Non-clinical Models to Determine Drug Passage into Human Breast Milk

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Running title: Non-clinical models for drug milk excretion.

Abstract

Background: Successful practice of clinical perinatal pharmacology requires a thorough understanding of the pronounced physiological changes during lactation and how these changes affect various drug disposition processes. In addition, pharmacokinetic processes unique to lactation have remained understudied. Hence, determination of drug disposition mechanisms in lactating women and their babies remains a domain with important knowledge gaps. Indeed, lack of data regarding infant risk during breastfeeding far too often results in discontinuation of breastfeeding and subsequent loss of all the associated benefits to the breastfed infant. In the absence of age-specific toxicity data, human lactation data alone are considered insufficient to rapidly generate the required evidence regarding risks associated with medication use during lactation.

Objective: To provide a state-of-the art regarding the non-clinical approaches that have been developed to explore the mechanisms underlying drug milk excretion.

Results: Several studies have reported methods to predict (to some extent) milk drug excretion rates based on physicochemical properties of the compounds. *In vitro* studies with primary mammary epithelial cells appear excellent approaches to determine transepithelial drug transport rates across the mammary epithelium. Several of these *in vitro* tools have been characterized in terms of transporter expression and activity as compared to mammary gland tissue. In addition, with the advent of physiology-based pharmacokinetic (PBPK) modelling, these *in vitro* transport data may prove instrumental in predicting drug milk concentration time profiles prior to availability of data from clinical lactation studies. *In vivo* studies in lactating animals have proven their utility in elucidating the mechanisms underlying drug milk excretion.

Conclusion: By combining various non-clinical tools (physicochemistry-based, *in vitro* and PBPK, *in vivo* animal) for drug milk excretion, valuable and unique information regarding drug milk concentrations during lactation can be obtained.

Key words

pharmacokinetics, lactation, breast milk, milk/plasma ratio, mammary epithelial cells, animal model, physiology-based pharmacokinetic modelling, relative infant dose.

Introduction

In recent years major efforts throughout Europe have aimed at increasing the rate of breastfeeding initiation as well to stimulate longer breastfeeding duration. These efforts are based on the overwhelming evidence regarding the multiple beneficial effects of breastfeeding on maternal and neonatal health [1]. Importantly these effects are not only short term. For instance, breastfeeding has been shown to substantially reduce the risk for development of diabetes mellitus and obesity at later age. However, the European statistics on (exclusive) breastfeeding practices are far from compliant with WHO guidelines [1]. Moreover, as recently reported in an editorial by Persson [2], the negative societal impact of non-breastfeeding appears largely underestimated by governments; at least one government referred to breastfeeding as a 'small matter', presumably in relation to 'more important' aspects in their perspective, such as for instance commercial interests driven by promotion of formula milk. However, as reported by Rollins et al. [3], global annual losses of about 300 billion $ have been calculated to be the result of cognitive deficits in relation to current lack of adherence to breastfeeding.

Several examples exist of drugs that are considered safe during breast feeding (see LactMed® database [4]). For instance, paracetamol can be safely used by nursing mothers as evidence from multiple independent studies shows that milk paracetamol levels at maternal therapeutic doses are very low and never exceed 5% of the maternal dose after normalizing for the infant body weight. In the case of the antibiotic ampicillin, milk levels also remain very low: for a maternal treatment regimen of 1.5-2 g/day, infant doses were estimated to be as low as 0.08-0.2 mg/day. Nevertheless, it is a confronting reality that for many other drugs used in lactating mothers it is currently still close to impossible to make evidence-based decisions on the risks associated with breastfeeding during medication use. The

implications of this reality are that breastfeeding women do not have access to life-saving medication and/or breastfed children do not benefit from breastfeeding when their mothers are prescribed medication for which data are lacking. This information gap is at least in part caused by a limited insight into the mechanisms governing drug transfer rates into human breast milk and the subsequent exposure of the infant. While physicochemical drug properties may provide a rough estimation of blood/milk partitioning kinetics for a given xenobiotic, the involvement of multiple underlying processes including milk protein binding, breast tissue protein binding, plasma protein binding and transporter-mediated excretion, complicates the complete understanding and hence reliable prediction for many drugs. Moreover, the impact of physiological, genetic and environmental covariates on those drug individual disposition processes has not been fully and/or systematically investigated. Besides the prediction of mean drug transfer rates into human breast milk, also the prediction of population variability in drug transfer rates (including identification of major covariates) poses a major risk. Importantly, the ultimate risk assessment on the breastfed child also urgently calls for a much more quantitative, mechanistic and integrated understanding of the factors determining systemic exposure in infants subsequent to oral intake of maternal medication excreted into human breast milk. In this context, integration or generation of information on the influence of maturational processes on intestinal drug absorption especially in neonates and young infants (up to 6 months) is needed.

