2*, *CYP17A1* and *HSD3B* in liver (25) and *HSD17B3* and *CYP17A1* in testis (53)) has been reported previously relative to different housekeeping genes or external standards. This data is shown again here relative to *TBP* to allow comparisons between tissues and additional fetal

liver samples have been included in the reported data. Fetal sex was confirmed by PCR of *ZFX* and *SRY* using genomic DNA and primers described in Supplementary Table 2 (54).

# **Isolation and incubation of human fetal testicular cells.**

Isolated fetal testes were treated with collagenase type I (Sigma Chemicals Co., St. Louis, USA) (1 mg/ml for 35 min at 37° C) and then disrupted mechanically. The testicular cells were collected by centrifugation at 300 g for 7 min, washed in Hank's balanced salt solution and resuspended in DMEM-F12 supplemented with 1 mg/ml BSA, 100 IU/ml penicillin and 100 µg/ml streptomycin. For incubation, 100 µl of a suspension containing 1.5x10<sup>5</sup> cells/ml was plated onto 96-well plates (Falcon, Franklin Lakes, NJ, USA) in the presence or absence of hCG (10 ng/ml) to stimulate Leydig cell activity. Cells were incubated for 24 hours at 37° C under 5% CO<sub>2</sub> and steroid levels in the culture media were measured by GC-MS/MS as described below.

# **Steroid extraction and quantification.**

Methods used to extract and profile steroid levels by GC-MS/MS in fetal plasma and culture media have been described elsewhere in detail (22;55;56). Briefly, fetal plasma samples (50 µl) or culture media samples (200 µl) were spiked with internal standards (stable isotope-labeled steroid analogues) and an enzymatic deconjugation of phase II metabolites was performed (Sulfatase, Sigma S9626 (100 U/ml) and β-Glucuronidase, Sigma G8132, (5 KU/ml)). Steroids were extracted twice with diethyl ether and a ChromP SPE cartridge was used for initial purification. Androgens and estrogens were separated by liquid/liquid partitioning with n-pentane and were further purified on a silica SPE cartridge. The androgen fraction was derivatized with MSTFA/TMIS/DTE and 2 µl of each extract was injected onto a Scion 436 gas chromatograph coupled to a Scion TQ triple quadrupole mass spectrometer



(Bruker, Fremont, CA, USA). Electron ionization (70 eV) was used and two diagnostic signals (SRM acquisition mode) were monitored for identification and quantification of the targeted compounds. Levels of circulating testosterone shown in detail here have been reported previously as mean levels (57).

Extraction and quantification of tissue steroid levels by LC-HRMS have been described previously (17). Tissue samples (25-65mg) from placenta, liver and adrenals or whole testes (8-20mg, one per fetus) were initially processed to isolate RNA/DNA/protein using Qiagen Allprep kits as above and steroids were then extracted from the column-eluates following addition of internal standards (stable isotope-labeled steroid analogues) (17). Following extraction, steroids were separated by reverse-phase liquid chromatography on an Accucore™ PFP column with trimethyl silane (TMS) endcapping (50 mm, 2.1 mm, 2.6 µm, Thermo Fisher Scientific) and using an UltiMate 3000 RSLCnano autosampler/pump (Thermo Fisher Scientific, Waltham, MA, USA). Steroids were then ionized by electrospray in positive mode and signals were recorded on a Q Exactive Orbitrap (Thermo Fisher Scientific) mass spectrometer. Sulfated steroids were detected in a second analysis after negative electrospray ionization. Levels of sulfated and non-sulfated DHEA have been combined in the reported results as have sulfated and non-sulfated androsterone. The fetal testes used in this part of the study were extracted in two batches and recovery of steroid sulfates in the first batch was poor so testicular DHEA and androsterone levels have only been reported for the second batch of 10 samples. For the second batch of testicular tissue an additional extraction step using chloroform/n-butanol was used to improve steroid sulfate extraction efficiency. As reported for other LC-MS methods (58), the electrospray ionization efficiency can be low for 5 $\alpha$ -reduced androgens which results in relatively high LOQ and LOD values for these compounds. In a separate study, therefore, testicular steroids from an

additional 6 fetuses were extracted and separation and quantification of steroids in these samples was carried out by GC-MS/MS as described above for plasma samples.

# **Statistics.**

Data were checked for normality and normalized by log-transformation as appropriate. Plasma steroid data was analyzed by 2-factor ANOVA with fetal age and maternal smoking as the factors. Correlations between steroid data and age were analyzed using Pearson's correlation coefficient. The effect of hCG on steroid secretion by the fetal testes was analyzed by t-test.

