HHS Public Access

Author manuscript

J Clin Pharmacol. Author manuscript; available in PMC 2023 September 01.

Published in final edited form as:

J Clin Pharmacol. 2022 September ; 62(Suppl 1): S94–S114. doi:10.1002/jcph.2117.

Predicting human fetal drug exposure through maternal-fetal PBPK modeling and *in vitro* or *ex vivo* studies

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Abstract

Medication (drug) use in human pregnancy is prevalent. Determining fetal safety and efficacy of drugs is logistically challenging. However, predicting (not measuring) fetal drug exposure (systemic and tissue) throughout pregnancy is possible through maternal-fetal (m-f) physiologically-based pharmacokinetic (PBPK) modeling and simulation (M&S). Such prediction can inform fetal drug safety and efficacy. Fetal drug exposure can be quantified in two complementary ways. First, the ratio of the steady-state unbound plasma concentration in the fetal plasma (or AUC) to the corresponding maternal plasma concentration (*i.e.* $K_{p,uu}$). Second, the maximum unbound peak ($C_{u,max,ss,f}$) and trough ($C_{u,min,ss,f}$) fetal steady-state plasma concentrations. We (and others) have developed a m-f PBPK model that can successfully predict maternal drug exposure. To predict fetal drug exposure, the model needs to be populated with drug specific parameters, of which transplacental clearances (active and/or passive) and placental/fetal metabolism of the drug are critical. Herein, we describe *in vitro* studies in cells/tissue fractions or the perfused human placenta that can be used to determine these drug-specific parameters. In addition, we provide examples whereby this approach has successfully predicted systemic fetal exposure to drugs that passively or actively cross the placenta. Apart from m-f PBPK models, animal studies also have the potential to estimate fetal drug exposure by allometric scaling. Whether such scaling will be successful is yet to be determined. Here, we review the above approaches to predict fetal drug exposure, outline gaps in our knowledge to make such predictions, and map out future research directions that could fill these gaps.

Keywords

Predicting fetal drug exposure; *in vitro* to *in vivo* extrapolation; perfused placenta; maternal-fetal PBPK model; transfected and placental cell lines; transporters and enzymes

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Conflicts of Interest

Authors declare no conflict of interest.

Introduction

Pregnant women often take medication (drugs) to treat pre-existing conditions (*e.g.,* epilepsy, depression, hypertension, bacterial or viral infections) or health complications produced by pregnancy (*e.g.*, preeclampsia, nausea, gestational diabetes). In some cases, pregnant women are prescribed medication to treat the maternal-fetal dyad (*e.g.*, progression/prevention of HIV infection) or just the fetus (*e.g.*, antenatal corticosteroids to prevent neonatal morbidity/mortality from preterm labor, digoxin to treat fetal tachycardia). Consequently, 80% of pregnant women take medication throughout their pregnancy.[1, 2] Despite prevalent use of medication in pregnancy, more than 90% of clinically approved drugs prescribed to pregnant women are used 'off-label' and lack appropriate information on their pharmacokinetics (PK), safety and efficacy in pregnancy.[2] In fact, 98% of the industry-sponsored clinical trials in the USA actively excluded pregnant subjects due to legal and practical considerations, and only 1% were specifically designed for pregnant women.[3] The above problem is compounded by the fact that determining the safety (especially long-term) and efficacy of drugs in pregnancy is logistically challenging. For example, the use of diethylstilbestrol in pregnancy was found to result in increased risk of breast cancer in mothers and in development of genital tract abnormalities, breast cancer and melanoma in the offspring.[4] Administration of thalidomide for pregnancy-induced nausea caused severe limb deformities (phocomelia) in thousands of offsprings.[5] For the purposes of this review, the word drug refers to a small molecule and not a large molecule, such as a protein.

Since measuring fetal safety and efficacy of a drug is logistically challenging, measuring a surrogate marker of these endpoints, namely fetal drug exposure, can inform assessment of fetal drug safety and efficacy. While systemic fetal drug concentration can be determined at birth by sampling umbilical vein (UV)/artery (UA) blood, such sampling is impossible earlier in pregnancy. And, as we have detailed before[6], a single UV sample at the time of birth does not provide an estimate of fetal drug exposure. To assess such exposure, one must determine the plasma/blood fetal steady-state drug concentration or the area under the plasma concentration-time curve (AUC) (at steady-state or after a single dose). Because fetal drug concentrations are driven by maternal drug concentrations, to take into account the inter-individual variability in the latter, the fetal steady-state plasma concentration (or AUC) is best expressed relative to the corresponding maternal value (*i.e.,* K_p). And, since it is the unbound drug that produces the pharmacological effect of a drug, it is the average fetal steady-state unbound plasma drug concentration ($C_{u,av,ss,f}$) relative to the average maternal steady-state unbound plasma drug concentration ($C_{u,av,ss,m}$), namely, $K_{p,uu}$, that should be estimated. In addition, where the plasma drug concentrations can fluctuate significantly during a dosing interval, it is also important to determine the unbound peak ($C_{u,max,ss,f}$) and trough ($C_{u,min,ss,f}$) fetal steady-state plasma drug concentrations. Ideally, one should measure fetal tissue drug concentrations where the drug's efficacy and toxicity is likely to manifest. In practice, neither this nor repeated blood sampling of the fetal circulation (to estimate systemic fetal drug exposure) is possible. Therefore, the only recourse to estimate fetal drug exposure (systemic or tissue) is by maternal-fetal (m-f) physiologically-based pharmacokinetic (PBPK) modeling and simulation (M&S).

PBPK models are physiologically relevant mathematical models wherein individual or grouped organs are depicted as compartments interconnected by relevant blood flow. These models are mechanistic in nature and incorporate intrinsic or system dependent physiological parameters (such as blood flows, tissue volumes, enzyme/transporter abundance), and associated population variability which allows prediction of disposition of any drug in the population of interest.[7] The mechanistic nature of PBPK models allows incorporation of the fetus and fetal tissues into the model as well as gestational-dependent changes in both maternal and fetal physiology. Thus, such models can be used to predict maternal-fetal disposition, throughout pregnancy, of any drug administered to the mother provided the models are populated with extrinsic, drug-specific parameters (physicochemical and absorption, distribution, metabolism, elimination (ADME) properties). We[8–11], and others[12–16] have developed a m-f PBPK model that can successfully predict maternal drug exposure. However, to predict fetal drug exposure, the model needs to be populated with drug specific transplacental clearances (active and/or passive) and placental/fetal metabolism, along with fetal and placental parameters that can affect the fetal drug exposure such as fetal fu, pH of blood, placental binding, ion trapping in the placenta.

In this review, first we describe factors that determine fetal drug exposure and define PK parameters to assess fetal drug exposure. Second, we describe *in vitro* studies in cells/tissue fractions or in the perfused human placenta that can be used to determine drug-specific disposition parameters in the placenta and the fetal liver. Third, we provide examples whereby these studies, combined with m-f PBPK M&S, have successfully predicted systemic fetal exposure to drugs that passively or actively cross the placenta. Apart from m-f PBPK models, animal studies also have the potential to estimate human fetal drug exposure by allometric scaling. Therefore, we discuss the potential for such scaling to be successful. Finally, we close by highlighting gaps in our knowledge to predict fetal drug exposure and suggest future directions to fill these gaps.

Placental physiology, abundance and location of placental drug transporters and metabolizing enzymes

Any drug administered to the mother will reach the fetus through multiple routes (Fig. 1a). Among these, transplacental transfer is the most important once blood flow to the placenta has been established (late first trimester)[17–19]. The placenta is of fetal origin and separates the maternal and fetal blood. Maternal blood from the uterine artery floods the intervillous space which acts like a “bath-tub” and bathes the fetal villi lined with the multinucleated syncytiotrophoblast formed by fusion of mononuclear cytotrophoblastic stem cells. This cellular arrangement does not allow paracellular diffusion of drugs across the syncytiotrophoblast layer (Fig. 1b).[20] Therefore, placental drug transfer can occur only by transporter-mediated active uptake/efflux and/or passive diffusion. Lipophilic drugs that are unionized at physiological pH generally have high passive diffusion clearance across the placenta provided they are not substrates of efflux transporters. Many drugs taken by pregnant women are substrates of the two most highly expressed efflux transporters in the human placenta, namely P-glycoprotein (P-gp), and Breast Cancer Resistance Protein