Based on this, the importance of more systematic and reliable data generation on drug disposition during breastfeeding can hardly be overestimated. Indeed, in the FDA-sponsored workshop on medication use during lactation held in April 2016 [5], it was recognized that decisions regarding safety of medicines used during lactation should be based not only on human lactation data, but also on all other available data such as: physicochemical

properties of the drugs, drug excretion mechanisms, covariates at the level of the infant. Clearly, non-clinical models are expected to represent a unique asset in unraveling drug milk excretion mechanisms, even though extrapolation of *in vitro* data or animal *in vivo* findings to the clinical context remains a specific point of attention. Nevertheless, combining non-clinical data with PBPK modelling and simulation [6], is emerging as a powerful strategy to make reliable and quantitative predictions of *in vivo* drug behavior. Such a strategy has for instance been applied repeatedly for quantitative prediction of numerous drug-drug interactions ([7-16]) or drug disposition in specific populations (e.g. pediatrics) [17-22]. This aim of this mini-review is to provide a state-of the art summary regarding the non-clinical methods that can be applied to gain a better mechanistic understanding of the pharmacokinetic process that is unique to the lactation state: blood-milk drug excretion. Clearly, also maternal ADME processes in general may be influenced by the physiological changes due to pregnancy and lactation, however this was beyond the scope of this manuscript and has been reviewed elsewhere for instance in the context of psychotropic medication [23].

Anatomy, physiology and cell biology of the mammary gland in relation to drug milk excretion

Based on histological examinations, adipose and glandular tissue represent 1:1 or 1:2 ratios of the human breast tissue mass in non-lactating and lactating state, respectively. Interestingly, milk production does not seem to depend on the number or size of ducts nor on the relative presence of glandular or fat tissue. Human milk is produced by the mammary (secretory) epithelial cells (or lactocytes) that line the mammary alveoli (small cavities) [24]. These alveoli are clustering in breast lobules, of which 15-20 are present in a human breast. Milk produced and stored by the alveoli is drained towards the nipple surface via milk ducts. Based on ultrasound imaging, Ramsay et al. [25] reported the presence of an average nine milk ducts (range 4-18) in the human breast, confirming reports of other investigators. The same study revealed milk duct diameters ranging between 1 and 4.4 mm. Transport rather than storage is the main function of the milk ducts.

The cell biological and physiological aspects of milk secretion have been described in a review by McManaman et al. [24], referring to 4 transcellular and 1 paracellular route responsible for secreting lipids, proteins, nutrients and water across the mammary epithelial cells into milk. The exocytotic route mediates secretion of water-soluble milk components including milk proteins (e.g. casein), lactose and Ca^{++}. The second route represents milk fat secretion as membrane bound fat globules (2-3 µm) that are also metabolically active (e.g. xanthine oxidoreductase). Vesicular transcytosis has been described as a third route and is responsible for secretion of immunoglobulins, transferrins and prolactin in milk. A fourth route is supported by the presence of transporters (e.g. for glucose), pores and ion channels in both membrane domains of the lactocyte. The paracellular channels between mammary epithelial cells are a fifth pathway. During lactation there appears to be a more pronounced

restriction of this pathway by tight junctional complexes, thus limiting this transport route to the movement of small hydrophilic compounds. Medicines can be expected to rely on one or more of these routes when appearing in milk subsequent to their presence in plasma. In addition, based on findings in other epithelia, passive membrane permeation is likely another important drug transport mechanism, especially for compounds that are relatively lipophilic. Less lipophilic drugs will also share transporters with endogenous compounds. Once present in milk, drugs may bind to proteins and/or lipids that constitute major fractions of milk composition [26].

A first detailed review on expression of transporters in the epithelial cells of the mammary gland was published about 15 years ago by Ito and Alcorn [27]: this compilation already included organic cation transporters (OCT), peptide transporters and nucleoside transporters. In addition, also members of the ATP-binding cassette (ABC) superfamily of transporters had been detected. Transporters displaying the highest expression in these isolated cells included: OCT1, OCTN1/2, SLCO2B1 (formerly “OATPB”), SLCO3A1 (formerly “OATPD”), PEPT2 and SVCT. In addition, OCTN1 and PEPT2 showed remarkable upregulation in lactating state. Meanwhile, specifically Breast Cancer Resistance Protein (BCRP/*ABCG2*) has been demonstrated to show high expression in mammary glands of human, bovine, mouse and pig (see Table 2). More recently, BCRP function was verified *in vivo* since it was found to mediate the transfer of several drugs into milk of dairy cows [28]. Table 2 provides an updated overview of mRNA/protein expression as well as activity of drug transporters described for mammary tissue and derived cell lines of various species. Besides the presence of typical drug transporters, also vitamin transporters such as sodium vitamin C (ascorbate) transporter (SVCT) and riboflavin transporter (RFVT) have been described in the mammary epithelium, with increased levels during lactation.

Relevant PK parameters in the context of drug milk excretion

The (PK) parameters relevant to medication use during lactation are listed in Table 1. The relative infant dose (RID) has become a widely used parameter to provide an assessment about the safety of a particular maternal drug for a nursing infant. RID reveals a relative measure of the dose the infant is exposed to. Dependent on the source, a 10% or even 5% is used as cut-off above which a significant risk should be considered. The major disadvantage of RID is the arbitrary character of the cut-off. RID should not be calculated for drugs that are also used in infants. In this case the infant daily dosage via milk (see Table 1) is compared directly to the standard neonatal or infant dose.

The milk to plasma (M/P) ratio represents the milk concentration relative to the plasma concentration. Importantly, this M/P ratio can be extremely time-dependent, and the intrinsic variability of the ratio will be very high for drugs that: (i) have a short plasma half-life; (ii) distribute extensively to milk; and (iii) are primarily distributing to the red blood cells. The M/P point ratio is only 'robust' when considering trough concentrations for a drug with a long half-life. For these reasons the M/P ratio should reflect the ratio of AUC's in milk and plasma to be useful. Some groups have developed models (pH partition and phase distribution) to predict the M/P ratio based on physicochemical parameters (e.g. log P, pKa), but these models can be considered superseded by the (semi-)mechanistic and PBPK models described below. As reviewed in details by Anderson and Sauberan [6], numerous studies have developed Quantitative Structure-Property Relationship (QSPR) models to predict the M/P ratio of a given drug candidate based on molecular/chemical properties of compounds.