# Author contributions

PJOS and PAF designed the study; M-LM, AM, US, ZCJ, NW, IS, DH and PF carried out the research; PJOS, PAF, KS, DH, OS, JPA, BLB, MB and SB analyzed data; POS and PAF wrote the paper.

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Table 1 Fetal tissue weights during the second trimester

<u>Tissue</u>	<u>Tissue weight (mg)*</u>	<u>Fold-differences vs combined testis weights</u>	<u>Reference</u>
Liver	700 – 14,800	117 – 220	our data and (59)
Testis (combined)	6 – 67	1 – 1	our data
Adrenal (combined)	50 – 1,300	8 – 19	our data
Genital tubercle	3 – 98	0.5 – 1.5	our data
Placenta	14,000 – 115,000	2,300 – 1,716	(60)

\* Range of tissue weights from the start to the end of the second trimester in the human

**Table 2 Steroids in fetal testis measured by GC-MS/MS\***

<u>Fetal ID</u>	A	B	C	D	E	F		
Fetal Age (weeks)	15	15	16	19	19	19		
Testis Weight (mg)	23.4	19.1	23.7	19.5	24.7	29.1		
	Steroid concentrations (ng/mg tissue)						<u>mean</u>	<u>sem</u>
Pregnenolone	1.282	2.421	1.216	1.551	0.841	0.676	1.331	0.2531
DHEA	0.544	0.553	0.196	0.367	0.094	0.053	0.301	0.0898
Progesterone	0.057	0.274	0.198	0.153	0.029	0.079	0.132	0.0383
Androstenedione	0.129	0.184	0.155	0.106	0.041	0.057	0.112	0.0227
Testosterone	0.810	0.660	1.877	0.943	0.372	0.526	0.865	0.2186
Androstanedione	0.052	0.050	0.030	0.034	0.047	0.041	0.043	0.0036
DHT	0.046	0.037	0.075	0.064	0.102	0.091	0.069	0.0103
Allopregnanolone	0.026	0.135	0.023	0.024	0.026	0.063	0.050	0.0181
Androsterone	0.011	0.018	0.014	0.013	0.018	0.022	0.016	0.0017
Androstenediol (5 $\alpha$ -androstane 3 $\alpha$ , 17 $\beta$ diol)	0.036	0.054	0.043	0.038	0.064	0.059	0.049	0.0047

\* Testicular steroid concentrations in six individual fetuses is shown.

## Figure 1

Canonical and alternative (backdoor) pathways of dihydrotestosterone synthesis. The canonical pathway has potential  $\Delta^4$  and  $\Delta^5$  sub-pathways. The enzymes which catalyze each step are indicated within the arrows. Enzymes written in black on a grey arrow are essential components of the canonical pathway and some appear in both canonical and backdoor pathways (e.g. CYP17A1). Enzymes written in black on a green background are specific to the backdoor pathway. Enzymes in grey text will carry out the described conversion but they may not be the principal enzyme involved. Other enzymes, not shown, may also be involved in components of the backdoor pathway (61).

Abbreviations: DHEA, dehydroepiandrosterone; Androstenediol, androst-5-ene-3 $\beta$ ,17 $\beta$ -diol; Androstenedione, 5 $\alpha$ -androstane-3,17-dione; Androstenediol, 5 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol; 5 $\alpha$ DHP, 5 $\alpha$ -dihydroprogesterone; 17OHDHP, 17 $\alpha$ -hydroxydihydroprogesterone; DHT, dihydrotestosterone.

## Figure 2

Concentrations of both canonical and backdoor steroids in the plasma of human male fetuses during the second trimester. Steroid levels from 39 or 42 individual fetuses are shown in each graph, arranged as in the pathways shown in Fig 1. The number of samples which were non-detectable (ND) for each steroid are shown and, where appropriate, the limit of detection (LOD) is shown as a red dotted line. Data shown in grey was above the LOD but below the formal LOQ which means that the quantified data shown for these samples is less reliable than for data shown in blue which was above the LOQ. The plasma used in these studies was from fetuses aged between 12 and 19 weeks. Abbreviations used are the same as those in Figure 1.

