Scaling in vitro activity of CYP3A7 suggests human fetal livers do not clear retinoic acid entering from maternal circulation

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All-trans-retinoic acid (atRA), the active metabolite of vitamin A, is a critical signaling molecule during embryonic and fetal development and is necessary for maternal health. Fetal exposure to endogenous atRA is tightly regulated during gestation in a tissue specific manner and maternal exposure to exogenous retinoids during pregnancy is teratogenic. The clearance of atRA is primarily mediated by the cytochrome P450 (CYP) 26 enzymes, which play an essential role in controlling retinoid gradients during organogenesis. We hypothesized that CYP26 enzymes in the human fetal liver also function as a protective barrier to prevent maternal atRA reaching fetal circulation. Using human fetal liver tissue, we found that the mRNA of CYP26A1 and CYP26B1 enzymes is expressed in the human fetal liver. However, based on inhibition studies, metabolite profiles and correlation of atRA metabolism with testosterone hydroxylation, clearance of atRA in the fetal livers was mediated by CYP3A7. Based on in vitro-to-in vivo scaling, atRA clearance in the fetal liver was quantitatively minimal, thus providing an insufficient maternal-fetal barrier for atRA exposure.
Results

Detection of CYP26 mRNA in human fetal livers. The mRNA expression of CYP26A1, CYP26B1 and CYP26C1 together with CYP3A7 was measured in fetal livers from 18 individual donors (Fig. 1) and in five control adult human livers (data not shown). CYP26A1 and CYP26B1 mRNAs were detected and quantified in 14 and 11 of the 18 fetal livers, respectively, while CYP26C1 mRNA was not detected in any of the 18 fetal livers. Considerable inter-individual variability, up to 125-fold, was observed in the expression of CYP26A1 in the fetal livers while the expression of CYP26B1 was less variable (Fig. 1). Two of the fetal livers had no detectable expression of any CYP26 mRNAs and in both of these livers robust CYP3A7 mRNA expression was detected. In fact, CYP3A7 expression was relatively high (Ct values 26–34) in 17 of the 18 fetal livers. One fetal liver had very low CYP3A7 expression (Ct, value 38) and this liver showed the highest CYP26A1 expression among all 18 fetal livers. The overall Ct values of CYP26A1 were higher in the fetal livers (one with Ct value 32 and others 37–38) than in the adult livers analyzed (Ct 27–36) while CYP26B1 Ct values were generally lower in the fetal livers (Ct, 36–39) than adult livers (CYP26B1 was only detectable in one adult liver). Due to differences in housekeeping gene expression between adult and fetal livers, no quantitative comparisons were made between fetal and adult liver mRNA expression. CYP26C1 mRNA was not detected in any of the adult human livers while CYP3A7 mRNA was detected in 3 of the 5 adult livers (Ct values 30–37).

atRA metabolism in human fetal livers. To explore whether the metabolites formed from atRA in fetal liver resembled either the metabolite profile observed from atRA with recombinant CYP3A7 or CYP26 enzymes, the metabolites formed from atRA by CYP3A7, CYP26A1 and CYP26B1 and by fetal liver S9 fractions were characterized (Fig. 2). Consistent with previous data, recombinant CYP26A1 and CYP26B1 hydroxylated atRA at multiple sites, while CYP3A7 only formed 4-OH-atRA and 4-oxo-atRA. The metabolite profile in human fetal livers was similar to that observed with CYP3A7. Of the atRA metabolites, only the formation of 4-OH-atRA and its metabolite 4-oxo-atRA was observed (Fig. 2). No formation of the CYP26 specific metabolite 16-OH-atRA was observed in fetal livers. The fetal liver metabolite profile corresponded to that observed with CYP3A7 and suggests lack of significant CYP26 contribution to atRA metabolism in fetal liver.

The metabolism of atRA was quantified in 27 individual fetal livers from gestational ages between 72 and 137 days (Table 1, Fig. 3). The metabolite formation velocity ranged from 24 ± 11 to 124 ± 7 pmol/min/mg protein for 4-OH-atRA, from 1.6 ± 0.1 to 16 ± 1 pmol/min/mg protein for 4-oxo-atRA and from 28 ± 11 to 134 ± 8 pmol/min/mg protein for the sum of 4-OH-atRA and 4-oxo-atRA. There was no correlation between gestational age and the formation of 4-OH-atRA (r² = 0.04, p = 0.33), 4-oxo-atRA (r² = 0.02, p = 0.49), or their sum (r² = 0.04, p = 0.33), and the metabolite formation velocity did not differ between weeks of gestation (Fig. 3, Table 2). There were no significant differences in metabolite formation between fetal sexes or maternal races (Table 2).