The Exposure Index (EI) also considers the eliminating capacity of the infant on top of the dose. The latter is obtained by multiplying the daily milk intake (10 mL/min/kg) with the M/P ratio. As for RID, a 10% cut-off is also used for decision making based on EI.

In silico models based on physicochemistry

Although the physicochemical properties of active pharmaceutical ingredients (APIs) in medication can be (and have been) used to determine the extent of drug transfer to breast milk, even qualitative prediction of this process based on physicochemistry alone is difficult to justify due to ignorance of: (i) transporter-mediated processes of drug excretion; (ii) the intrinsic pharmacodynamic / toxicodynamic profile of the API. In relation to the latter, a suggestion made at the FDA 2016 workshop summary on medication use during lactation was to consider data obtained in developmental toxicity studies in an attempt to provide guidance on the infant risk associated with low Relative Infant Dose (RID) levels. In terms for predicting the role of individual transporters in vectorial transport of drug substrates, QSPR models will certainly contribute to future strategies enabling prediction of transport rates across mammary epithelial cells [6]. However, this will require integration of these QSPR models in mechanistic models for drug disposition at the cellular level, and this represents an active domain of research requiring further development time.

Nevertheless, as illustrated by Koshimichi et al. (see below) [29], integration of physicochemical parameters within a mechanistic model is one way forward to improve future predictions of drug milk excretion relying at least partly on this type of drug-specific information such as MW and lipophilicity (log P). It is not surprising that physicochemical drug properties do correlate substantially with drug milk excretion in view of: (i) passive membrane permeation representing a major transepithelial transport route at the

mammary gland ; (ii) the pronounced impact of physiological properties such as the acidic pH of the milk, promoting the excretion of basic (and at least moderately lipophilic) drugs from blood as explained by the pH partition theory (also referred to as ion trapping) [6].

In vitro models to determine transport of drugs across mammary epithelial cells

Relying on the hypothesis that the mammary epithelium represents the single most important barrier (or gate) for drug excretion from blood into breast milk, the development and application of *in vitro* models relying on cultured mammary epithelial cells implicitly carry a significant promise in terms of bio-relevance and *in vivo* predictivity, while at the same time controlling model complexity. Indeed, the validity of such an approach has been shown extensively for many other epithelial or endothelial barriers throughout the human body (intestinal mucosa, renal tubules, blood-brain barrier, placenta). For instance, the Caco-2 model [30-33], mimicking the intestinal epithelial barrier of (primarily) enterocytes, is probably the single most widely used *in vitro* model for studying drug transport rates and mechanisms. The Caco-2 model, despite its limitations (e.g. no CYP3A4 expression, cancerous phenotype, lack of mucus production), but due to its reliability, has long been accepted as a robust *in vitro* model supporting decisions regarding the oral absorption potential of drug candidates.

In 2006, Kimura et al. [34] were the first to describe the development of a human culture model with mammary epithelial cells. The purpose of their study was to characterize and qualify this cell culture system for studying drug transfer between blood and milk including transporter-mediated processes (a feature that had been remarkably lacking in physicochemistry-based models for drug transfer during lactation). Kimura and co-workers applied a cell-culture technique that encompassed making normal human mammary

epithelial cells (HMEC) trypsin-resistant before growing them on Matrigel®-coated inserts for conducting drug transport studies. The advantage of this approach was that the most trypsin-resistant monolayers showed a tighter epithelial phenotype as evidenced by TransEpithelial Electrical Resistance (TEER) values of around 200 Ohm.cm^2, which is comparable with TEER measured across many other common *in vitro* epithelial cell models (e.g. Caco-2, Madin-Darby Canine Kidney - MDCK) [35]. As opposed to non-trypsin resistant HMECs, trypsin resistance also associated with detectable mRNA levels of β-casein, which was considered consistent with differentiation of these cells to a 'lactating state' phenotype. The HMEC also displayed bidirectional transport of the model anion *p*-aminohippurate (PAH) and the model cation tetraethyl ammonium (TEA), established substrates of the Organic Anion / Organic Cation Transporter (OAT/OCT) families, respectively. Interestingly, only the transport of TEA exhibited polarity, with about 45% higher transport in the secretory direction. Consistently, the authors demonstrated mRNA levels of both OCT1 and OCT3, with OCT1 levels exceeding OCT3 levels in trypsin-resistant (i.e. differentiated) cultures (see Table 2). This finding is consistent with the substantial upregulation of OCT1 in mammary epithelium of animals and human during lactation (see Table 2).

The cell culture technique for inducing trypsin resistance described above was also described more recently by Jaeger et al. [36]. They purified initial heterogenous cultures of mammary epithelial cells with fibroblasts and adipocytes, thereby taking advantage of the fact that the latter two cell types dislodge more quickly from cell culture surfaces upon trypsinization, leaving behind only the desired islands of epithelial cells.

In vitro models relying on mammary epithelial cells have also been established based on cells obtained from animal tissue. For instance, Bataineh et al. demonstrated functional activity of OCT and OAT isoforms in immortalized bovine mammary epithelial cells [37,38].