## Figure 3

Fetal tissue levels of steroid intermediates involved in the canonical and backdoor synthesis of DHT. Tissue levels of steroids from the placenta and fetal liver (n=20; placentas and fetuses were from the same pregnancies), fetal adrenal (n=30) and fetal testis (n=10 (for DHEA and androsterone) or 25 (for all other steroids)) are shown as individual points in each graph and arranged in the pathways shown in Fig 1. Levels of 17 $\alpha$ -hydroxylated intermediates were not measured in this part of the study. The number of samples which were non-detectable (ND) for each steroid are shown and, where appropriate, the limit of detection (LOD) is shown as a red dotted line. Data shown in grey was above the LOD but below the formal LOQ which means that the quantified data shown for these samples is less reliable. The LOD for each sample (in ng/mg tissue) depended on the mass of tissue extracted and the lines drawn are based on the average mass of each tissue used. Green arrows indicate that the relevant enzymes are detectable (as mRNA transcripts) in that tissue while red arrows indicate that the presumed enzyme is not detectable (based on data in Figure 4). Abbreviations used are the same as those in Figure 1.

## Figure 4

Fetal tissue levels of mRNA transcripts encoding enzymes involved in the synthesis of DHT through the backdoor pathway. Data shows levels of transcripts in testis (n=22), adrenal (n=21), liver (n=45), genital tubercle (n=10), liver (n=46) and placenta (n=20) from individual male fetuses during the second trimester. Transcript levels have been measured relative to the housekeeping gene *TBP*. For each transcript the Y axis has been maintained constant for all 5 tissues so that direct comparison of transcript levels can be made. The number of non-detectable (ND) samples is shown on each graph. The horizontal red bar indicates mean expression and, in cases where levels are very low, the mean is also provided in text on the graph.

## Figure 5

Proposed steroidogenic pathways leading to androsterone (An) synthesis and masculinization in the second trimester human male fetus. Steroid hormone conversion is shown by wide green arrows with the converting enzymes written within the arrow. Red arrows show transport between organs in the fetal circulation. The blue double-headed arrow indicates that exchange is also taking place between the placenta and the maternal circulation. Most circulating progesterone in the fetal circulation is likely to come from the placenta and this will be reduced to 5 $\alpha$ -dihydroprogesterone (5 $\alpha$ DHP) by SRD5A1 in the placenta, fetal liver and fetal testis with the fetal liver likely to be the major site. Allopregnanolone (AlloP5) production by AKR1C2 is also most likely to occur in the placenta and fetal liver because the substrate is present in those tissues and they express the highest total levels of enzyme transcript. Some conversion may also occur in the testis. Significant levels of androsterone are only detectable in the placenta and adrenal so they are the likely source of the circulating steroid. The adrenal lacks other intermediates in the backdoor pathway so AlloP5 must come from other tissues. The placenta lacks CYP17A1 so androsterone production is likely to depend on adrenal DHEA as substrate. Testosterone (T) from the fetal testes also acts as an essential substrate for DHT synthesis at the external genitalia.

Abbreviations: P5, pregnenolone; P4, progesterone; 5 $\alpha$ DHP, 5 $\alpha$ -dihydroprogesterone; AlloP5, allopregnanolone; DHEA, dehydroepiandrosterone; An, androsterone; T, testosterone; DHT, 5 $\alpha$ -dihydrotestosterone.