Identification of CYPs that metabolize atRA in human fetal livers. To quantify which CYP enzymes contribute to atRA clearance in fetal liver, selective inhibitors of CYP3A7 and CYP26 were used. Fluconazole has been previously shown not to inhibit CYP2616. The inhibition of CYP3A7 mediated 4-OH-atRA formation by fluconazole (300 μM) was confirmed using recombinant CYP3A7. Inhibition of CYP26A1 by talarozole (200 nM) was reproduced with recombinant CYP26A1 (Fig. 4). The specificity of talarozole (200 nM) towards CYP26 was confirmed using recombinant CYP3A7. Talarozole did not significantly inhibit CYP3A7 mediated 4-OH-atRA formation (Fig. 4). Together these data show that fluconazole and talarozole can be used as specific inhibitors of CYP3A7 and CYP26 respectively. In human fetal liver S9 fractions, atRA metabolite formation was inhibited 30–60% by fluconazole and 30–40% by talarozole (Fig. 4), suggesting that both CYP26 enzymes and CYP3A7 contribute to atRA clearance in human fetal liver. Ketoconazole inhibits both CYP26 enzymes and CYP3A716. Consistent with this inhibition profile, ketoconazole caused a >95% decrease in atRA metabolite formation. However, these inhibition experiments do not unequivocally define which CYP enzyme is predominant in atRA clearance in human fetal liver. Therefore, to further define the importance of CYP3A7 in atRA clearance in fetal liver, correlation analysis between atRA metabolite formation and the formation of the CYP3A7 specific testosterone metabolite 6β/10β-testosteron was conducted (Fig. 3). atRA oxidation (the sum of 4-OH-atRA and 4-oxo-atRA formation) and 6β/10β-testosteron formation from testosterone correlated significantly in the fetal livers tested (r² = 0.38, p < 0.05) suggesting that CYP3A7 plays a major role in atRA metabolism in human fetal liver.
Prediction of atRA clearance in the human fetal liver. To define the quantitative role of the fetal liver in atRA clearance and in potentially serving as a barrier to maternal-fetal retinoid transfer, the overall organ clearance and extraction ratio of atRA metabolism was calculated for the fetal liver for the gestational ages studied. The overall intrinsic clearance of atRA by the whole fetal liver (Cl_{infl}) increased with gestational age due to the growth of the fetal liver. The intrinsic clearance per mg S9 protein was independent of gestational age (Fig. 3). The predicted fraction of atRA removed from fetal circulation by the fetal liver (extraction ratio) was very low, ranging from 0.01 to 0.05 (Fig. 3F). This suggests minimal extraction of maternal atRA by the fetal liver.
Discussion

dtRA is a key developmental morphogen, and distinct concentration gradients of dtRA within the developing embryo and fetus are crucial for regulation of cellular differentiation. Embryonic development requires gestational age and tissue-type specific regulation of dtRA concentrations. Based on this, we hypothesized that a barrier, such as the fetal liver or the placenta, exists between the mother and the fetus to prevent maternal endogenous dtRA passing to the fetus and enabling autonomous regulation of fetal dtRA concentrations. Previous studies have shown CYP26 mRNA expression in fetal liver. Hence, we hypothesized that the CYP26 enzymes, which are generally believed to be the main human retinoic acid hydroxylases, would constitute a maternal-fetal barrier for maternal dtRA. Based on the mRNA analysis of individual fetal livers, however, CYP26 enzymes appeared relatively insignificant in the human fetal liver both in terms of observed mRNA expression and apparent activity. At the same time, CYP3A7 mRNA was abundant in the fetal livers, in agreement with past studies. The detection of high CYP26A1 mRNA in one fetal liver that had very low CYP3A7 expression is of particular interest. If CYP3A7 is predominantly responsible for dtRA clearance in the fetal liver, low expression or lack of this CYP would result in increased dtRA concentrations in fetal liver. These increased dtRA concentrations should in turn induce the expression of CYP26A1 in the fetal liver leading to the observed mRNA expression pattern.