Ex vivo models of drug milk excretion

Several *ex vivo* studies have been performed to model the biology of mammary gland in animal models, and rodent tissue explants containing whole alveoli date back to 1957 [39]. MEC from dairy cow (BMEC) have been extensively used to investigate xenobiotic transfer in milk utilized for human consumption since the first protocol was established [40].
The use of isolated bovine udder as an *ex situ* model was also validated. Perfused mammary glands from slaughtered cows remain viable up to six hours and can be used as model of percutaneous drug absorption capable of metabolizing drugs *in vitro* [41-43].

Predicting drug milk transfer based on (semi-)mechanistic models

Mechanistic models for drug milk transfer recapitulate purely physiological (e.g. milk volume) as well as compound-specific parameters (such protein binding) with the purpose to predict PK parameters that inform about the extent of drug excreted in milk. For instance, Koshimichi et al. [29] developed such a mechanistic model (see Figure 1) to predict milk/plasma exposure (AUC) ratio's, that is by using clearance values in both directions between milk and plasma, corrected for unbound fractions in both compartments (f_p and f_{milk}). After selection of drugs for which reliable data sets were available regarding plasma and milk concentration profiles, curve fitting was applied to retrieve values for secretory clearance (Cl_{sec}) and reuptake clearance (Cl_{re}). Multiple linear regression was subsequently applied with the purpose to derive empirical equations with physicochemical parameters [e.g. Log P, Polar Surdace Area (PSA), MW,…] of the drugs describing the observed Cl values. Importantly, their model started from the hypothesis that rapid equilibrium between blood and milk is generally not occurring. This implies the assumption of a permeability-limited

model for blood-milk transfer, i.e. the rate of transfer across the epithelium rather than the mammary blood flow determines the milk drug excretion rates. A schematic representation of the applicable transfer model is shown in Figure 1 and illustrates the importance of knowledge of drug binding in plasma and in milk next to bidirectional clearance values between blood (plasma) and milk for a given drug. It should be noted that although their model was developed based on data for drugs with Cl_{re} values of not more than 5000 mL/h (i.e. rapid equilibrating drugs were excluding), the authors conclude that their model is likely applicable also for these rapidly equilibrating drugs, i.e. the Cl_{re} and Cl_{sec} values for these compounds can be predicted based on the empirical equations developed in this study (which rely on a set of physicochemical parameters).

In the model developed by Koshimichi et al., it is also intriguing that although Cl_{sec} and Cl_{re} are comparable for most drugs, different sets of physicochemical properties were found to predict the actual clearance values. The authors pointed out that this may be due to the fact that in particular for compounds with Cl_{re} values < 100 mL/h, there was a poor correlation between both clearance values. Furthermore, with the exception of these compounds with low Cl_{re} values, the involvement of a transporter may be suspected when the model yields dissimilar values for Cl_{sec} and Cl_{re}. Indeed, deviation for a given compound from the linear correlation established between these opposite Cl terms involves the presence of 'additional transfer' mechanisms (although this would not hold true for drugs with low Cl – see above). This is due to the fact that this correlation was established solely based on drugs showing passive permeation across mammary epithelia.

Another important assumption made in the model by Koshimichi et al. is the fact that the milk volume is fixed at 500 mL, simply based on the knowledge that nursing would reduce the breast milk content by not more than 150 mL. However, the breast milk volume is a

dynamic parameter, which results from the balanced effects of milk production (15-20 mL/h on average, peaking at 60 mL/h) and lactation. Clearly, future improvements in this type of model-based prediction of drug transfer into milk could imply taking into account a non-fixed milk volume.

Furthermore, Koshimichi et al. also acknowledge the burden of lack of measured values for free drug in milk (f_{milk}) as opposed to f_p values (which are available for instance in Drugbank). This implies there is an urgent need to set up assays for experimental determination of f_{milk} values for marketed drugs. The success of such an expedition will depend on the development of sensitive and selective bioanalytical assays for these drugs along with a standardized protocol executed in a single laboratory. The latter will also include exploring the influence of milk composition (e.g. lipid content) on f_{milk}, which likely depends on the physicochemical parameters of the drug. Time- and event-dependent as well as physiologically-based changes in milk lipid content (e.g. due to nursing) but also milk pH (varying between 6.7 and 7.3 [6]) can then be incorporated into future physiology-based pharmacokinetic (PBPK) models.

Implementing in vitro data into PBPK models

A major asset of PBPK models for predicting drug milk transfer ('PBPK-milk' models) is that the majority of the existing (even semi-quantitative) knowledge regarding drug milk excretion (mechanisms) can be incorporated into a single concept, which is then amenable for iterative fine-tuning as additional insights are gained. Examples of existing information to be included are: plasma (and milk) protein binding, time and subject-dependent milk lipid composition and pH, milk lipid content, milk-blood drug (re)equilibration rates (rapid/slow).

A second major type of input for (true) PBPK models are the *in vitro* data obtained in

biorelevant experimental model systems. Such model systems typically reflect the local tissue physiology and function, yielding data describing the rates (including saturability) of drug-specific disposition processes (e.g. metabolism or transport across the intestinal epithelium). In the case a of PBPK-milk model, required *in vitro* data would include bidirectional clearance values for the milk-blood barrier (see above). In this context, the importance of proper scaling of *in vitro* data to the *in vivo* context and units is crucial and this so-called *In vitro-in vivo* Extrapolation (IVIVE) step does often not get sufficient attention. Application of IVIVE to *in vitro* data reflecting drug-specific permeability of the milk-blood barrier would encompass establishing accurately the *in vitro-in vivo* surface area ratio across which transport (and also milk production *in vivo*) is occurring.