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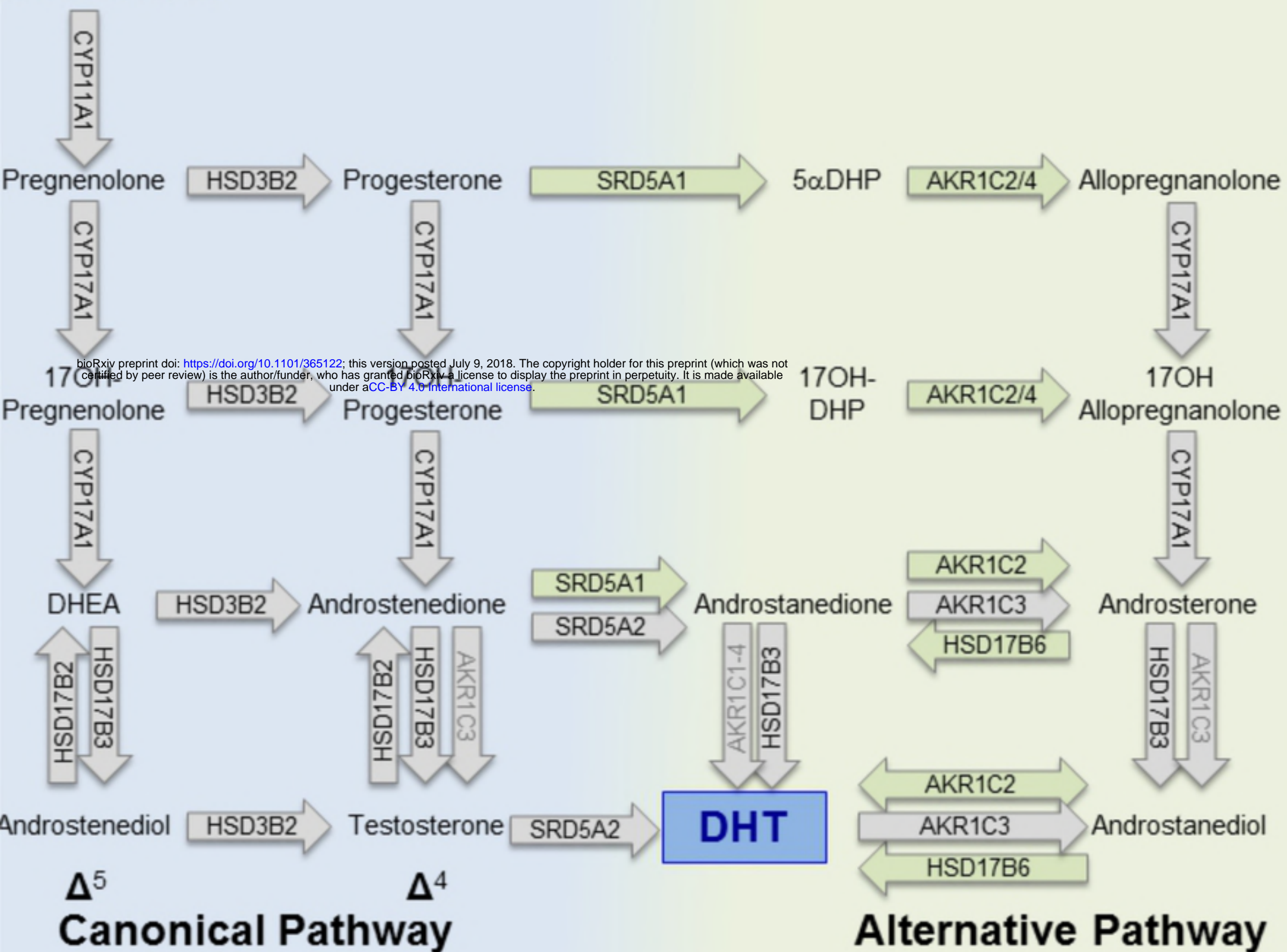
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# Cholesterol