Figure 2. Identification of the metabolites formed from dtRA by recombinant CYP26A1, CYP26B1, CYP3A7 and by human fetal livers. Panels A–D show representative chromatograms of metabolite standards (A) and incubations with recombinant CYP26A1 (B) CYP26B1 (C) and CYP3A7 (D). Panels E and F show the metabolite formation in S9 fractions from two representative human fetal livers (hFL). The individual donors are indicated with the sample number (18 and 38 listed in Table 1). The incubations were conducted as described in materials and methods. The m/z transitions of 315 > 253 Da (4-OH-RA and 18-OH-RA; black line), 313 > 269 Da (4-oxo-RA; blue line) and 315 > 241 Da (16-OH-RA; red line) were monitored by LC-MS/MS and the observed peaks are labeled in each panel.
Although this observation is from a single donor, it is consistent with the prevailing notion that CYP26A1 expression is responsive to RA concentrations and its expression is induced by increased RA concentrations\(^{17,28}\).

Despite the detection of CYP26 isoform mRNA in human fetal livers, all the data presented here (the metabolite profiles, CYP inhibition data and correlation of 4-OH-RA formation with 6\(\beta\)OH-TST formation) support the role of CYP3A7 as the main human fetal liver RA hydroxylase with only a minor contribution of CYP26 enzymes. This was surprising, as in adult human liver CYP26A1 was previously found to be the main RA hydroxylase despite the activity of CYP3A4 and CYP3A5 as RA hydroxylases\(^{13}\). Yet, even in the adult human liver, CYP3A4 and CYP3A5 expression did significantly correlate with RA hydroxylation activity. In particular in livers with low CYP26A1 expression, CYP3A enzymes were likely to be significant RA hydroxylases\(^{13}\). Although CYP3A7 constitutes 30–85% of the fetal liver CYPs\(^{29}\) we expected that the >1,000 fold higher intrinsic clearance of RA by the CYP26 enzymes in comparison to CYP3A7\(^{13,15,30}\) would translate to an important contribution of CYP26s to fetal liver RA clearance, even if their expression was much lower than CYP3A7. However, the data collected does not support this hypothesis and we conclude that CYP3A7 is the main human fetal liver RA hydroxylase. This finding is likely to translate to other RA isomers, 13-\textit{cis} RA and 9-\textit{cis} RA as well, as they have similar clearance profiles by CYP26s as RA\(^{30}\) and are metabolized by CYP3A7\(^{25}\). The lack of change in RA hydroxylation rates in three fetal livers from gestational days 96–109 when compared to livers from days 54–89 of gestation\(^{23}\). The previously observed change with gestational age is likely due to the small sample size that did not completely capture inter-individual variability in RA clearance.

This is the first study to scale the observed microsomal or S9 protein activity to the entire fetal liver. This scaling shows that the metabolic capacity of the fetal liver towards RA is unlikely to contribute significantly to fetal RA clearance, and thus this metabolism is not quantitatively sufficient to protect the developing fetus. While the intrinsic clearance of RA metabolism per mg of fetal liver protein did not change with gestational age, our analysis shows that when the growth of the liver and increase in umbilical blood flow is accounted for, the overall metabolic clearance of RA increases significantly with gestational age. However, the extraction ratio is unchanged with gestational age, and the predicted extraction ratio (0.01–0.05) suggested that maximum 5% of maternal RA could be extracted by fetal liver at any gestational age. The scaling methods used here, the analysis