Another advantage of PBPK-based approaches is that also maternal variations in drug exposure for instance related to pharmacogenetics can also be incorporated when predicting not only the median infant exposure but also the variability in this exposure. Indeed, for most drugs on the market, covariates determining (adult) systemic exposure variability have been identified and this information should not be ignored when making predictions about exposure ranges in infants.

Anderson and Sauberan [6] conclude in their 2016 review article on '*Modelling Drug Passage into Human milk*' that "*at the current state of technological development, modelling is not a substitute for breast milk and concentration analysis, but might be used for helping to simulate drug passage based on plasma and concentration data in studies'.* Although this exactly reflects the purpose and current scope of population pharmacokinetic modelling (which is a top-down approach), the statement seems to underestimate the utility of PBPK modelling (being a bottom-up approach). When applied to drug milk excretion, PBPK modelling should provide the ability to predict *in vivo* drug milk levels without having

analysed clinical milk and blood samples. Indeed, PBPK modelling by definition relies on *in vitro* data in combination with population-specific physiology to derive concentration time profiles in the systemic circulation, various tissues and excreta including breast milk. It is important to stress that PBPK modelling should not be designated as 'pure modelling' since the underlying *in vitro* data are obtained in model systems recapitulating the cellular physiology (e.g. at an epithelial barrier) that is also present *in vivo*. This also implies that the validation of such PBPK models should not be reduced to just a comparison between model-based predictions and clinical data obtained from a single study. Model identifiability remains an important question to be addressed and this should be pursued by providing evidence for the mechanistic basis of the PBPK model.

In vivo animal models

Performing in vivo lactation studies in different animal species may provide the opportunity to mechanistically explore drug milk excretion, taking into account all the variables determined by systemic metabolism and disposition. Nonetheless, current literature reports very few examples of *in vivo* trials involving drugs and milk. One of the possible reasons for such lack in non-clinical *in vivo* trials may be related to the fact that the lactation process is extremely variable amongst animal species. Parameters that fluctuate include, among others, the anatomy of the udder itself (position, number of teats and glands per teat), the rate of milk production, the qualitative/quantitative composition of milk and the duration of lactation [44]. All of the above-mentioned physiological patterns make the translational value difficult to forecast. Another critical point is represented by drug metabolism itself. Indeed different animal species, if not different breeds within the same species, can show extreme variability in both enzymatic asset and metabolic pathways [45,46]. This is why the

only way to fully explore animal models' potential and achieve the highest translational value in drug discovery, would be enrolling different species in the same experimental protocol and combining the results on the basis of the physiological variability.

The analysis of the existing scientific literature regarding animal models of lactation for drug transfer has highlighted a prevalence of research aimed to obtain information in two main fields: translational medicine and food safety.

Generally speaking, the use of rodent species in such translational context, has led to the common idea that "animal data are generally not useful in predicting drug concentrations in human milk" [5]. Rats and mice are indeed preferred in the first phases of biomedical research protocols because of their cost-effectiveness, ease of husbandry and reproduction, short lifespan that allows to evaluate different life phases in a short time frame and lower neurological development. However, their metabolic and digestive patterns are quite different from humans, potentially leading to substantial differences in drug metabolism and milk composition [47,48]. Mice are approximately 3000 times smaller than humans, with a much slower basal metabolic rate but, when corrected per gram of body weight, it is seven times greater, with relevant implications in drug blood levels and lactational transfer. Moreover, in such species sexual maturation is quite early and reproduction/lactation happen in a narrow time window with large litter size, in total contrast with humans, that show relatively late sexual maturation, broad reproductive span and small litter size [49]. Nonetheless, it has to be acknowledged that extensive information regarding rodents' mammary glands have been collected as often enrolled as non-clinical models in studies specifically designed to clarify mammary cancer mechanisms [50].

It is by now clear how enrolling an animal model in any experimental trial is a matter of compromising, as the "perfect" model does not exist, but the biggest concern, with respect

to the specific research question to be addressed, always has to be its translational value, tailored on the basis of the research field and the peculiar similarities between the model and humans.

This is why, especially in the last decades, the interest of the scientific community has shifted toward the use of “large” animal models. When it comes to lactation studies and large animal models, it is obvious how ruminants are thoroughly investigated for food safety reasons, as milk is widely used for human consumption.

For such purposes, ovine [51] and bovine [28] are the two most representative species, as food-producing animals routinely undergo medical treatments for different clinical symptoms, and drug transfer rate is investigated to avoid xenobiotic presence in milk. Many papers have investigated the direct or receptor-mediated lactational transfer in dairy cows, sheep, goat and water buffalos. Therefore, collected data (frequently related to veterinary drugs that share with human drugs the active principle) may also be used for translational purposes. Unfortunately, when the aim is to analyze transfer of drugs and exogenous compounds through the mammary gland, ovine and cows show some limitations. It is indeed very well recognized that feeding habits strongly correlate with metabolic capabilities and processes, and these animals, as cranial fermenters herbivores, are quite different from humans.

From the anatomical point of view, mammalian species show both diversity and similarities of structure [52]. Human breast consists of approximately 10 ducts terminating in clusters of alveoli, each related to a specific lobule. In cows a more complex organization of ducts is present with only some of them participating in the oxytocin-induced milk ejection. Secretory tissue in the udder is organized into lobes, with each lobe made up of many lobules each containing 150-220 alveoli.