(BCRP).[21–24] These ATP-binding cassette transporters serve to prevent entry of drugs into the placenta (and therefore the fetal compartment) by acting as gatekeepers. They serve this function by effluxing drugs as soon as the drugs attempt to cross the lipid bilayer of the syncytiotrophoblast. These transporters can also efflux drugs from the intracellular compartment. P-gp transports mostly lipophilic, unionized cationic drugs (*e.g.* HIV protease inhibitors) and some neutral drugs (*e.g.* digoxin), while BCRP can transport anionic (*e.g.* nitrofurantoin), cationic (*e.g.* cimetidine) lipophilic and hydrophilic compounds and their conjugates.[25] A substantial number of drugs available in market have some affinity for P-gp. Additionally, both P-gp and BCRP have overlapping substrate selectivity. Therefore, both these efflux transporters, act either alone or in tandem, to reduce fetal exposure to drugs. This has been demonstrated in *in vitro*, perfused placenta as well as in *in vivo* (animals) studies. For example, P-gp-mediated transport of calcein-AM and vinblastine was inhibited by P-gp inhibitors in Transwell® assays with human placental choriocarcinoma derived BeWo cells.[26] In the dually perfused placenta, the transplacental clearance of cyclosporin A (P-gp substrate) was significantly increased when co-administered with the P-gp inhibitor quinidine or chlorpromazine.[27] In an *in vivo* mouse study, the fetal/maternal plasma concentration ratios of P-gp substrates digoxin, saquinavir, and paclitaxel was significantly greater in *Mdr1a/1b*(−/−) fetuses than in the *Mdr1a/1b*(+/+) fetuses of the same dam.[28] Furthermore, the P-gp inhibitor cyclosporin A was found to significantly increase the fetal liver distribution of [^{11}C]-verapamil in a PET imaging study in macaques.[29] Similarly, in membrane vesicles prepared from human term placenta, BCRP was shown to mediate transport of mitoxantrone[30] and glyburide[31]. BCRP was observed to significantly limit the maternal-to-fetal transport of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)[32] and glyburide[33] in the perfused human placenta. Additionally, increased fetal exposure of topotecan[34], nitrofurantoin[35] and glyburide[36] was observed in *Bcrp1*(−/−) mice as compared to the wild-type pregnant mice. Apart from P-gp and BCRP, multidrug resistance proteins 1–3 and 5 (MRP1–3 and 5) are also important in transplacental transport, but to a lesser extent.[23, 25] The placenta also expresses other efflux transporters as well as influx transporters (see Table 1). However, studies to demonstrate their functional role *in vivo* in determining fetal drug exposure, through *in vitro* or perfused human placenta studies, are limited. The placental transporters show gestational age-dependent abundance/expression *e.g.* the abundance/expression of P-gp, BCRP, Organic Anion Transporting Polypeptide 1A2 (OATP1A2), OATP2B1, OATP1B1, Organic Cation/Carnitine Transporter 2 (OCTN2) decreases from first trimester to term[21, 22, 37–45] while that of Multidrug Resistance Protein 2 (MRP2), Organic Anion Transporter 4 (OAT4) and Organic Cation Transporter 3 (OCT3) increases as gestation progresses.[22, 46–49] The abundance/expression of OATP4A1, MRP1, MRP3 and Multidrug And Toxin Extrusion 1 (MATE1) shows no change throughout pregnancy.[37, 43, 46, 48, 49] It is important to note that where the abundance of transporters per gram of placental tissue decreases (*e.g.* P-gp), the overall abundance of the transporters in the whole placenta increases with gestational age because the placenta significantly increases in size with gestational age. Unfortunately, literature reports are controversial regarding the ontogeny of several placental transporters (*e.g.,* P-gp, MRP1, OATP1B1, OCT3)[21, 37, 46, 48–50] and additional studies are needed to clarify their ontogeny.

In addition to drug transporters, the placenta also expresses a variety of drug metabolizing enzymes including cytochrome P450s (CYPs), UDP-glucuronosyltransferase (UGTs), sulfotransferases (SULTs), glutathione-S-transferases (GSTs) (Table 2). Among them, CYP19A1 (aromatase) is the predominant CYP.[51] It is involved in the metabolism of both endogenous compounds (conversion of androgens to estrogens) as well as xenobiotics (such as aflatoxin B1[52], glyburide[53], buprenorphine[54], and methadone[55]). Multiple drugs such as zidovudine[56], glyburide[57], methadone[55], oxcarbazepine[58], warfarin[59] undergo metabolism in human placental microsomes. Additionally, perfused placental studies have demonstrated the metabolism of bupropion[60], oxcarbazepine[61], buprenorphine[62], tenofovir and emtricitabine[63]. However, it is important to note that for placental drug metabolism to affect $K_{p,uu}$, it must be significant relative to the other clearance pathways in Eq 2 (below). Whether this is the case for the drugs cited above has not been determined. Placental expression of CYP isozymes is generally higher in the first trimester than term.[64] In contrast, the expression of UGTs generally remains at about the same levels in the placenta throughout pregnancy.[65] It is important to reiterate here that though the abundance of an enzyme per gram of placental tissue may decrease, its total abundance in the whole placenta may increase with gestational age due to the significant gestational age-dependent increase in placental weight. Multiple maternal and environmental factors, such as drug abuse, smoking, alcohol consumption, polluted air, and contaminated food can affect the activity of placental enzymes.[66] For example, the activity of UGT and CYP1A is significantly higher in placentae of mothers who smoke and is greatest in women who smoke and consume alcohol.[67] In contrast, the catalytic activity of CYP19A1 decreases in mothers who smoke but its protein abundance is not significantly altered.[68]

The fetal liver also expresses drug metabolizing enzymes such as CYPs, UGTs, SULTs, xanthine oxidase (Table 2). The most prominent among them is CYP3A7 which accounts for almost 30% of the total fetal CYP content. Its expression increases with gestational age and reaches highest at postnatal days 1 and 7.[69] All other CYPs have lower expression in the fetal liver vs. adult liver.[70] Among the SULTs, SULT1A1, SULT1A3, SULT2A1, SULT1E1 and SULT1A3 are expressed in the fetal liver.[71–74] The SULT enzymes are believed to play a major role in the metabolism of endogenous substances (such as catecholamines, steroids like dehydroepiandrosterone, estrogens) important for fetal development.[71–73, 75] The expression of UGTs has also been detected in the fetal liver (Table 2).

The drug reaches the amniotic fluid through urinary excretion by the fetus and by diffusion from the placenta. This drug can be reabsorbed through the intestine when the fetus swallows the amniotic fluid.[76] The fraction reabsorbed determines the extent of intestinal reabsorption. This recycling of the drug is likely the highest for hydrophilic drugs that tend to be renally excreted. Irrespective of the extent of drug excretion into the fetal urine, due to this drug recycling, this pathway is akin to distribution into a peripheral fetal compartment rather than irreversible loss from the fetal compartment. Additionally, the fetal skin is also permeable to drugs before keratinization begins at gestational week (GW) 20)[77], allowing direct entry of the drug from the amniotic fluid into the fetal circulation (Fig. 1a).

Pharmacokinetic parameters that describe fetal drug exposure

Here we describe four key PK measures that are important to describe fetal drug exposure namely, $K_{p,uu}$, the unbound steady-state average ($C_{u,av,ss,f}$), peak ($C_{u,max,ss,f}$) and trough ($C_{u,min,ss,f}$) fetal plasma concentrations. Each is defined in detail below along with factors that determine their value. For the purposes of this discussion, we have assumed that the drug is administered to the mother to steady-state.

$K_{p,uu}$ and fetal $C_{u,av,ss}$ plasma concentration

$K_{p,uu}$ is independent of the drug dose or maternal plasma concentration provided the drug follows linear transplacental and fetal disposition PK (Eq. 1).

$$K_{p,uu} = \frac{f_{u,f} \times AUC_f}{f_{u,m} \times AUC_m} = \frac{f_{u,f} \times C_{av,ss,f}}{f_{u,m} \times C_{av,ss,m}} \quad \text{(Eq. 1)}$$

Where, $f_{u,f}$ and $f_{u,m}$ are unbound drug fractions in fetal and maternal plasma respectively, AUC_f and AUC_m are area under total plasma concentration-time curves in the fetus and the mother respectively, $C_{av,ss,f}$ and $C_{av,ss,m}$ are the total plasma steady-state concentrations in the fetus and the mother, respectively.

While it is important to know the absolute $C_{u,av,ss,f}$ plasma concentration, *in vivo*, this value is primarily determined by the corresponding maternal concentration. Therefore, the ratio of this concentration and the corresponding maternal concentration, $K_{p,uu}$, is usually predicted. Once $K_{p,uu}$ is predicted, $C_{u,av,ss,f}$ can be calculated for any corresponding maternal concentration.

$K_{p,uu}$ is determined by a number of factors (Eq. 2) that determine transplacental and fetal clearance of the drug, namely: i) unbound passive placental diffusion clearance (CL_{PD}); ii) unbound active placental efflux (CL_{PM}), influx (CL_{MP}), or placental metabolic drug clearance (CL_{p0}) and iii) unbound fetal clearance (CL_{f0}) (*e.g.,* fetal hepatic clearance).[6]

$$K_{p,uu} = \frac{CL_{PD} + CL_{MP}}{CL_{PD} + 2CL_{f0} + CL_{p0} + CL_{PM} + \left(\frac{CL_{p0} + CL_{PM}}{CL_{PD}}\right) * CL_{f0}} \quad \text{(Eq. 2)}$$

As indicated above, the placenta is not well-endowed with enzymes that can extensively metabolize drugs (*e.g.,* CYPs).[7,8] While the placenta expresses influx transporters, to date there is no evidence of drugs that are actively transported into the placenta (and therefore the fetal compartment) via such transporters. Thus, CL_{MP} and CL_{p0} can usually be considered negligible. But, this is not the case with efflux transporters such as P-gp and BCRP which are highly expressed in the apical membrane of the placental syncytiotrophoblast[22] and prevent entry of drugs into the placenta (and therefore the fetal compartment). In the fetus, the drug can be eliminated from the fetal circulation by fetal metabolism in the fetal liver. However, due to its size and selective expression of CYPs (primarily CYP3A7), the fetal liver is usually not a significant determinant of drug metabolism and therefore fetal drug exposure (*i.e.,* CL_{f0} is usually negligible relative to the transplacental CL_{PD}). Therefore, in

general, the fetal $K_{p,uu}$ is usually dependent on the magnitude of CL_{PM} relative to CL_{PD} (note all clearance values in Eq 2 and 3 are the unbound clearance values) (Eq. 3):

$$K_{p,uu} = \frac{CL_{PD}}{CL_{PD} + CL_{PM}} \quad \text{(Eq. 3)}$$

When there is negligible placental efflux ($CL_{PM} = 0$) or placental/fetal elimination, $K_{p,uu}$ will be 1 because passive transplacental diffusion clearances should be equal in both directions. In this event, fetal unbound AUC will approximate maternal unbound AUC and can be predicted from the latter.[79] But, when a drug is actively effluxed (*i.e.*, $CL_{PM} > 0$), $K_{p,uu}$ will be less than 1. In that event, the fetal unbound AUC will be less than the maternal unbound AUC and cannot be estimated solely based on maternal drug concentration data. Instead, to predict $K_{p,uu}$, one needs the values of all the transplacental clearances or the ratio of the values shown in Eq. 3. Later in this article, we address how these values can be determined through *in vitro* cell or perfused placenta studies.