| Fetal Liver code | Gestational Age (Day) | Sex Of fetus | Race       | Yield of S9 protein as mg/g fetal liver | Fetal liver weight (g) | Umbilical vein flow (mL/min) |
|------------------|-----------------------|-------------|------------|----------------------------------------|------------------------|-----------------------------|
| FL 30            | 72                    | M           | n/a        | 27                                     | 0.4                    | 0.45                        |
| FL 20            | 74                    | M           | n/a        | 70                                     | 0.5                    | 0.45                        |
| FL 21            | 74                    | n/a         | Asian      | 79                                     | 0.5                    | 0.45                        |
| FL 36            | 80                    | n/a         | Caucasian  | 43                                     | 0.9                    | 0.78                        |
| FL 39            | 80                    | F           | Asian/Hispanic | 53                                 | 0.9                    | 0.78                        |
| FL 24            | 85                    | n/a         | Asian      | 75                                     | 1.2                    | 1.6                         |
| FL 37            | 85                    | M           | n/a        | 43                                     | 1.2                    | 1.6                         |
| FL 14            | 87                    | F           | n/a        | 47                                     | 1.4                    | 1.6                         |
| FL 12            | 91                    | F           | Asian      | 52                                     | 1.9                    | 2.6                         |
| FL 31            | 96                    | M           | Caucasian  | 46                                     | 2.6                    | 2.6                         |
| FL 13            | 98                    | M           | n/a        | 32                                     | 3.0                    | 4.8                         |
| FL 27            | 98                    | F           | Caucasian  | 36                                     | 3.0                    | 4.8                         |
| FL 18            | 98                    | n/a         | Asian      | 33                                     | 3.0                    | 4.8                         |
| FL 34            | 100                   | M           | n/a        | 48                                     | 3.4                    | 4.8                         |
| FL 40            | 101                   | M           | Caucasian  | 68                                     | 3.7                    | 4.8                         |
| FL 33            | 101                   | M           | n/a        | 93                                     | 3.7                    | 4.8                         |
| FL 15            | 101                   | n/a         | n/a        | 37                                     | 3.7                    | 4.8                         |
| FL 23            | 105                   | M           | Alaskan    | 46                                     | 4.7                    | 7.8                         |
| FL 38            | 105                   | F           | Caucasian  | 63                                     | 4.7                    | 7.8                         |
| FL 19            | 108                   | F           | Hispanic   | 60                                     | 5.6                    | 7.8                         |
| FL 28            | 108                   | F           | Asian      | 67                                     | 5.6                    | 7.8                         |
| FL 29            | 108                   | F           | n/a        | 42                                     | 5.6                    | 7.8                         |
| FL 26            | 113                   | n/a         | n/a        | 44                                     | 7.4                    | 11.1                        |
| FL 25            | 113                   | M           | n/a        | 57                                     | 7.4                    | 11.1                        |
| FL 17            | 115                   | n/a         | n/a        | 47                                     | 8.3                    | 11.1                        |
| FL 16            | 120                   | M           | Caucasian  | 42                                     | 10.7                   | 15.6                        |
| FL 22            | 137                   | M           | Alaskan    | 62                                     | 23.2                   | 26.8                        |

Table 1. Donor characteristics and fetal liver physiological parameters used. Each fetal liver (FL) is identified with a numerical sample code. The fetal liver weights listed are based on reported data for gestational days 72–137\(^{39,40}\). The umbilical vein blood flow was calculated based on the reported value of 111.7 mL/min/kg fetal weight\(^{42}\).
of the extraction ratio and the prediction of the overall fetal liver metabolic clearance are likely useful for future estimations of the role of the fetal liver in clearance of therapeutic drugs and toxins which are metabolized by fetal liver CYPs or glucuronidation enzymes.

Collectively, the data presented here are in agreement with the current consensus that fetal tissue exposure to atRA and tissue atRA concentrations and signaling are regulated at the individual organ level and not via a maternal-fetal barrier. It is also important to note that atRA signaling is most significant during early embryogenesis, a window of gestation that cannot be feasibly studied in human tissues. As such, concordance of the findings from this study with animal models will need to be established in studies in model organisms at gestational time.

Figure 3. Quantitative analysis of atRA and testosterone (TST) metabolism in human fetal livers. Formation of 4-OH-RA (A), 4-oxo-RA (C) and the sum of 4-OH-RA and 4-oxo-RA (E) in individual human fetal livers from different gestational ages is shown. The insets list the correlation analysis between gestational age and product formation velocity. Panel D shows the correlation between atRA oxidation and 6β-OH-TST formation in individual human fetal livers. Panel E shows the intrinsic clearance of atRA oxidation in the fetal livers (Cl_{int,FL}) based on whole organ scaling from the S9 fraction data as described in the methods section. The calculated extraction ratio of atRA at different gestational ages is shown in panel F for individual human fetal livers.
analyzed in duplicates and the q-RT-PCR repeated on three separate occasions. For samples that were undetected mRNA was extracted from 18 fetal livers and five adult human livers as previously described \(^28\). 50–70 mg of liver was quantified using StepOnePlus™ q-RT-PCR (Applied Biosystems; Carlsbad, CA, USA). All samples were -actin CYP26A1 reagents (Applied Biosystems, Carlsbad, CA). The mRNAs of as a control gene, and CYP3A7 β-Fisher Scientific, Waltham, MA). cDNA was synthesized from 1 manufacturer’s recommendations. Total RNA was quantified using a Nanodrop 2000c Spectrophotometer (Thermo TRI reagent (Invitrogen; Grand Island, NY, USA). Homogenization was conducted using Omni Bead Ruptor 24 was homogenized in 2 ml Omni Hard Tissue Homogenizing tubes containing 1.4 mm ceramic beads and 1 ml of Human fetal liver tissues (n\(=\) 187) ranging from reported gestational days 67 to 137 were collected by the Birth Defects Laboratory at the University of Washington and flash frozen upon collection using liquid nitrogen and stored at \(-80\) °C until ready for use. The gestational days were based on self-reports and exact dates of conception are not known. Donated tissues were from elective abortions and all donor moms signed informed consent for donating the tissues. Tissues from fetuses whose mothers had known drug use were excluded from this study. Adult liver tissues (n\(=\) 5) were from de-identified donors from University of Washington human liver bank.