In mice, the tree-like system is very simple at birth and starts increasing in complexity at puberty (4 weeks) reaching a diffuse presence into the stroma after some oestrous cycle (10 weeks) but the formation of secretory alveoli only occurs during a secondary growth, with the onset of pregnancy. At pregnancy, ducts and alveoli are spread within the connective tissue stroma and are quite similar to human. At the completion of involution, the murine mammary ducts and lobules return to a state resembling that of the nulliparous state. In the nulliparous adult human breast, lobules can exist in various states of differentiation depending on the menstrual cycling history. Moreover, the distribution of oestrogen and progesterone receptors is different between the two species and suggest that there are fundamental differences in hormone sensitivity [53].

Another critical point when choosing the animal model is the role and the presence of different transporters involved in drug milk excretion. [54].

The veterinary medicine domain is fully aware of this problem and a comparative approach among different species is started to investigate the function of biological barriers and modulation of drug absorption, distribution and elimination in the intestine, liver and kidney for clinical purposes [55,56], as well as translational medicine and for food safety ones [57]. In such scenario, the porcine species may represent a more suitable model, with better anatomical and physiological, thus metabolic, similarities [58]. Moreover, literature provides an extensive characterization of this species in a wide array of fields, making for a good standardization and comprehension of the model [59]. Pigs have an average of 6-7 pairs of complex glands. The secretory tissue of each gland is independent. A detailed investigation of transcriptomics of some drug transporters in swine has been also performed [60] (see Table 2). The lactation of the sow is sensibly shorter than the one in humans, and this could represent an advantage in analyzing all its different phases in a reasonable amount of time,

the milk could be easily collected and analyzed without interfering with the lactation process and the piglets can be analyzed individually for the possible effect of the lactational transfer. Referring to the above-mentioned principle of “compromising” when choosing an animal model, it has to be acknowledged that swine placenta differs from human placenta for both morphology and histology. It is indeed classified as diffuse/folded, with villi distribution over the entire surface of the chorionic bag forming folds, and epitheliochorial, as the trophoblast is apposed on the intact epithelium of the uterus and cohesion is achieved by interdigitations with the apical microvilli of cell membranes [61]. Such difference is obviously pivotal when the focus is on maternal/fetal drug transfer during pregnancy, as differences in placentation determine different exchange mechanisms. When the focus is on lactational transfer, differences in placentation still have to be acknowledged, as colostrum composition will be different especially for immunoglobulins, but less important throughout the entire time of lactation.

Focusing on porcine models, piglets are an important additional resource because, both for economical zoo-technical purposes and scientific translational research, much information is available on blood biochemistry [62] and on the maturity of their intestinal barrier and transport mechanisms [63]. Moreover, the availability of different handling and sampling techniques make piglets an important resource for data collection [64-66] and they have already been proposed as human pediatric surrogates for PK/PD [67]. Finally, having the chance to obtain data from piglets throughout a longer time span, will also allow to investigate effects of lactational drug transfer on maturational changes.

It is also important to mention that, in order to bypass the limitations related to the size of such species, biomedical minipigs have been developed and proposed for pharmacological and toxicological assessments[68,69], and have already been used for PBPK modelling [70].

Minipigs, and in particular Göttingen, show dramatically different growth rates when compared to domestic swine breeds, with a weight range of 12 -45 kg [71] that makes handling and husbandry easier. From a reproductive point of view, the main differences are represented by sexual maturation, relatively earlier in minipigs (usually 3-6 months of age) [72], and litter size that is generally lower [71] . Despite an extremely high level of characterization of minipigs model for toxicology, data regarding qualitative and quantitative composition of the milk, as for the domestic pig, are lacking.

Discussion including future perspectives

Even though a limited number of drugs has been studied, non-clinical models for investigating ADME during lactation provide the potential to unravel **not only mechanisms of drug milk excretion but, along with PBPK modelling, to also support quantitative prediction of medication concentrations in human milk**. The value of such predictions can hardly be overestimated, especially in view of the challenges associated with the conduct of clinical lactation studies.

Nevertheless, there remains a lot of work to be done to further develop, optimize and validate the non-clinical models, especially in terms of translatability. Indeed, apart from understanding mechanisms and making median or mean predictions of drug milk concentrations and excretion rates, also the (often substantial) **population variability in these lactation-specific disposition processes needs to be explored**. Furthermore, for instance genetic polymorphisms in maternal CYP enzymes may contribute to significant interindividual variability in maternal drug exposure, consequently leading to altered distribution to the foetus or changed concentrations in breast milk during lactation. In this context, reference is made to a case of morphine intoxication in an infant due to the ultra-rapid metabolizing phenotype of the mother taking codeine [73].

From a non-clinical model development and validation perspective, more attention should go to **adequate scaling of variability in non-clinical findings to uncertainty figures for predicted clinical values**. Specifically, with respect to determining population variability, PBPK modelling carries the promise that variability may be better explained in terms of physiological parameters some of which are time-dependent. For instance, the lower fat content in foremilk versus hindmilk will have opposite influences on the milk partitioning of

hydrophilic and lipophilic drugs. Careful implementation of such physiological processes into PBPK models will support the prediction of drug milk concentrations profiles in the absence of drug-specific clinical data.