Although $K_{p,uu}$ provides an estimate of fetal exposure at steady-state or over a dosing interval, its major limitation is that it does not provide information about the dynamic variations in fetal unbound plasma concentrations with time. In other words, the value of the peak or trough unbound steady-state fetal plasma drug concentration. However, this limitation can be overcome by incorporating into a m-f PBPK model the exact magnitude of the various transplacental clearances and the volume of distribution of the drug in the fetal compartment. The approach is discussed in detail later in this article.

While $K_{p,uu}$ is independent of protein binding of the drug, K_p is not. K_p changes with gestational age because the plasma protein concentration in the maternal-fetal compartments change with gestational age.[80] For example, fetal α1-acid glycoprotein concentrations increase from almost undetectable level at 12 weeks to 0.3–0.4 g/l at >GW 35, while maternal α1-acid glycoprotein concentrations fluctuate between 0.38 and 1.05 and show no trend with advancing gestational age. These changes lead to an increase in α1-acid glycoprotein fetal/maternal serum concentration ratio from 0.057 at week 12 to 0.37 at >GW 35.[80] On the other hand, maternal serum albumin concentration decreases during gestation, while that in the fetus increases with gestational age. As a result, the fetal/maternal serum albumin concentration ratio gradually rises from 0.38 at 12 weeks to 1.20 at term.[80] Although Eq. 1 may mathematically suggest that $K_{p,uu}$ is dependent on the total fetal drug plasma concentration, that would be incorrect. In fact, it is the other way round. To illustrate this point, consider a drug that passively crosses the placenta, is extensively bound to albumin, and is not metabolized in the placenta or the fetal liver. The $K_{p,uu}$ of this drug will be unity (as only the unbound drug crossed the placenta) irrespective of the gestational age. However, its K_p value will change with gestational age due to changes in albumin concentration in the maternal-fetal compartment.

Fetal and maternal PK parameters that determine fetal unbound peak ($C_{u,max,ss,f}$) and trough ($C_{u,min,ss,f}$) steady-state plasma concentrations

Fetal unbound peak ($C_{u,max,ss,f}$) and trough ($C_{u,min,ss,f}$) steady-state plasma concentrations are determined in manner analogous to factors that determine plasma concentrations in a non-pregnant individual after oral absorption of drugs. Thus, when the rates of entry and exit of the drug into and from (back to the mother and by fetal elimination) the fetal compartment are equal, $C_{u,max,ss,f}$ will be achieved. For this reason, $C_{u,max,ss,f}$ usually occurs later than the $C_{u,max,ss,m}$. However, the greater the transplacental clearance, the faster and higher the $C_{u,max,ss,f}$ is reached. In addition, the magnitude of $C_{u,max,ss,f}$ will depend on the volume of distribution of the fetal compartment and fetal clearance (via the placenta or by metabolism). The smaller the fetal volume of distribution, the larger the $C_{u,max,ss,f}$ and vice-versa. Likewise, the smaller the clearance from the fetal compartment the larger the $C_{u,max,ss,f}$. Similarly, $C_{u,min,ss,f}$ will be determined by the magnitude of fetal drug clearance during the dosing interval. Because maternal concentrations drive fetal concentrations, the higher the maternal concentrations, the higher both $C_{u,max,ss,f}$ and $C_{u,min,ss,f}$ will be (see Zhang et al., 2017[6] for details). Finally, the corresponding total $C_{max,ss,f}$ and $C_{min,ss,f}$ will be determined by the protein binding of the drug in the fetal compartment.

Predicting fetal systemic and tissue drug concentrations from animal and *in vitro* studies

As indicated above, measuring fetal (systemic or tissue) exposure to drugs is logistically challenging, especially before term. However, such exposure could be predicted using animal studies or *in vitro* studies combined with PBPK M&S. Below, we discuss both these approaches.

Animal studies

Several animal models ranging from small rodents such as mice, rats, and guinea pigs to larger animals like sheep and non-human primates have been used to study the transplacental passage and fetal tissue distribution of drugs. The smaller animals (*e.g.,* mice) can be genetically modified to study the role of specific transporters in fetal exposure to drugs. For example, Zhang et al. demonstrated that presence of murine *Bcrp* significantly limits fetal distribution of nitrofurantoin in pregnant mice while exerting only a minor effect on the systemic clearance of the drug.[35] However, small animal studies have some limitations. Due to the small fetal size, fetal blood samples are difficult or impossible to obtain. Thus, both maternal (plasma) and whole fetus and placental tissue drug concentrations are obtained only once (at the time of sacrifice) after drug administration. Consequently, multiple pregnant dams need to be sacrificed and the data pooled to characterize the entire AUC of the drug concentration-time profile. Fortunately, due to inbreeding, inter-animal variability tends to be low and this “naive-pooled” approach to data analysis works relatively well.[35, 36, 81, 82] When interpreting the fetal concentrations data, it is important to keep in mind that these concentrations are not in fetal blood or plasma but in the entire fetus. Therefore, for those drugs that bind extensively to fetal tissues, the ratio of the whole fetus/maternal plasma AUC may be greater than unity and should not be interpreted as transport

of the drug into the fetal compartment. Also, it is often not possible to determine the drug concentrations in individual fetal tissues. In addition, placental/fetal tissue concentrations of the drug should be corrected for the amount of blood in the organ, especially for drugs that extensively bind to blood components.

In larger animals (sheep and non-human primates), intravascular catheters can be placed both in the dam and the fetus to obtain matched fetal and maternal plasma drug concentration-time profiles over a period sufficient to characterize the corresponding AUCs. The pregnant non-human primates are similar to humans in that they have a discoid placenta with multivillous fetal-maternal interdigitation and a hemo-monochorionic barrier.[83] These animals were used to determine the rate and extent of the placental transfer of anti-HIV drugs zidovudine, didanosine, zalcitabine and stavudine.[84] These *in vivo* animal data strongly correlated with those from the perfused human placenta. However, these drugs cross the placenta by passive diffusion and a corresponding correlation for drugs which are actively transported across the placenta or extensively metabolized in the placenta/fetal liver has not been investigated.

The major limitation associated with animal models is the large inter-species differences in (i) placental anatomy, metabolism, and transport, (ii) fetal metabolism and transport (iii) substrate selectivity of drug metabolizing enzymes and transporters and (iv) the abundance of placental/fetal transporters and metabolizing enzymes.[83, 85–87] These differences may lead to variations in fetal drug exposure, thus preventing meaningful extrapolation to humans.[81, 88] Nevertheless, whether animal data can be used to predict human fetal drug exposure is an open question that has yet to be answered.

In vitro cell and perfused human placenta studies combined with PBPK M&S

Over the years, multiple maternal-fetal (m-f) PBPK models have emerged which show progressive success in predicting maternal and fetal drug exposure.[89] Initial m-f PBPK models were focused on estimating variability in maternal drug exposure, with limited emphasis on fetal drug exposure. This was due to lack of curation of gestational age-dependent fetal and placental physiological parameters (*e.g.,* gestational age dependent tissue blood flow) and lack of availability of the abundance of drug transporters and metabolizing enzymes which determine drug CL_{MP}, CL_{PM}, CL_{p0}, and CL_{f0}. However, we[6, 22, 79, 90, 91] and others[92–98] have begun to fill these gaps in knowledge by generating/curating relevant physiological data (*e.g.* blood flow, organ sizes, plasma protein concentrations) and describing their gestational-age dependent changes with models that allow interpolation for any gestational age. In addition, we have quantified the abundance of placental transporters at various gestational ages.[22] To determine the various parameters which govern fetal exposure, we need to populate the m-f PBPK models with drug-specific CL_{PD}, CL_{MP}, CL_{PM}, CL_{p0}, and CL_{f0}. These parameters can be determined through *in vitro* cell studies and/or *ex vivo* perfused human placenta studies. As discussed earlier, there is no evidence of influx transport of drugs across the human placenta (CL_{MP}), and because CL_{p0} and CL_{f0} usually contribute minimally to fetal drug exposure, these parameters can be discounted. Therefore, as described below, the remaining two parameters namely CL_{PD} and CL_{PM}, need to be estimated, to populate the m-f PBPK model. However, if CL_{MP}, CL_{p0}

and CL_{f0} are significant relative to the net transplacental CL, they can be taken into account using the approaches described below, in combination with metabolic depletion studies in placental and fetal liver homogenates or microsomes[99], to predict fetal drug exposure.