### Methods

#### Chemicals and Reagents.

- **atra**, ketoconazole, flunonazole, and talazolozole were purchased from Sigma-Aldrich (St. Louis, MO), 4-oxo-RA-d\(_3\) and *atra*-d\(_3\) were purchased from Toronto Research Chemicals (North York, Ontario).
- 4-OH-RA, 4-oxo-RA, and 18-OH-RA were synthesized as previously described\(^{15,16}\). Optima LC/MS-grade water, optima LC/MS-grade acetonitrile, ethanol and ethyl acetate were purchased from Fisher Scientific (Pittsburg, PA). Recombinant CYP3A7 supersomes coexpressed with P450 reductase were purchased from BD Gentest. Recombinant CYP26A1 and CYP26B1 were expressed in baculovirus infected insect cells and membrane fractions prepared via ultracentrifugation as previously described\(^{15,20}\).

#### Collection of Human livers.

The study was approved by the Institutional Review Board (IRB) at the University of Washington and the studies were conducted in accordance with the guidance of Office of Human Research Protections. Human fetal liver tissues (n = 27) ranging from reported gestational days 67 to 137 were collected. Adult liver tissues (n = 5) were from de-identified donors from University of Washington human liver bank.

#### Analysis of CYP26 mRNA expression.

To explore the presence of CYP26 enzymes in human fetal liver, mRNA was extracted from 18 fetal livers and five adult livers as previously described\(^{15,20}\). 50–70 mg of liver was homogenized in 2 ml Omni Hard Tissue Homogenizing tubes containing 1.4 mm ceramic beads and 1 ml of TRI reagent (Invitrogen; Grand Island, NY, USA). Homogenization was conducted using Omni Bead Ruptor 24 (Omni International; Kennesaw, GA, USA) and mRNA was extracted with TRI reagent according to the manufacturer’s recommendations. Total RNA was quantified using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 1 µg mRNA using TaqMan reverse transcription reagents (Applied Biosystems, Carlsbad, CA). The mRNAs of CYP26A1, CYP26B1 and CYP26C1 were quantified as target genes, CYP3A7 as a control gene, and β-actin, GAPDH and GUSB were evaluated as housekeeping genes. Based on the variability in the gene expression, β-actin was chosen as the housekeeping gene. mRNA expression was quantified using StepOnePlus™ q-RT-PCR (Applied Biosystems; Carlsbad, CA, USA). All samples were analyzed in duplicates and the q-RT-PCR repeated on three separate occasions. For samples that were undetected in one of the three experiments (5 samples for CYP26A1, 4 for CYP26B1 and 1 for CYP3A7) a Ct value of 40 was assigned to the undetected run and the mean of the three experiments was calculated. For samples that were undetected in two of the three experiments, samples were considered as target gene undetected (2 samples for CYP26A1 and 6 for CYP26B1). The relative abundance of CYP26A1, CYP26B1, CYP26C1 and CYP3A7 mRNA expression was analyzed by the ΔΔCt method using β-actin as a housekeeping gene and the data are presented as a fold difference in comparison to the mean value for each gene. No comparisons for expression between genes and between adult and fetal livers were done. Human primer and probe pairs for CYP26A1 (Hs01075675_m1, FAM), CYP26B1 (Hs01011223_m1, FAM), CYP26C1 (Hs01595345_m1, FAM), CYP3A7 (Hs00426361_m1, FAM), ACTB (Hs01060665_g1, FAM), GUSB (Hs00939627_m1, FAM) and GAPDH (Hs02786624_g1, FAM) were obtained from Applied Biosystems (Carlsbad, CA, USA).