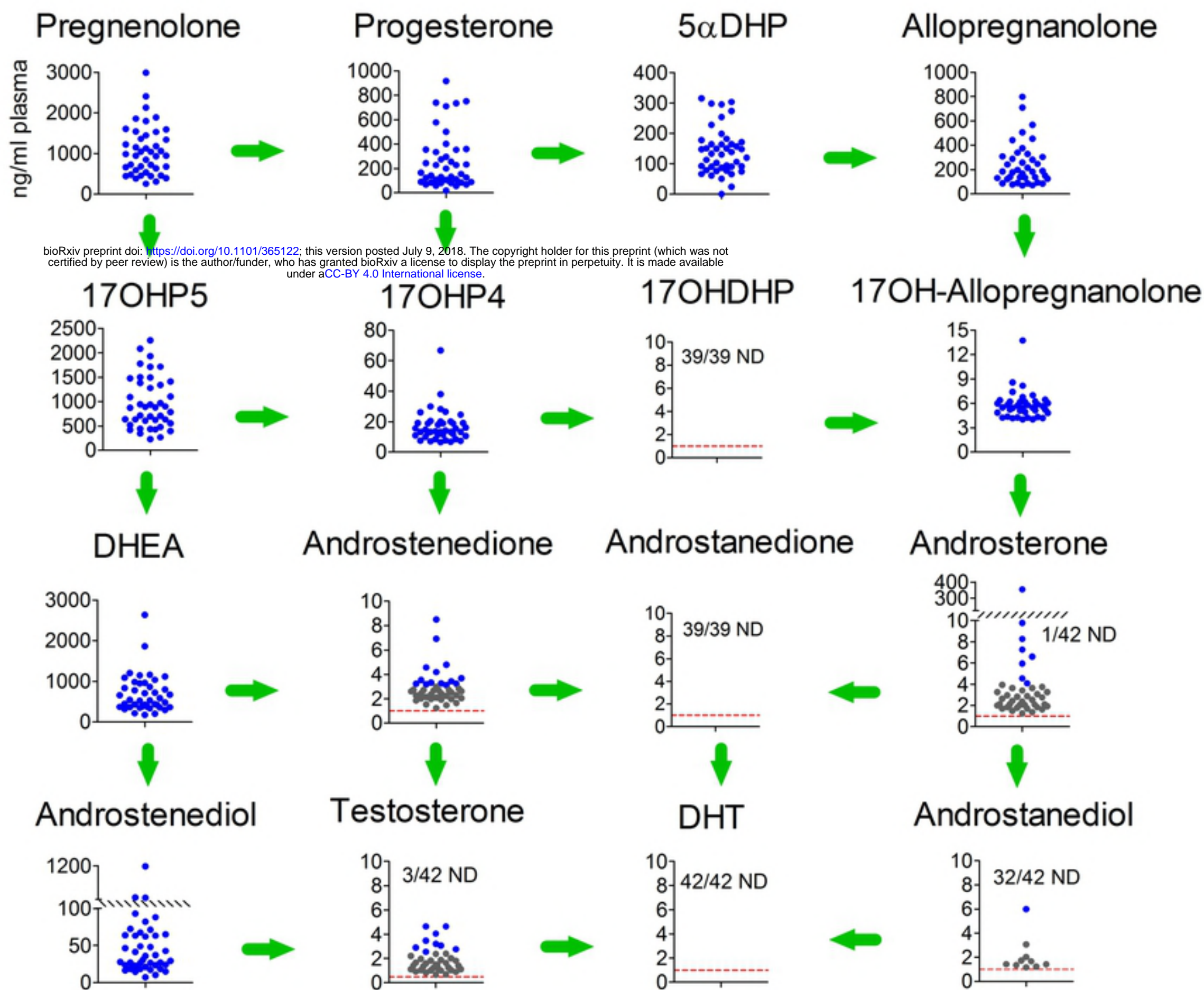


**Figure 1**

Canonical and alternative (backdoor) pathways of dihydrotestosterone synthesis. The canonical pathway has potential  $\Delta^4$  and  $\Delta^5$  sub-pathways. The enzymes which catalyze each step are indicated within the arrows. Enzymes written in black on a grey arrow are essential components of the canonical pathway and some appear in both canonical and backdoor pathways (e.g. CYP17A1). Enzymes written in black on a green background are specific to the backdoor pathway. Enzymes in grey text will carry out the described conversion but they may not be the principal enzyme involved. Other enzymes, not shown, may also be involved in components of the backdoor pathway (61).

Abbreviations: DHEA, dehydroepiandrosterone; Androstenediol, androst-5-ene-3 $\beta$ ,17 $\beta$ -diol; Androstenedione, 5 $\alpha$ -androstane-3,17-dione; Androstenediol, 5 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol; 5 $\alpha$ DHP, 5 $\alpha$ -dihydroprogesterone; 17OHDHP, 17 $\alpha$ -hydroxydihydroprogesterone; DHT, dihydrotestosterone.