Two approaches can be utilized to estimate CL_{PD} and CL_{PM}, namely the efflux ratio-relative expression factor (ER-REF) approach using cells (*i.e.,* placental or transporter-transfected cells) or the perfused human placenta (Fig. 2). The former can be used only when the transporter(s) involved are known. While the latter can be used even when the transporter(s) involved are not known. Both these approaches utilize the *in vitro* to *in vivo* extrapolation (IVIVE) strategy to scale the magnitude of the unbound bi-directional placental clearance determined in the respective system to that *in vivo*. The IVIVE scalar for the ER-REF approach is called REF, which accounts for the differences in transporter abundance between the *in vitro* cell model and *in vivo* (*i.e.* the entire placenta). In the case of the perfused placenta, the bi-directional placental clearance determined in a single perfused cotyledon needs to be scaled to the entire placenta (*i.e.,* physiological scaling). This is done by utilizing the average number of cotyledons in the placenta or by scaling the cotyledon weight or volume to the total placental weight or volume. We would like to emphasize here that the data generated by both approaches (cell and perfused placenta studies) can be used to predict $K_{p,uu}$, as detailed below, independent of m-f PBPK models provided placental transporter and/or metabolism (and not fetal metabolism) is the major determinant of fetal exposure to drugs. However, the dynamic changes in fetal exposure can be predicted ONLY if these two approaches are combined with the m-f PBPK models.

ER-REF combined with the m-f PBPK approach—This approach requires determination of two parameters, ER and REF (Fig 2, left panel). REF is the ratio of the abundance of the transporter of interest in the placenta and the cell line. The magnitude of drug efflux (or influx) by placental transporters is measured as ER in the Transwell® assay using either placental cell lines or transporter overexpressing cell lines (see below for details). Since the transporter expression in these cell lines is likely to differ from that in the human placenta, the ER measured in these cells needs to be corrected for this difference in abundance using the REF. Unless the abundance on the cell membrane is measured using a method such as biotinylation, combined with quantitative targeted proteomic[100], the REF approach assumes that the total abundance of the transporter measured is that on the cell membrane and the transporters that are present there are functional. The advantage of the REF approach (over that of the perfused placenta approach) is that the abundance of various placental transporters in the human placenta of different gestational ages is available. Therefore, the ER-REF approach can be used to predict fetal exposure to drugs once blood flow to the placenta has been established.[91]

ER determination:

In the Transwell® assay, the polarized cells over-expressing the transporter of interest, are seeded onto a Transwell® insert to create the apical and basal compartments corresponding to the maternal and fetal blood, respectively (Fig. 2, left panel). Then, the drug is added either to the apical or basal chamber (*i.e.,* the donor chamber) and drug concentration in the contralateral receiver chamber is sampled at various times points. The ratio of the apparent

drug permeability from the basal-to-apical (B→A) ($P_{app(B→A)}$) and apical-to-basal (A→B) ($P_{app(A→B)}$) chambers is the ER that represents the magnitude of *in vitro* transport by the overexpressed transporters across the cell monolayer (Eq. 4).[91]

$$ER = \frac{P_{app(B \to A)}}{P_{app(A \to B)}} \equiv \frac{CL_{int(B \to A)}}{CL_{int(A \to B)}} = \frac{cA_{A(R)} \times AUC_{A(D)}}{cA_{B(R)} \times AUC_{B(D)}} \quad \text{(Eq. 4)}$$

Assuming that the surface area is the same in both directions, the P_{app}'s can be set equivalent to $CL_{int(B→A)}$ and $CL_{int(A→B)}$, the intrinsic clearances of drug in the B→A and the A→B directions, respectively; $cA_{A(R)}$ and $cA_{B(R)}$ are cumulative amounts of drug in corresponding receiver compartment; $AUC_{A(D)}$ and $AUC_{B(D)}$ are AUC of the drug in corresponding donor compartment. AUC, rather than initial donor drug concentration, is used to compensate for any depletion of drug in the donor compartment. Proteins are usually not included in the donor chamber. If included, the P_{app} needs to be determined for the unbound drug.

Then, to predict the *in vivo* $K_{p,uu}$ (*i.e.* $K_{p,uu,IVIVE}$), the ER determined in the cell line is scaled (Eq 5)[91] by REF as follows:

$$K_{p,uu,IVIVE} = \frac{1}{\left(ER_{Inh(-)} - ER_{Inh(+)}\right) * REF + 1} \quad \text{(Eq. 5)}$$

where $ER_{Inh(-)}$ and $ER_{Inh(+)}$ is the efflux ratio in the absence and presence of complete inhibition of the transporter of interest, respectively. The difference in ER in absence and presence of complete inhibition of transporters provides the magnitude of ER associated with active transport. Alternatively stated, the bi-directional passive diffusion clearance of the drug cancels out when estimating the net active transport ER. In the absence of transport, the ER in the absence and presence of the transporter inhibitor will be identical. Therefore, as predicted, $K_{p,uu,IVIVE}$ will be 1. For efflux transporter drug substrates, the difference in the ERs will be >1 and $K_{p,uu,IVIVE}$, as expected, will be <1. Thus, when $K_{p,uu,IVIVE}$ is estimated using Eq. 5, it is independent of the absolute magnitude of passive diffusion clearance across the placenta. This is because in this equation only the relative value of the active and passive clearance is taken into consideration. We would like to emphasize again that the $K_{p,uu,IVIVE}$ can be estimated independent of any m-f PBPK model. However, this value does not provide an estimate of the dynamic exposure of fetal exposure to a drug (i.e. $C_{u,max,ss,f}$ and $C_{u,min,ss,f}$). To do so, one must estimate the absolute value of each bi-directional clearance pathway (active and passive) of the drug, as discussed below under the subheading Transporter-transfected cell lines. Parenthetically, the fraction of a drug transported (*e.g.,* $ft_{P\text{-}gp}$), in vivo, can be estimated as:

$$ft_{P-gp} = 1 - K_{p,uu,IVIVE} \quad \text{(Eq. 6)}$$

The choice of the cell line to be used in the Transwell® assay should be one that expresses the transport of interest for the drug in question. Although other transporters are also present in placenta, P-gp and BCRP are the most abundant and many drugs are substrates of one

or both these transporters. However, of these two transporters, P-gp plays a more prominent role in governing fetal exposure to drugs that are taken by pregnant women. Hence, the choice of the cells to use in the Transwell® assay could be either a human placental cell line or one that is engineered to overexpress the transporter of interest (*e.g.,* MDCKII cells overexpressing P-gp). These two options are discussed below.

Placental cell lines:

The human placental choriocarcinoma trophoblast cell lines (BeWo, Jar, Jeg-3 and ACH-3P) have been used to address transplacental transport of drugs.[101, 102] While BeWo, Jar, and Jeg-3 cells are derived from a trophoblastic tumor, the ACH-3P cell line was derived by fusing primary trophoblast cells from week 12 placenta with human choriocarcinoma cells.[103] The BeWo, Jar and Jeg-3 cell lines express relevant efflux drug transporters (P-gp, BCRP, MRP1, etc.) at the mRNA and protein level.[46, 104, 105] However, P-gp activity in BeWo cells is low. Jar cells lines have low activity of both P-gp and BCRP.[104, 106–108] Of the cell lines, several can form tight junctions[109], including a subclone of BeWo cells, a property necessary for use in Transwell® assays to determine drug transport.[110] However, the BeWo b30 cell monolayer tight junctions are not easy to establish and maintain for an extended period of time.[110] While xenobiotic transport has been shown in these cells, their ability to predict placental transport accurately has not been demonstrated. Therefore, they could be used to identify potential transporters relevant for *in vivo* transport of drugs.[102] However, there are multiple limitations associated with the placental cell lines (Table 3). Due to these limitations, they have not been used for IVIVE of fetal exposure to drugs. Instead, non-placental cell lines, such as Madin-Darby Canine Kidney (MDCKII) cells, transfected with human transporters, have been used as a surrogate.

Transporter-transfected cell lines:

MDCKII cells can overcome the major limitations of trophoblast-derived cell lines in that they can form tight junctions and therefore limit paracellular drug transfer which should be absent in the syncytiotrophoblast (a single cell barrier). Additionally, these cells, after transfection, can overexpress the major human placental transporters (*e.g.*, P-gp and BCRP), and thus, allows easy detection of transporter activity *in vitro*. If a drug is a substrate of both P-gp and BCRP, cells expressing these transporters individually can be used to predict the $K_{p,uu,IVIVE}$ of such a drug. And, the ft via each transporter can be estimated to allow prediction of possible drug-drug interactions or polymorphisms that could modulate fetal drug exposure. Furthermore, it is also possible to remove any confounding contribution from endogenous transporter by knocking it out (*e.g.*, canine P-gp is knocked out in hMDR1-MDCK$^{cP\text{-}gpKO}$). However, the MDCK cell monolayer does not represent the complex physiology of the placenta and lacks secondary cell populations (*e.g.*, endothelial cells) where transport (though minor contribution) can occur.[23]

As indicated before, the ER-REF approach considers ONLY the transport-mediated ER or clearance and is independent of the absolute magnitude of CL_{PD} of the drug. Also, it does not provide the absolute value of the maternal-fetal or the fetal-maternal drug clearance. But, to dynamically predict fetal drug exposure, an estimate of the active transport vs. passive

clearance is needed. Therefore, we have proposed an approach to estimate the CL_{PD} of any drug by using the *in vivo* midazolam CL_{PD} as a calibrator (Eq. 7).

$$CL_{PD}\ of\ test\ drug = \frac{P_{app}\ \text{of test drug}}{P_{app}\ \text{of midazolam}} * CL_{PD}\ of\ midazolam \quad \text{(Eq. 7)}$$

Where, P_{app} represents the apparent permeability in MDCKII or another mammalian cell line where the drug is not transported. Once the absolute value of the CL_{PD} has been estimated, the magnitude of the transport clearance, as a fraction of the CL_{PD}, can be obtained from $K_{p,uu,IVIVE}$.[91]

Validation of the ER-REF plus the m-f PBPK model approach:

Any approach or model needs to be validated prior to applying it to prospectively predict fetal drug exposure to a drug for which such data are not available. Therefore, it is logical to ask if this ER-REF approach, combined with the m-f PBPK model, has been validated? It has for P-gp transported drugs[91] but needs to be validated for placental transport mediated by BCRP or other transporters. For validation, rich *in vivo* UV and simultaneously obtained MP concentrations in multiple maternal-fetal dyads that span the AUC profile of the drug are required to assess model prediction performance. But such *in vivo* data are limited and available for only a limited number of drugs for which the transporters involved (or the lack thereof) have been definitively identified. Using these inclusion criteria, we have successfully validated the ER-REF plus m-f PBPK model approach for four P-gp substate drugs namely dexamethasone DEX, betamethasone BET, darunavir DRV and lopinavir LPV.[91] Such validation for BCRP substrates and dual P-gp/BCRP substates is ongoing in our laboratory.