The ultimate potential of PBPK modelling in general is that drug-specific clinical data would not be needed anymore for every drug (candidate) to accurately predict *in vivo* behavior of drug candidates. However, in order to achieve this level of accuracy, patient- or at least population-specific physiological data will be needed at high resolution, in addition to drug-specific data related to interactions with relevant biological targets. The latter can be determined based on *in vitro* experimentation. Clearly, in particular for drug disposition during lactation, such accurate PBPK-based predictions would represent a major breakthrough.

Regarding the **utility of animal data**, the FDA workshop report published by Wang et al. [5] only discusses the utility of animal data from the perspective of direct prediction of human milk concentrations, which is maybe a somewhat narrow perspective. The added value of *in vivo* animal studies can be situated at various levels:

(i) ***in vivo* animal studies (as most other non-clinical studies) are almost uniquely powered to provide insight into drug disposition mechanisms**. For instance a key mechanistic aspect of drug milk distribution that appears underappreciated in the clinical setting [6] is the phenomenon of drug reuptake from milk to blood. Indeed, awareness on reuptake rates for specific drugs, i.e. those showing rapid distribution across the milk/blood barrier would significantly improve advice to nursing mothers with respect to optimal nursing times in relation to maternal medication administration.

(ii) by combined interpretation of animal *in vitro* and *in vivo* data, valuable information may be gained regarding *in vitro in vivo* extrapolation (IVIVE), an approach that is a prerequisite for reliable PBPK model development. As for the role of different transporters involved in drug milk excretion in animals and humans, it should be noted that apart from differences (e.g. different isoforms) similarities exist as well, for instance in terms of substrate specificity of the ABC-type transporters such as P-glycoprotein (*MDR1, ABCB1*) and those belonging to the MRP (*ABCC*) family [74,75]. In addition, Table 2 illustrates that upregulation of BCTP/Bcrp in mammary gland epithelium during lactation is observed across the various species evaluated.

(iii) Animal studies may also prove useful in starting to unravel the influence of lactation-specific covariates in drug milk excretion rates. Examples of such factors include:

- milk composition (e.g. protein versus lipid, creamatrocrit [25]);
- relative timing between maternal dosing and breastfeeding;
- absolute timing of milk collection (early/late);
- drug-drug interaction potential at the level of drug milk excretion;
- relative importance of passive versus active excretion processes in relation to drug physicochemistry.

Especially in view of the future potential of PBPK modelling approaches, we can also expect **growing interest in and implementation of *in vitro* cellular models with animal mammary epithelial cells**. Although direct/simple extrapolation of *in vitro* findings in these models to *in vivo* predictions (even in animals) should not be done [26], these cellular models can have significant value in detecting polarity in transport and thus providing indication for the involvement of active transport mechanisms. Clearly, as recently also acknowledged by

Wang et al. [5], incorporation of transporter-mediated processes in non-clinical approaches for drug milk excretion is one of the most important assets of the *in vitro* systems relying on cultured HMECs. As described above, transporter-overexpressing cell lines (e.g. for BCRP) have also been successfully used in combination with other approaches (for instance based on physicochemistry) to successfully predict milk drug transfer including the role of that specific transporter [76]. However, in order to take into account the possible roles of all transporters expressed in the mammary gland (see Table 2 for current knowledge), *in vitro* models such as those relying in HMECs are preferred. A prerequisite for this remains the full characterization of transporter functions in cultured HMECs in comparison with native tissue. A comparative quantitative proteomic analysis, as recently and currently being applied to many tissues has significant added value here too (especially also in light of building a more robust PBPK platform).

It should be noted that several important aspects related to medication use during lactation were not covered in this manuscript, notably:

(i) effect of medication use on physiological functions related to pregnancy (placental effects of drugs) and lactation (milk production);

(ii) infant factors affecting risks associated with exposure to maternal medication.

The importance of the latter was also reflected in the conclusion made by Anderson and Sauberan in their 2016 review article on 'Modelling drug passage into human milk', i.e. assessment or prediction of (variability of) drug concentration-time profiles in human milk is not sufficient for reliable risk assessments regarding nursing infants during maternal drug therapy. In this context, several efforts are ongoing [77] to document the state of the art regarding the ontogeny of drug disposition pathways in humans and animals – with focus

on neonates - and incorporating such information into PBPK models for predicting drug disposition in pediatric populations particularly neonates. As recently reported by Gasthuys et al. [67], piglets could also be a promising animal model to explore the impact of maturational parameters on pharmacokinetics and pharmacodynamics. For the purpose of PBPK model verification, the utility not only of drug concentrations in milk, but also systemic infant exposure should be emphasized. Indeed, although the burden for systemic infant sampling is (very) high, opportunistic sampling approaches should be feasible as well as more non-invasive PK assessments (e.g. urine, saliva). It should be noted that verification of PBPK models predicting infant drug exposure via breast milk can also be achieved with (existing) PK data obtained after dosing neonates or infants directly with the maternal drug of interest. An important parameter to be considered when predicting infant drug exposure is the infant age-dependency of the ingested milk volume [78]. According to this study, the average daily intake beyond the first week of life is 150 mL/kg, albeit with a CV% above 20%.

At the time of writing of this manuscript an IMI project, named ConcePTION, has been approved to address some of the challenges described in this review article. ConcePTION is expected to generate major breakthroughs in the broad field of pharmaco-epidemiology of drug use during pregnancy and lactation. Work package 3 of ConcePTION in particular addresses the much-needed development and validation of non-clinical methods for drug excretion to breast milk. Those methods, as also discussed in this manuscript, include animal lactation studies, *in vitro* transport studies across mammary epithelial cells and PBPK modelling. The work package also describes the cross-validation of these methods in close

collaboration with activities in work package 4 which describes the conduct of human lactation studies including biobanking and analysis of milk samples.