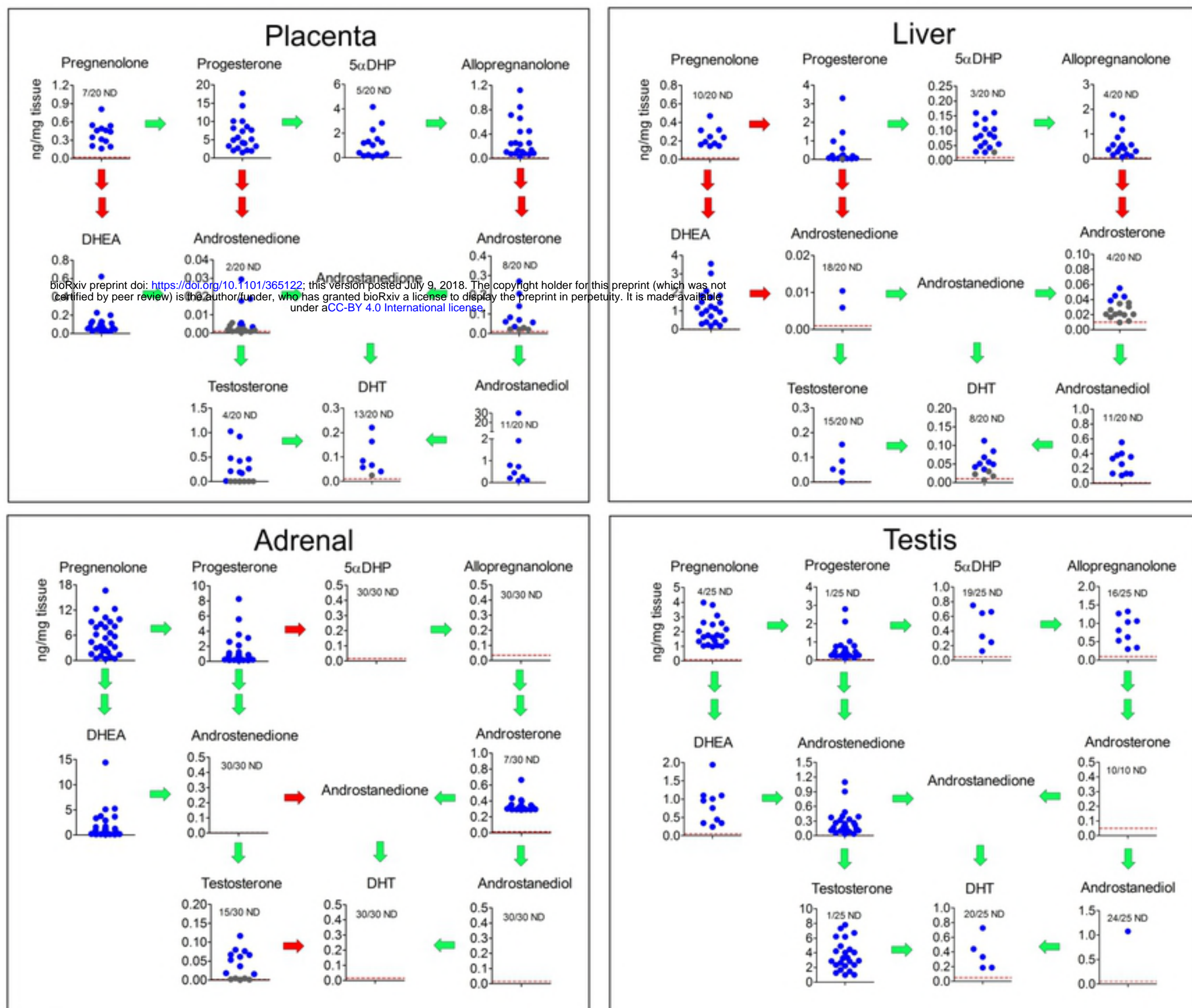




**Figure 2**

Concentrations of both canonical and backdoor steroids in the plasma of human male fetuses during the second trimester. Steroid levels from 39 or 42 individual fetuses are shown in each graph, arranged as in the pathways shown in Fig 1. The number of samples which were non-detectable (ND) for each steroid are shown and, where appropriate, the limit of detection (LOD) is shown as a red dotted line. Data shown in grey was above the LOD but below the formal LOQ which means that the quantified data shown for these samples is less reliable than for data shown in blue which was above the LOQ. The plasma used in these studies was from fetuses aged between 12 and 19 weeks. Abbreviations used are the same as those in