Now that our ER-REF/m-f PBPK model approach has been validated, it can be used to predict fetal drug exposure to other drugs that are P-gp substrates. Moreover, this approach is versatile in that it can be used to predict fetal exposure to P-gp substrate drugs at earlier gestational ages as we have done before.[91] The m-f PBPK model purposed by us[6], and others (Simcyp version 19 onwards), incorporates multiple fetal organs allowing us to dynamically predict fetal tissue drug concentration such as fetal brain. To do so successfully, one would have to have information (currently lacking) on transporter abundance at the fetal tissue:blood barrier. Plus, the approach would have to be validated for a select number of transporter-selective substrate drugs. The former is possible, but the latter is logistically challenging. Future research should be directed to accomplishing these goals.

Ex-vivo perfused human placenta combined with the m-f PBPK model approach—This model was initially developed by Maurice Panigel and Henning Schneider[111, 112] The placental perfusion model allows for the study of multiple pharmacokinetic factors such as passive diffusion, active transport, metabolism, and tissue binding. Unlabored placentas are preferred for this approach since placental cell death and other complications (*e.g.* infection with exposure to meconium and increased prostaglandin concentrations with inflammation and hypoxia) increase with labor duration.[113] In this model, a single cotyledon of the placenta is dually perfused through both the intervillous space and a cannulated fetal artery to represent the maternal and fetal blood flow,

respectively. The outflow perfusate is then collected from the intervillous space and the cannulated fetal vein to represent circulating blood returning to the maternal and fetal circulation, respectively. Perfusate buffers are commonly oxygenated with a 95% oxygen and 5% carbon dioxide gas to increase duration of placentae viability. Additionally, proteins (*e.g.,* 2.0 g/L bovine serum albumin) are added to the buffer to allow binding to plasma proteins and to increase drug solubility. In a given cotyledon, the drug is included in the donor perfusate (*i.e.,* either the maternal or the fetal perfusate) and drug concentration is sampled in both the donor and the receiver outflow. The drug perfusion can be set up in two ways: 1) recirculating or 2) single-pass. For the recirculating approach, any outflow from the vein that is not collected for determination of drug concentration is re-circulated. However, for the single-pass approach, the outflow that is not collected for determination of drug concentration goes to waste and the cotyledon is continuously perfused with fresh buffer (with the drug contained in only in the donor perfusate).

Both approaches can be used to evaluate placental drug transfer, but the major difference between them is that the single-pass approach reaches steady-state quicker than the recirculating approach, however the difference in time to steady state is reduced for higher permeability compounds and smaller reservoirs.[114] Also, potentially toxic cellular metabolic waste products build up in the re-circulated perfusate while they do not in the singe-pass perfusate. For the re-circulation approach (but not the single-pass approach), the *ex vivo* fetal/maternal concentration ratio (provided steady-state has been reached) can be directly compared to the *in vivo* $K_{p,uu}$. If steady-state has not been reached or if the single-pass approach is used, the unbound drug clearance ratio (maternal-fetal/fetal-maternal) needs to be estimated to translate to *in vivo* $K_{p,uu,IVIVE}$ (see below). Thus, the single-pass method requires both maternal-fetal and fetal-maternal perfusions to estimate *in vivo* $K_{p,uu}$ while the re-circulation approach needs perfusion in only one direction, but to steady state. To apply the above approaches to estimate the *in vivo* $K_{p,uu}$, fetal CL would have to be negligible.

Maternal-fetal drug clearance can be estimated when drug is introduced into the maternal perfusate and collected from the fetal venous outflow, and vice versa for the fetal-maternal clearance estimation (*i.e.* drug is introduced into the fetal perfusate and collected from maternal venous outflow).[115] To estimate clearance in both directions, two individual cotyledons must be perfused, one for each clearance estimation. The clearance values estimated from the model can be scaled to the whole placenta, either by scaling the average number of cotyledons in the placenta or by scaling the cotyledon weight or volume to the total placental weight or volume. However, in using this scaling approach, several assumptions are made: 1) the abundance and activity of the transporters and enzymes in the perfused placentae are identical to that *in vivo;* 2) the entire cotyledon is perfused *ex vivo* as it is *in vivo*. The latter assumption is doubtful because in placental perfusion experiments this is usually not the case; part of the cotyledon is not perfused to prevent leakage. Also, because only one fetal artery is cannulated, it is unlikely that the perfused surface area is the same in both directions, maternal-to-fetal and fetal-to-maternal. There will also be inter-cotyledon variability in the surface area perfused. All these factors will likely result in mis-prediction of the i*n vivo* drug placental CL. To determine the contribution of transporters or enzymes in drug transfer, the perfusions can be conducted in the absence and presence of a selective inhibitor of the transporter or enzyme.

It is important to note that perfused placenta can provide an estimate of $K_{p,uu}$ (provided fetal clearance of drug is negligible) but to predict the dynamic fetal drug exposure, it needs to be combined with a m-f PBPK model which must be populated with the $CL_{MP,}$ CL_{p0} and CL_{PM}. This combined approach has been employed to dynamically predict fetal exposure to drugs that passively cross the placenta (*e.g.* acetaminophen[116], dolutegravir[117], sildenafil[118]) and are possibly metabolized in the placenta (*e.g.* tenofovir and emtricitabine)[63]. This approach has also been used to successfully predict fetal exposure to a drug (darunavir) that is actively transported by the placenta.[119] While the authors attributed the higher fetal to maternal clearance vs. maternal to fetal clearance of darunavir to a difference in the physiological blood flows, we believe the difference is due to active P-gp efflux of the drug. As to whether the perfused placenta, combined with PBPK M&S, can predict fetal drug exposure to a wider array of drugs that are transported by the placenta is yet to be determined. It should be noted here that it is not necessary to conduct perfused placenta studies to predict fetal exposure to drugs that passively cross the placenta as such dynamic predictions can be made without conducting these costly and challenging studies (see Zhang et al. 2017)[6].

The overall merits and limitations of the above two approaches (ER-REF and perfused placenta) combined with m-f PBPK M&S are provided in Table 3.

Expert opinion on future directions and overall conclusions

There are multiple knowledge gaps in our ability to predict fetal exposure to drugs. First, fetal physiological data for early gestation (<GW9) are incomplete and therefore the present m-f PBPK models cannot be used to predict fetal exposure to drugs prior to GW9. Second, it is impossible to validate fetal drug exposure (systemic or tissue) predictions earlier in gestation because prenatal fetal sampling is not ethical or logistically possible. Third, there is limited information about the ontogeny of non-CYP enzymes in the placenta and the fetal liver. Likewise, for the ontogeny of enzymes and transporters in fetal tissues. Fourth, the ER-REF-PBPK approach is validated for only for P-gp substrates and has yet to be validated for other placental transporters. Fifth, fetal drug exposure estimated by m-f PBPK models at term has been validated for only a limited number of P-gp substate drugs due to paucity of the necessary UV/MP data for such drugs. For the same reasons, fetal tissue drug concentration prediction by the m-f PBPK models cannot be validated. Sixth, the current m-f PBPK models do not incorporate potential absorption of drugs from the amniotic fluid through the skin, which could be important earlier in gestation when the fetal skin is highly permeable.[120]

It is important to emphasize that, besides factors listed above, fetal drug exposure is also driven by maternal exposure. Therefore, the absolute magnitude of fetal drug exposure cannot be predicted without accurately predicting maternal drug exposure. This can be done using our m-f PBPK model as we have previously shown[6, 8–11, 79, 90, 91], if relevant data on transporter and enzyme abundance are available for both healthy pregnant women and those with diseases (*e.g.* hepatic impairment). In this regard, the impact of pregnancy on the abundance and activity of non-CYP enzymes (such as UGTs, SULTs, carboxylesterases) and hepatic transporters (such as SLC and ABC transporters) are lacking for both healthy

pregnant women and those with diseases. Moreover, such data for CYPs are limited to a few isoforms (*e.g.,* CYP1A2, CYP2D6 and CYP3A4) and, where available, they are available mostly for the 3rd trimester (healthy pregnant women only).[121, 122] The effect of pregnancy on activity or abundance of metabolic enzymes and transporters in non-hepatic tissues of pregnant women such as intestine, are not available. Such data are particularly important for drugs that are metabolized or transported in the intestine such as CYP3A and BCRP. In contrast, data are available about the effect of pregnancy on renal transporters.[123–125] Thus, targeted enzyme and transporter probe drug studies in pregnant women (at all gestational ages) are needed to fill these knowledge gaps to predict both maternal and fetal drug exposure without conducting *in vivo* studies for every drug administered to pregnant women.