#### Preparation of liver S9 fractions.

Human fetal liver S9 fractions containing cytosol, cell membranes including microsomes, and small mitochondria and other small cell organelles were prepared to evaluate *atra* metabolism in human fetal liver. For this 300 µL of 50 mM Potassium phosphate (KPi) buffer (pH 7.4) containing

| gestational week 10–12 (n = 5) | 4-OH-RA formation (pmol/min/mg protein) | 4-oxo-RA formation (pmol/min/mg protein) | 4-OH-RA + 4-oxo-RA formation (pmol/min/mg protein) | 4-oxo-RA/4-OH-RA (ratio) |
|---|---|---|---|---|
| 61 ± 23 | 5.2 ± 3.8 | 66 ± 25 | 0.09 ± 0.04 |
| gestational week 12–14 (n = 8) | 75 ± 34 | 7.1 ± 3.7 | 82 ± 37 | 0.10 ± 0.05 |
| gestational week 14–16 (n = 9) | 73 ± 30 | 6.9 ± 4.3 | 80 ± 33 | 0.09 ± 0.03 |
| gestational week 16–20 (n = 5) | 77 ± 24 | 6.6 ± 4.6 | 84 ± 28 | 0.08 ± 0.04 |
| Female Fetus (n = 8) | 80 ± 25 | 7.5 ± 3.6 | 87 ± 28 | 0.09 ± 0.03 |
| Male Fetus (n = 11) | 75 ± 27 | 7.4 ± 4.7 | 83 ± 31 | 0.10 ± 0.05 |
| Alaskan (n = 2) | 106 ± 9 | 14.8 ± 1.5 | 121 ± 10 | 0.14 ± 0.00 |
| Asian (n = 6) | 76 ± 35 | 6.4 ± 3.6 | 82 ± 38 | 0.09 ± 0.04 |
| Caucasian (n = 6) | 73 ± 21 | 5.7 ± 3.1 | 79 ± 24 | 0.08 ± 0.03 |

Table 2. Descriptive analysis of *atra* oxidation in the different groups of fetal livers included in the analysis. There were no significant differences in *atra* oxidation rates between gestational age groups, sex, or race.
Figure 4. Inhibition of atRA metabolism by specific CYP inhibitors. Panel A shows the inhibition of 4-OH-RA formation by CYP3A7 inhibitor fluconazole (fluc, 300 μM) and the CYP26 inhibitor talarozole (tal, 200 nM). atRA concentrations were 10 μM and 500 nM in the CYP3A7 and CYP26 incubations. Panels B and C show the percentage inhibition of 4-OH-RA (B) and 4-OH-RA + 4-oxo-RA (C) formation in four representative human fetal livers (hFL, numbers correspond to sample identifiers) by fluconazole (black bars), talarozole (grey bars), and ketoconazole (striped bars) in comparison to vehicle controls (white bars). The stars (*** indicate a significant difference between the incubations with the indicated inhibitor and vehicle control. The numbers indicate the donor codes for the fetal liver donors.
250 mM sucrose, 1 mM EDTA and 1 mM PMSF was added to 0.1–0.3 g of fetal liver sample and the tissue was homogenized in 2 mL Omni Hard Tissue Homogenizing tubes containing 1.4 mm ceramic beads using a 2×20 sec cycles with an Omni Bead Ruptor 24 containing dry ice in acetone (Omni International, Kennesaw, GA). The homogenates were then centrifuged at 9,000 g for 20 min to pellet cell nuclei, large organelles and unbroken cells and the resulting supernatant (S9 fraction) was stored at −80 °C. The overall protein concentration was determined using albumin as the calibration standard and a Pierce BCA Protein Assay (Thermo Fisher Scientific, Inc., Rockford IL).