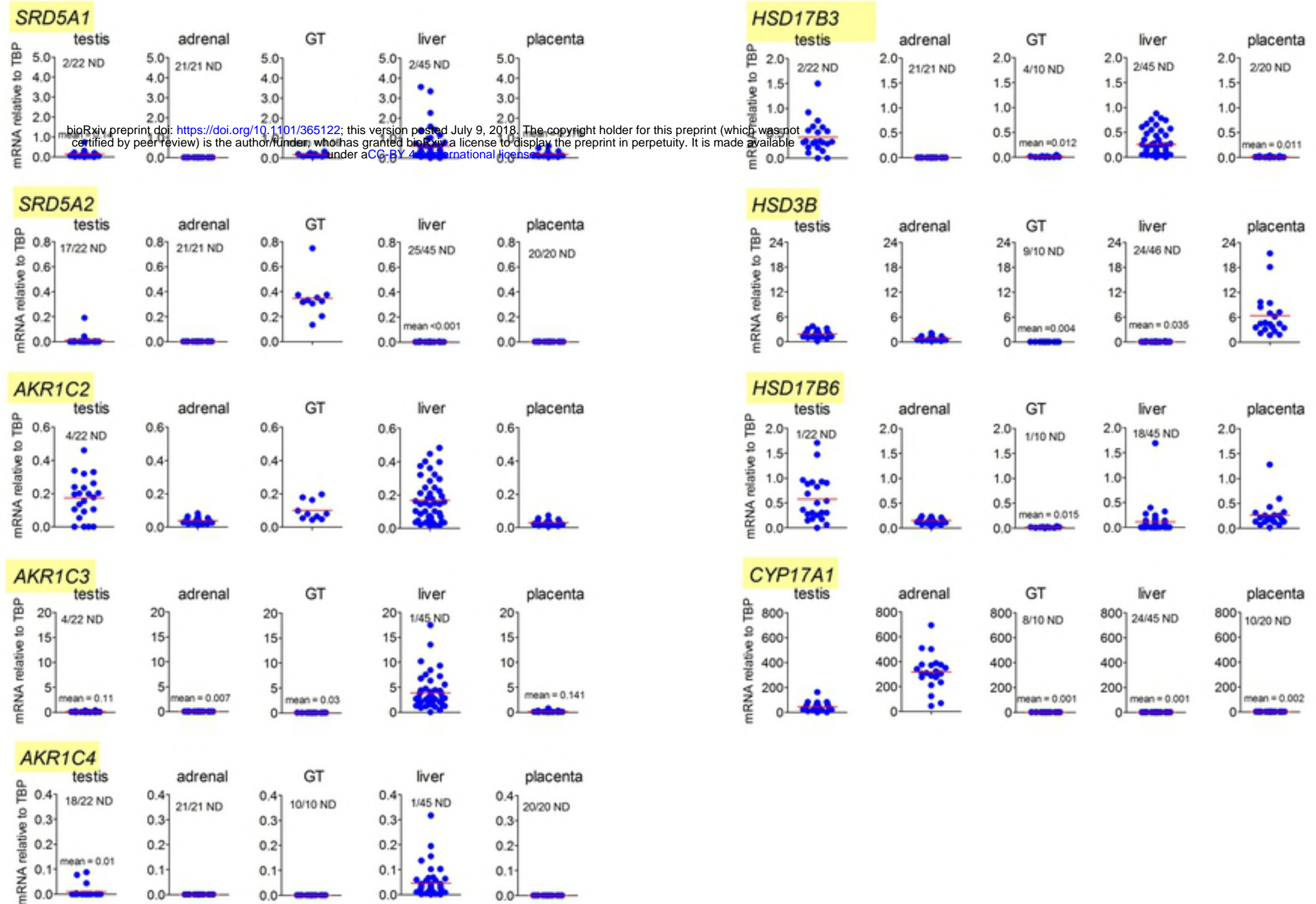




**Figure 3**

Fetal tissue levels of steroid intermediates involved in the canonical and backdoor synthesis of DHT. Tissue levels of steroids from the placenta and fetal liver (n=20; placentas and fetuses were from the same pregnancies), fetal adrenal (n=30) and fetal testis (n=10 (for DHEA and androsterone) or 25 (for all other steroids)) are shown as individual points in each graph and arranged in the pathways shown in Fig 1. Levels of 17 $\alpha$ -hydroxylated intermediates were not measured in this part of the study. The number of samples which were non-detectable (ND) for each steroid are shown and, where appropriate, the limit of detection (LOD) is shown as a red dotted line. Data shown in grey was above the LOD but below the formal LOQ which means that the quantified data shown for these samples is less reliable. The LOD for each sample (in ng/mg tissue) depended on the mass of tissue