Despite the above gaps, there has been enormous progress in predicting maternal-fetal drug exposure. Mechanistic m-f PBPK models have been developed, populated with much of the relevant physiological data. These model, together with newly devised or refined approaches (*e.g.* ER-REF), have successfully predicted dynamic fetal exposure to drugs that cross the placenta passively[79] and actively[90, 91]. The next frontier in this area of research is to predict and validate fetal tissue drug exposure. We have already shown that the ER-REF approach is successful in predicting human adult brain and liver drug concentrations where the drugs are actively transported across the tissue:blood barrier.[126, 127] Thus, there is every reason to believe that this approach will be successful in predicting fetal tissue drug exposure provided the relevant data on the ontogeny of transporters and enzymes in these tissues are available.

Data Sharing

Since this is a review paper, there are no original data to share.

Funding statement

This work was supported by the National Institutes of Health grant P01DA032507 (JDU), the Bill and Melinda Gates foundation grant INV-006678 (AB, JDU), and the National Institutes of Health grant TL1TR002318 (ARK).

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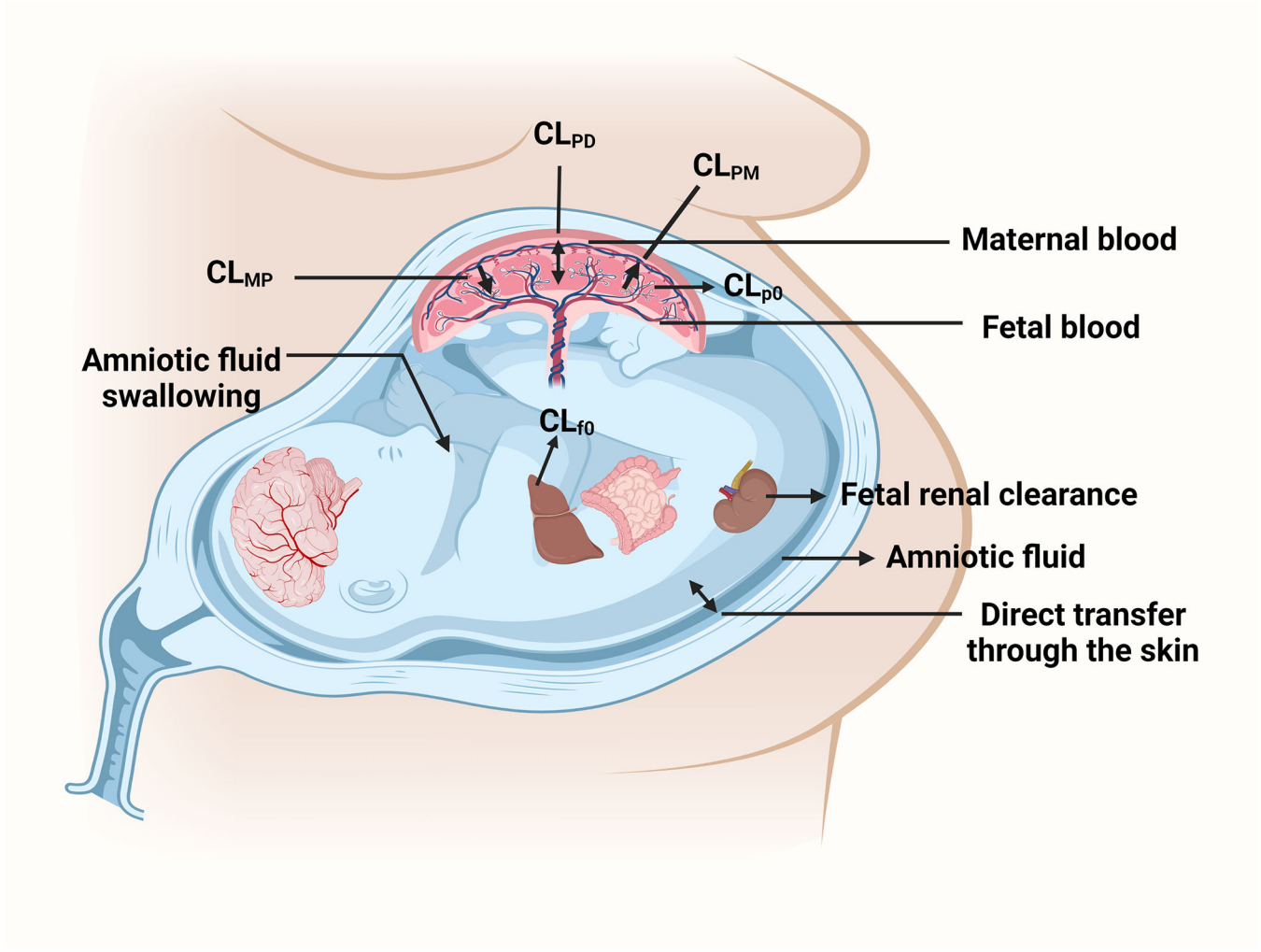

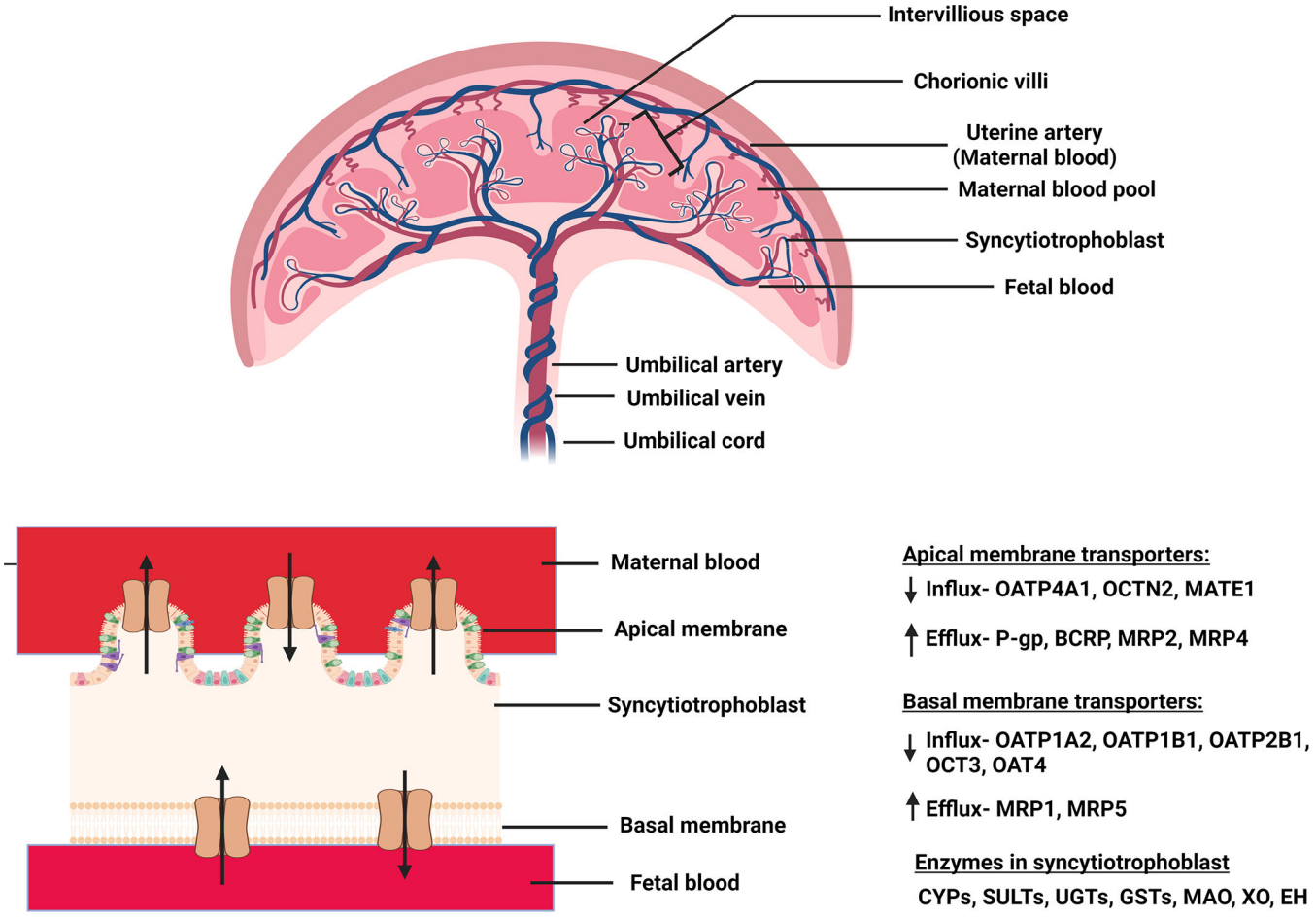

Figure 1:

(a) Routes by which drugs distribute into or are cleared from the fetal compartment. (b) Expanded view of the placenta with magnification of the syncytiotrophoblast layer and examples of transporters and enzyme expressed there. Abbreviations: CL_{PD}: passive bi-directional placental clearance; CL_{MP}: transporter-mediated influx clearance; CL_{PM}: transporter-mediated efflux clearance; CL_{p0}: placental metabolic clearance; CL_{f0}: fetal metabolic clearance; CYPs: cytochrome P450s (CYPs); UGTs: UDP-glucuronosyltransferases; SULTs: sulfotransferases, GSTs: glutathione-S-transferases; MAO: monoamine oxidase; XO: xanthine oxidase; EH: epoxide hydrolase. Details of other transporters and enzymes present in the placenta are provided in Table 1.