**Evaluation of atRA metabolism via in vitro Incubations.** The metabolism of atRA in human fetal livers and the enzymes responsible for atRA metabolism were first qualitatively evaluated by standard incubation methods as previously described by us. The specific metabolites formed from atRA by recombinant CYP26A1, CYP26B1 and CYP3A7 in comparison to human fetal liver S9 fractions from representative donors was assessed. 5 pmols of CYP26A1, CYP26B1 and CYP3A7 in 1 mL of 100 mM Potassium phosphate (KPi) buffer (pH 7.4) were incubated for 2 mins (CYP26A1) and 10 mins (CYP26B1 and CYP3A7) with 5 µM atRA at 37 °C, respectively. The incubations were quenched with ethyl acetate, metabolites extracted as previously described and the product formation was then measured by LC-MS/MS as described below. For fetal liver S9 incubations two representative livers were chosen based on the mRNA expression levels of CYP3A7 and CYP26A1. In brief, 0.3 mg S9 protein in 1 mL 100 mM KPi buffer were incubated at 37 °C with 5 µM atRA. After a pre-incubation of 5 min the reactions were initiated with the addition of NADPH (1 mM final concentration) and allowed to proceed for 10 min.

The formation of atRA metabolites was measured in individual human fetal liver S9 fractions from 27 donors as previously described. All of the incubations were conducted under confirmed linear range of time and protein content. Of the atRA metabolites monitored, only 4-OH-RA and 4-oxo-RA were detected in fetal liver S9 fractions and therefore the formation of 4-OH-RA and 4-oxo-RA was quantified in the incubations. To correct the metabolic rates for the sequential formation of 4-oxo-RA from 4-OH-RA CYP3A7 and Talarozole was added to the amount of 4-OH-RA formed for analysis of 4-OH-RA formation rate. For each fetal liver, atRA (500 nM) was incubated with 0.1 mg S9 protein/mL of 100 mM KPi buffer (pH 7.4) in 37 °C. After a pre-incubation of 10 min the reactions were initiated with the addition of NADPH (1 mM final concentration) and allowed to proceed for 10 min. The reactions were terminated with 3 mL ethyl acetate, internal standard (atRA-d5, 50 nM) was added and the metabolites extracted by liquid-liquid extraction. The ethyl acetate layer was collected and dried under a stream of nitrogen, and the dry residue reconstituted in 100 µL acetonitrile for MS/MS analysis.

To determine the relative contributions of CYP26 and CYP3A7 enzymes in atRA oxidation in the fetal livers, CYP selective inhibitors were used to inhibit the target enzymes in incubations with S9 fractions from four representative donors. Fluconazole was chosen as the CYP3A7 specific inhibitor as it has been shown to not inhibit CYP26A1. Talarozole was chosen as the CYP26 inhibitor based on previous characterization of its potency towards CYP26A1 and CYP26B1. Ketocnazole was included in the analysis as it is a well characterized pan-CYP inhibitor with high potency both towards CYP3A7 and CYP26. The inhibition of atRA metabolism by fluconazole (with CYP3A7) and by talarozole (with CYP26A1 and CYP3A7) was confirmed as previously described. In brief, for fluconazole inhibition, atRA (10 µM) was incubated for 10 min with CYP3A7 (5 pmol/mL) in the presence and absence of fluconazole (300 µM) and the percent decrease in 4-OH-RA and 4-oxo-RA formation was quantified. For talarozole inhibition, atRA (500 nM) was incubated for 10 mins with CYP3A7 (5 pmol/mL) or 2 mins with CYP26A1 (2 pmol/mL with added 4 pmol/mL P450 reductase) in the presence and absence of talarozole (200 nM). All of the samples were extracted and analyzed by HPLC-MS/MS. Based on the data collected using recombinant enzyme systems, fluconazole was used at 200 nM to selectively inhibit CYP26 in human fetal liver S9 fractions from four representative donors. In addition, ketocnazole was tested at 10 µM concentration as a pan-CYP inhibitor but it is also likely more potent inhibitor of CYP3A7 than fluconazole. atRA concentration was 500 nM. Incubations were performed as described above for fetal livers and analyzed for metabolite formation by HPLC-MS/MS. The percent inhibition was calculated by comparing the metabolite formation velocity in the presence of the inhibitor to vehicle control.