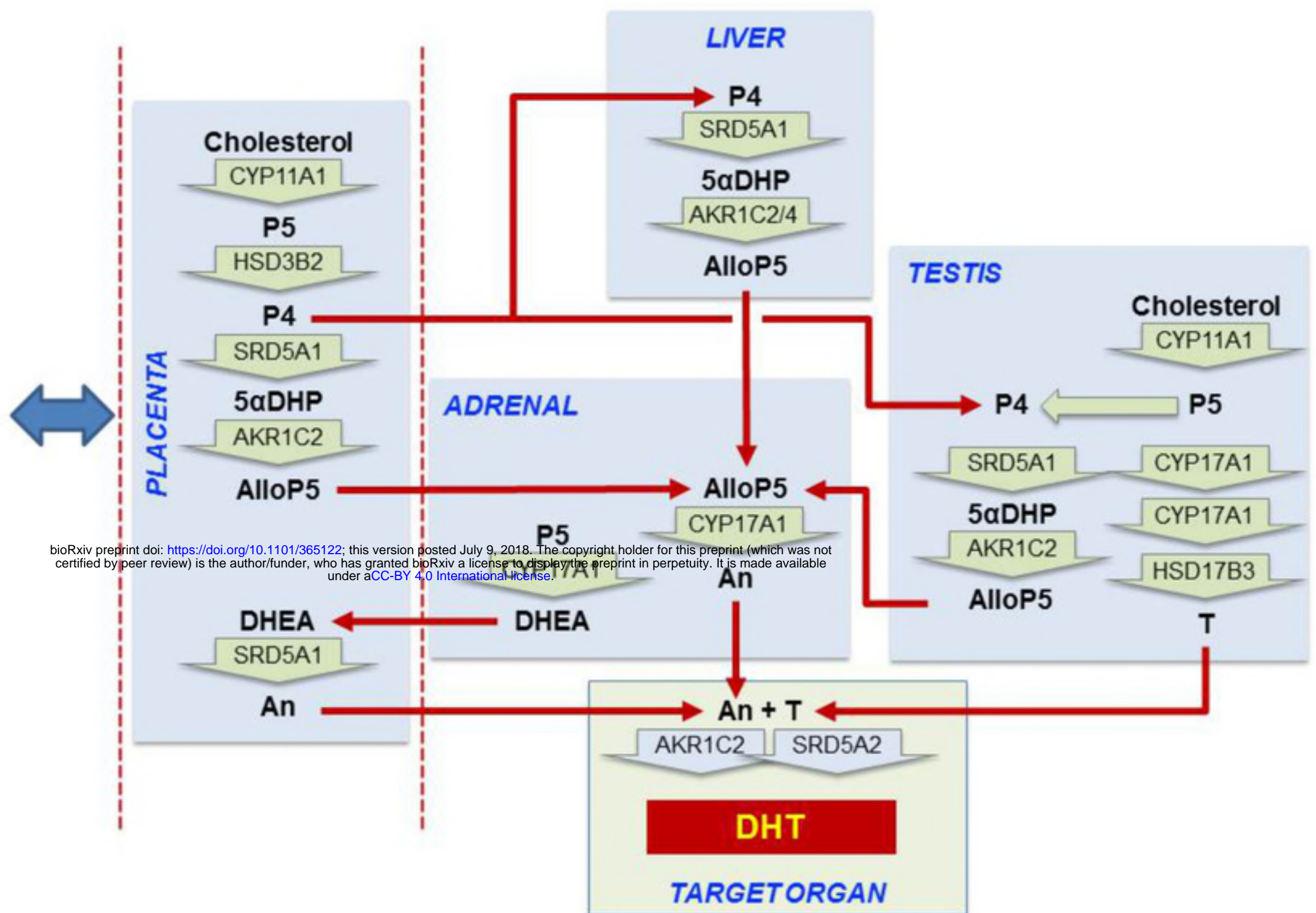




**Figure 4**

Fetal tissue levels of mRNA transcripts encoding enzymes involved in the synthesis of DHT through the backdoor pathway. Data shows levels of transcripts in testis (n=22), adrenal (n=21), liver (n=45), genital tubercle (n=10), liver (n=46) and placenta (n=20) from individual male fetuses during the second trimester. Transcript levels have been measured relative to the housekeeping gene *TBP*. For each transcript the Y axis has been maintained constant for all 5 tissues so that direct comparison of transcript levels can be made. The number of non-detectable (ND) samples is shown on each graph. The horizontal red bar indicates mean expression and, in cases where levels are very low, the mean is also provided in text on the graph.





**Figure 5**

Proposed steroidogenic pathways leading to androsterone (An) synthesis and masculinization in the second trimester human male fetus. Steroid hormone conversion is shown by wide green arrows with the converting enzymes written within the arrow. Red arrows show transport between organs in the fetal circulation. The blue double-headed arrow indicates that exchange is also taking place between the placenta and the maternal circulation. Most circulating progesterone in the fetal circulation is likely to come from the placenta and this will be reduced to 5α-dihydroprogesterone (5αDHP) by SRD5A1 in the placenta, fetal liver and fetal testis with the fetal liver likely to be the major site. Allopregnanolone (AlloP5) production by AKR1C2 is also most likely to occur in the placenta and fetal liver because the substrate is present in those tissues and they express the highest total levels of enzyme transcript. Some conversion may also occur in the testis. Significant levels of androsterone are only detectable in the placenta and adrenal so they are the likely source of the circulating steroid. The adrenal lacks other intermediates in the backdoor pathway so AlloP5 must come from other tissues. The placenta lacks CYP17A1 so androsterone production is likely to depend on adrenal DHEA as substrate. Testosterone (T) from the fetal testes also acts as an essential substrate for DHT synthesis at the external genitalia.

Abbreviations: P5, pregnenolone; P4, progesterone; 5αDHP, 5α-dihydroprogesterone; AlloP5, allopregnanolone; DHEA, dehydroepiandrosterone; An, androsterone; T, testosterone; DHT, 5α-dihydrotestosterone.