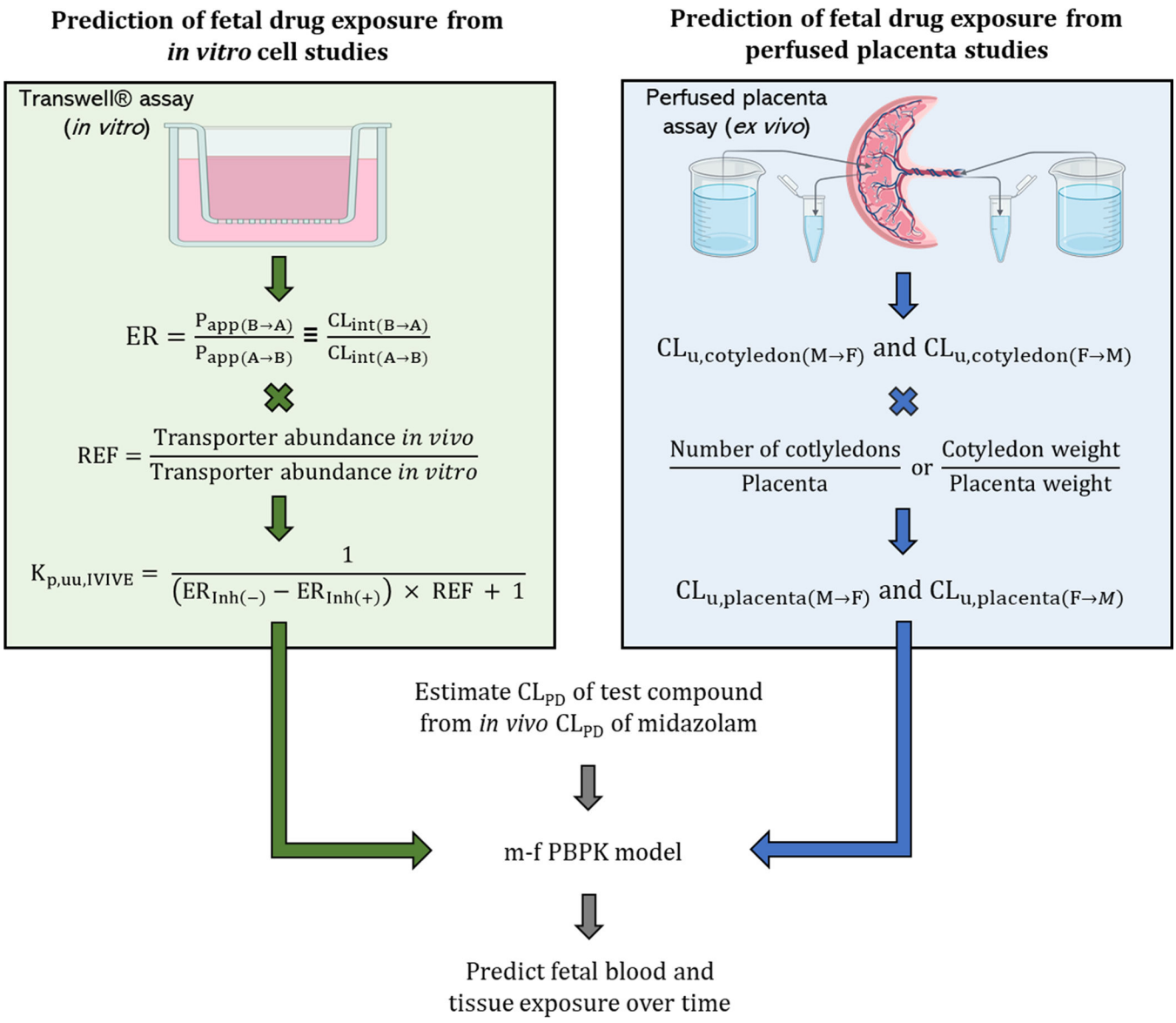

Figure 2:

Prediction of fetal drug exposure using the m-f-PBPK approach combined with data from (a) the ER-REF studies (left panel) or (b) the perfused placenta studies (right panel). Although the perfused placenta approach is shown in a single-pass mode, the re-circulation mode could also be used. CL_u - unbound clearance; $CL_{int(B\rightarrow A)}$ and $CL_{int(A\rightarrow B)}$ - intrinsic drug clearance in the B→A and the A→B direction, respectively; ER - efflux ratio; $P_{app(B\rightarrow A)}$ and $P_{app(A\rightarrow B)}$ - apparent drug permeability in the B→A and the A→B direction, respectively; F→M - fetal to maternal; M→F - maternal to fetal; REF - relative expression factor.

Table 1:

Drug transporters expressed in the human placenta

Name of transporter*	Gene/ Alternative name	Technique used for detection/Quantification			Localization in the syncytiotrophoblast	Transporter type	Direction of net transport	Potential effect on fetal drug exposure	Reference
		First trimester	Second trimester	Third trimester/term					
P-gp +	ABCB1, MDR1	R, P	R, P	R, P, A	Apical membrane	Efflux	Fetal to maternal	Reduced	21, 22, 37–40, 128–131
BCRP +	ABCG2, MXR	R, P	R, P	R, P, A	Apical membrane	Efflux	Fetal to maternal	Reduced	21, 22, 37, 38, 41, 128–130, 132
MRP1 #	ABCC1	R	NA	R, P, A	Basal membrane	Efflux	Maternal to fetal	Enhanced	37, 46, 132–135
MRP2 ^	ABCC2, CMRP, CMOAT	R, P	R, P	R, P, A	Apical membrane	Efflux	Fetal to maternal	Reduced	37, 46, 47, 129–131, 135–137
MRP3 #	ABCC3	R	NA	R, P, A	Apical membrane	Efflux	Fetal to maternal	Reduced	37, 46, 135–137
MRP4	ABCC4	NA	NA	R, P	Apical membrane	Efflux	Fetal to maternal	Reduced	137
MRP5	ABCC5	R	R	R, P	Basal membrane	Efflux	Maternal to fetal	Enhanced	46, 138
OATP1A2 +	SLCO1A2, SLC21A3, OATP-A	R, P	NA	R, P, A	Basal membrane	Influx	Fetal to maternal	Reduced	43, 129, 139, 140
OATP1B1 +	SLCO1B1, OATP-C, LST1	R	NA	NA	Basal membrane	Influx	Fetal to maternal	Reduced	43, 140
OATP2B1 +	SLC21A9, OATP-B	P	P	R, P, A	Basal membrane	Influx	Fetal to maternal	Reduced	22, 42, 141
OATP4A1#	SLC21A12, OATP1, OATP-E	R, P	NA	R, P	Apical membrane	Influx	Maternal to fetal	Enhanced	43, 139
OCT3 ^	SLC22A3, EMT	R, P	R, P	R, P, A	Basal membrane	Influx	Fetal to maternal	Reduced	22, 48, 49, 142–144
OCTN2 +	SLC22A5	NA	NA	R, P, A	Apical membrane	Influx	Maternal to fetal	Enhanced	44, 45, 145
OAT4^	SLC22A9, SLC22A11	R, P	R, P	R, P	Basal membrane	Influx	Fetal to maternal	Reduced	22, 141, 146, 147

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Name of transporter*	Gene/ Alternative name	Technique used for detection/Quantification			Localization in the syncytiotrophoblast	Transporter type	Direction of net transport	Potential effect on fetal drug exposure	Reference
MATE1#	SLC47A1	R	NA	R	Apical	Influx	Maternal to fetal	Enhanced	48, 49

R = mRNA; P = protein; A = activity measured using the perfused human placenta; NA = not applicable

* - bolded transporters are those that are likely to be functionally most important in transplacental transfer of drugs

- no change in expression/abundance with gestational age, per mg protein

^ - increase in expression/abundance with gestational age, per mg protein

+ - decrease in expression/abundance with gestational age, per mg protein.

Transporters with no symbol indicate that the data are not available. Reference 22 contains quantitative information for transporters.