**LC-MS/MS methods of quantification of retinoid metabolites.** The formation of atRA metabolites was measured using an Agilent 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA) with an Agilent Zorbax C18 column (3.5 µm, 2.1 mm × 100 mm) and coupled to an AB Sciex API 5500 Q/IT mass spectrometer (AB Sciex, Framingham, MA) as previously reported with minor modifications on the chromatography. In brief, analytes were separated using a gradient elution as follows: starting from 100:90 acetonitrile: aqueous to 1:1 acetonitrile: aqueous over 0.5 min then increased to 85:15 acetonitrile: aqueous over 4 min and then after 0.1 min hold increased to 95:5 acetonitrile: aqueous held for 2.5 min. The aqueous phase contained 0.1% formic acid throughout. Analytes were detected using negative ion electrospray and monitoring MS/MS transitions of m/z 241 → 255 Da (atRA), m/z 259 → 273 Da (atRA-d5), m/z 315 → 253 Da (4-OH-RA), m/z 315 → 269 Da (4-oxo-RA), m/z 315 → 241 Da (16-OH-RA) and m/z 316 → 272 Da (4-oxo-RA-d5). Retinoid concentrations were quantified using Analyst software and peak height ratios between the analyte and the internal standard (4-oxo-RA-d5).

**Characterization of CYP3A7 specific activity and Testosterone Metabolism in fetal livers.** Testosterone hydroxylation (formation of 6α-OH-testosterone) was used as a CYP3A7 specific probe reaction and analyzed as previously described. All incubations were performed in triplicate. Testosterone (at 100 µM, final concentration) was incubated with 0.2 mg hFL S9 protein/mL of 100 mM KPi buffer (pH 7.4). All incubations were performed using 96-well plates with a total volume of 0.1 mL per well. The mixtures were pre-incubated for 10 minutes and reactions were initiated by the addition of NADPH (1 mM final concentration). After 10 minutes 80 µL of the incubations were added to 80 µL ice-cold acetonitrile to quench the reaction. The samples were then
In vitro to in vivo Scaling of arfRA metabolism and Statistical Analysis. Statistical analyses were performed using Prism v.5 (GraphPad Software, Inc., La Jolla, CA). Correlation between metabolite formation velocity from arfRA and testosterone as substrates in human fetal livers was tested using linear regression. Differences between arfRA metabolite formation in the presence and absence of inhibitors were tested by one-way analysis of variance. Differences in arfRA metabolism between fetal livers collected at gestational weeks 10–12, 12–14, 14–16, and 16–20, between different genders of the fetus, and different races were tested by one-way analyses of variance coupled with Bonferroni’s Multiple Comparison Test. A p value < 0.05 was considered significant for all statistical analyses.

The overall clearance of arfRA by fetal liver was predicted using standard in vitro-to-in vivo scaling methods15,36. First, the overall intrinsic clearance (Clint) of arfRA metabolism in the fetal liver was calculated by summing 4-OH-RA and 4-oxo-RA formation from arfRA in the fetal liver S9 fraction incubations for each individual donor and the Clint scaled to the whole liver using Eq. 1 as described previously for adult liver13:

\[ Cl_{int, FL} = Cl_{int per mg S9 protein FL} \times \frac{mg S9 protein_{FL}}{S_{FL}} \times \frac{total S_{FL}}{S_{FL}} \]  

Eq. 1

in which FL refers to fetal liver and the total g of fetal liver (liver weight) is specified for the specific gestational ages in Table 1. The hepatic extraction ratio (ERFL) by fetal liver for arfRA was calculated from Eq. 2 based on the well-stirred model of the liver37,38:

\[ ER_{FL} = \frac{f_{u} \times Cl_{int, FL}}{0.5 \times Q_{umbilical vein} + f_{u} \times Cl_{int, FL}} \]  

Eq. 2

in which fetal liver blood flow is calculated as 0.5 times the blood flow of the umbilical vein (Qumbilical vein) based on previous report13. The Qumbilical vein, for each gestational age is listed in Table 1. The plasma unbound fraction of arfRA (fu) used was 0.01 based on previous report39.

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Author Contributions
All three authors wrote the manuscript, and designed the research, Dr Ariel Topletz and Dr Guo Zhong conducted the experiments and all three authors wrote the manuscript. Parts of the work in this manuscript were included in Dr Ariel Topletz’s PhD thesis from University of Washington as partial fulfillment of the requirements for her PhD degree.

Additional Information
Competing Interests: Nina Isoherranen holds a US Patent number: 9963439 on CYP26 Inhibitors. She has received honorariums from Pfizer and Genentech and is a consultant for Boehringer Ingleheim. Ariel Topletz is currently an employee of Seattle Genetics.

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