Table 2:

Drug metabolizing enzymes expressed in the human placenta and fetal liver

Enzyme name	Placenta				Fetal liver			
	First trimester	Second trimester	Third trimester/ term	Reference	First trimester	Second trimester	Third trimester/term	Reference
CYP1A1 +	**R, P, A**	**ND**	**R, P, A**	64, 148–150	R, P, A	ND	ND	151
CYP1A2	R	ND	P	148, 150	ND	ND	ND	
CYP1B1	ND	ND	R	149	ND	ND	ND	
CYP2C8/9	P, R	ND	P	148, 152	ND	R, A	R, A	153
CYP2D6	R	ND	R	148, 149	ND	R	ND	154
CYP2E1	R	ND	R	64, 148, 149	ND	ND	ND	
CYP2J2	P	ND	R, P	149, 152	ND	ND	R	149
CYP3A4	R	ND	R	64, 148	ND	R	ND	154
CYP3A5	R	ND	R	64, 148	R, A	R, A	R, A	149, 155
CYP3A7 #	R	ND	ND	148	**P, A**	**R, P, A**	**R**	82, 149, 156
CYP19	**A**	**ND**	**A**	157, 158	A	ND	ND	157
SULT1A1	A, P	A, P	A, P	159	A	R, P, A	A	71, 72, 160, 161
SULT1A2	ND	ND	ND		ND	R	ND	72
SULT1A3	A, P	A, P	A, P	159	A	R, P, A	A	71, 72, 160
SULT1B1	ND	ND	ND		ND	R	ND	
SULT1C2	ND	ND	ND		ND	R, P	ND	72, 161
SULT1C4	ND	ND	ND		ND	R	ND	161
SULT1E1	A	A	A	159	ND	R, P, A	ND	72, 161
SULT2A1	A	ND	A	159	ND	R, A	ND	72, 161
SULT2B1	A	ND	R, A	159, 162	ND	R	ND	161
UGT1A1	ND	ND	R, P, A	163	ND	R	R	164
UGT1A4	ND	ND	**R, P, A**	163	ND	R	R	164
UGT1A6	ND	ND	**R, P, A**	163	ND	R	R	164
UGT1A9	ND	ND	R, P, A	163	ND	R	R	164

Enzyme name	Placenta				Fetal liver			
UGT2A2	ND	ND	ND		ND	R	R	164
UGT2A3	ND	ND	ND		ND	R	R	164
UGT2B4	P	ND	R, P,	65, 67		R	R	164
UGT2B7	P	ND	R, P	65, 67	R	R	R	164, 165
UGT2B10	ND	ND	R, A	65	ND	R	R	164
UGT2B11	ND	ND	R, A	65	ND	R	R	164
UGT2B15	ND	ND	R, A	65	R	R, P	R, P	164–166
UGT2B17	ND	ND	ND		R	R	R	164, 165
GST	ND	ND	R, A	167	ND	A	P	168, 169
MAO	ND	ND	**R, P, A**	170–172	ND	P, A	ND	173
X ^	ND	ND	**R, P, A**	174, 175	**A**	**A**	**A**	176
PCES1 – 3	ND	ND	R, A	177	ND	ND	ND	
CES	ND	ND	ND		ND	A	ND	178
Peptidases	P	ND	P	179	ND	ND	ND	
Epoxide hydrolase	R, P	R, P, A	R, P	152, 180, 181	ND	A	ND	181

R = mRNA; P = protein; A = activity was measured in microsomes, S-9, cytosol or homogenates of placental or fetal liver tissue; ND = not determined

* - bolded enzymes are those that are likely to be functionally most important in the metabolism of drugs

- no change in activity/expression with gestational age, per mg protein

^ - increase in activity/expression with gestational age, per mg protein

+ - decrease in activity/expression with gestational age, per mg protein.

Enzymes with no symbol indicate that the data are not available.

Table 3:

Merits and limitations of various approaches that can be used to estimate fetal drug exposure

	Merits	Limitations
Animal models		
	• Allows study of placental transfer and fetal toxicity of drugs at different gestational ages (limited gestational age range for small animals) • Rodents can be genetically modified to study the role of specific transporters in fetal distribution of drugs • Lower interindividual variation due to inbred animals (small animals only); pooling samples from multiple dams/fetuses is feasible • In large animals, both maternal and fetal plasma drug concentration-time profiles can be determined in the same animal	• Interspecies variability in placental anatomy and transporters, placental and fetal metabolism, substrate selectivity of placental/fetal drug metabolizing enzymes and transporters and abundance of placental/fetal drug metabolizing enzymes and transporters limits extrapolation to humans • Accurate extrapolation from animal models to humans has not been demonstrated • For small animals, due to the small size of the pups, blood samples from pups cannot be obtained. Instead, drug exposure in the entire fetus is determined by pooling multiple pups to generate the AUC of the total fetal drug concentration-time profile. These profiles are then compared with a plasma AUC profile generated in the dam by pooling plasma concentrations obtained by sacrificing the dam at different time points. This is costly and has the potential to increase variability in the data • For small animals, due to size, fetal tissue drug concentration cannot be determined except perhaps near term
m-f PBPK modeling *		
	• Dynamic profiling of fetal drug exposure is possible at term as well as early in gestation (> GW 9) • Prediction of fetal tissue drug exposure is theoretically possible provided the necessary data on fetal tissue enzyme and transporter activity or abundance are available • Can be used to predict fetal exposure to drugs that are effluxed by placental P-gp using the ER-REF or potentially the perfused placenta approach • Can be used to predict fetal exposure to drugs that passively cross the placenta without the need to use either the ER-REF or the perfused placental approach • Can be used to predict the effect of intrinsic (*e.g.* transporter/metabolic enzyme genetic polymorphisms, disease conditions) and extrinsic factors (*e.g.* smoking, drug interactions) on fetal drug exposure	• The limited availability of *in vivo* data for maternal-fetal exposure to probe drugs that are selectively transported or metabolized by a single transporter or enzyme precludes model validation for such drugs (*e.g.* those transported solely by BCRP). • As pre-term fetal sampling is not possible, prediction of fetal exposure at gestational ages other than at term cannot be validated • Likewise, prediction of fetal tissue drug exposure cannot be validated due to ethical and logistical constraints in obtaining such data • Lack of data on quantitative contribution and ontogeny of other routes of fetal drug exposure such as direct transfer of drug from the placenta to the amniotic fluid or transfer of drug from the amniotic fluid through the permeable fetal skin may affect model predictions • Lack of mechanistic and quantitative data on major factors that affect maternal drug exposure (and therefore fetal drug exposure) earlier in gestation (such as gestational age dependent changes in transporters, CYPs, UGT and non-CYP metabolizing enzymes) precludes prediction of maternal-fetal exposure to drugs transported or metabolized by these proteins • Lack of data on tissue specific changes in abundance and/or activity in transporters and enzymes in the maternal compartment (*e.g.* intestine) precludes prediction of maternal-fetal exposure to drugs that are transported or metabolized in these tissues
ER-REF approach	• Independent of magnitude of passive diffusion clearance in the *in vitro* system • Not constrained by high degree of technical expertise, limited cell viability and throughput • Approach is validated for P-gp substrates (using MDCKII transfected cell lines) but needs to be validated for other important transporters present in the placenta (*e.g.* BCRP) • Fraction transport (ft) by a specific transporter can be estimated which can be used to predict the effect of drug-drug interactions, transporter/metabolic enzyme genetic polymorphism and diseases that could modulate fetal drug exposure	• Assumes that all quantified transporters are present on the membrane and functional. Otherwise, abundance of transporters should be determined in the cell membrane (*e.g.* by biotinylation). • Identity of transporter(s) involved in the placental transfer of drug is required • Transporter and metabolizing enzyme abundance *in vitro* and *in vivo* should be available • Does not provide absolute measure of passive diffusion clearance (requires calibration with midazolam data) • Approach is validated only for P-gp substrates but not for other transporters

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	Merits	Limitations
	• Allows dynamic prediction of fetal exposure to drugs at earlier gestational ages by accounting for the ontogeny of placental transporter abundance and change in passive diffusion clearance due to size. To do so, ft, at term, via each transporter must be known *Placental cell lines* • More physiologically relevant • Useful to estimate placental transfer (or transport if transport activity is not low) and pharmacologic response to drugs • BeWo b30 cells can form cellular monolayers with tight junctions and hence can be used for flux studies provided transporter functional activity is significant (though it is problematic to establish and maintain the cells) • Can be used as an initial screen of placental transport to identify the transporters potentially relevant *in vivo* (provided selective inhibitors are available) *Transporter overexpressing cell lines (e.g.MDCKII)* • Forms tight junctions • Can overexpress the major human placental transporters (P-gp or BCRP), and allows easy detection of transporter activity *in vitro* • Can be used to predict placental transport of drugs *in vivo* that are substrates of multiple transporters	*Placental cell lines* • Does not represent the complex physiology of the placenta and lacks secondary cell populations (*e.g.*, endothelial cells) where transport can occur • Limited transporter expression decreases the sensitivity of the analysis to identify relevant drug transporter • Quantitative accuracy in prediction of *in vivo* transplacental clearance of drugs has not been validated • Difficult to predict transplacental transport of drugs that are substrates of multiple transporters unless a selective inhibitor of each transporter is available *Transporter overexpressing cell lines (e.g.MDCKII)* • Does not represent the complex physiology of the placenta and lacks secondary cell populations (*e.g.*, endothelial cells) where transport can occur • Requires studying each transporter separately to estimate the *in vivo* ft via a given transporter
Placental perfusion approach	• Most closely resembles the *in vivo* situation as it incorporates flow and retains placental anatomy • Allows simultaneous study of multiple PK parameters such as passive diffusion, active transport, metabolism, and tissue binding • Inter-individual variability in transplacental clearance can be determined • Does not require prior knowledge about the transporters involved in transplacental transfer • Does not require the assumption that all transporters are on the membrane and functional, as abundance is not quantified • Does not require REF for scaling and hence is free of the assumption that activity is proportional to abundance • Can be used to quantify placental drug accumulation	• Can determine drug transfer ONLY close to or at term and not at earlier gestational ages (i.e. trimester 1 and 2) • Ability to accurately predict fetal exposure to drugs that are transported by the placenta has not been thoroughly validated • Requires special equipment and technical expertise • Low throughput • The maximum perfusion duration is six hours, after which the tissue is no longer viable, which can pose an issue if insufficient drug has appeared in the receiver compartment within 6 hours • No standardized placenta viability criteria have been established • ft via individual transporters can be determined only if selective inhibitors are available.

* - Merits and limitations of just the m-f PBPK modeling approach are listed here. Those of the ER-REF and the placental perfusion approach are listed later in the table followed by the merits and limitations specific to the placental and transporter overexpressing cell lines