TABLES

Table 1: Parameters used to describe PK specifically related to drug milk excretion and/or drug disposition during lactation.

Parameter	**Equation**	**Description**
Infant Daily Dosage (mg/kg/day)	Infant Daily Dosage = Drug concentration in milk x daily volume of milk ingested	Equation to calculate the infant daily dosage
M/P ratio	Drug concentration in milk = M/P ratio x maternal plasma concentration	Milk to plasma concentration ratio
RID - Relative infant dose	$RID = \frac{Infant\ Daily\ Dosage}{Maternal\ Daily\ Dosage}$	RID represents the ratio of infant and maternal doses, both expressed in mg/kg/day.
EI, %	$EI = A \times \frac{M/P}{Cl_I} \times 100$ With A representing the milk intake of 0.10 mL/min/kg (or 144 mL/kg daily)	Exposure index, relying on infant (or even corrected adult) clearance to provide a relative idea about infant drug exposure

Table 2: Overview of mRNA/protein expression and/or activity of drug transporter isoforms that have been reported in mammary epithelial tissue, cells or derived cell lines of different species. The transporters were classified as belonging to the SLC, SLCO and ABC transporter families. When orthologous genes have been described in species other than human, they are listed with right alignment below the human gene shown in bold. Only in cases where no human ortholog has been described for a given isoform or subfamily, the animal gene is shown in bold. Gene symbols are shown in italics below the corresponding protein isoforms [79].

ISOFORM (SPECIES)	SOURCE & LEVEL (mRNA/PROTEIN/ACTIVITY)	PARAMETER VALUE	COMMENTS	REF
SLC family				
PEPT1 (*human*) ***SLC15A1***	mRNA in MEC in lactating versus non-lactating state	0.05/0.16	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Pept1 (*rat*) *Slc15a1*	mRNA in Sprague-Dawley rat mammary gland	~4-fold peak	During early lactation as compared to late gestation.	[81]
PEPT2 (*human*) ***SLC15A2***	mRNA in MEC in lactating versus non-lactating state	1.59/BLD	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
	mRNA in human milk	present	Comparable to kidney mRNA levels	[82]
Pept2 (*rat*) *Slc15a2*	mRNA in Sprague-Dawley rat mammary gland	~1.6-fold peak	During early lactation as compared to late gestation.	[81]
	mRNA in lactating Sprague-Dawley mammary gland	present	apical membrane of epithelial cells of ducts and glands	[82]

Mct1 (mouse) ***Slc16a1***	mRNA and protein expression in mammary glands of ddY mice	peaks	during lactation	[83]
Folate transporter 1 (*rat*) ***Slc19a1***	mRNA in Sprague-Dawley rat mammary gland	~6-fold reduction	By the end of lactation as compared to late gestation	[81]
OCT1 (*human*) ***SLC22A1***	mRNA in MEC in lactating versus non-lactating state	ß-actin ratio: 3.5/0.45	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Oct1 (*mouse*) *Slc22a1*	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary epithelial cell line	➢ ~1.5-fold increase ➢ Present	➢ in lactating state, relative to non-lactating state ➢ No effect of differentiating treatment	[57]
	➢ mRNA in murine mammary tissue (FVB mice) ➢ activity as reflected by M/P ratio of cimetidine, metformin, acyclovir and thiamine (vitamin B1)	➢ ß-actin ratio: 5-25 ➢ > 2-fold reduction	➢ upregulation at the onset of lactation ➢ reduced M/P ratio in Oct1/2 knockout mice	[84]
Oct1 (*rat*) *Slc22a1*	➢ mRNA in lactating rat mammary gland ➢ Activity as reflected by M/P ratio of cimetidine	➢ Present ➢ M/P ratio: 29	➢ lower expression as compared to liver/kidney ➢ Reduced by nitrofurantoin	[85]

	mRNA in Sprague-Dawley rat mammary gland	~17-40-fold increase	By end of lactation as compared to late gestation	[81]
Oct2 (*mouse*) ***Slc22a2***	mRNA in murine mammary tissue (FVB mice)	ß-actin ratio: 0.2-2	gradual downregulation during lactation	[84]
OCT3 (*human*) ***SLC22A3***	mRNA in MEC in lactating versus non-lactating state	ß-actin ratio: 0.16/0.48	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Oct3 (*mouse*) *Slc22a3*	mRNA in murine mammary tissue (FVB mice)	ß-actin ratio: 0.5-5	downregulation during lactation	[84]
Oct3 (rat) *Slc22a3*	mRNA in lactating rat mammary gland	present	lower expression as compared to kidney	[85]
OCT1/3 (*human*) ***SLC22A1/3***	mRNA expression and TEA transport in trypsin-treated HMEC cell line	Transport polarity factor [BL->AP/AP->BL)]: 1.44	Activity reflects a combined OCT1/3-mediated transport; OCT1 mRNA > OCT3 mRNA as measured in the same experiment	[34]
OCT (*bovine*) *SLC22A*	TEA transport across the BME-UV immortalized cell line	Transport polarity factor [BL->AP/AP->BL)]: 2.55	Polarized transport towards secretory direction [BL->AP]	[38]
OCTN1 (*human*) ***SLC22A4***	mRNA in MEC in lactating versus non-lactating state	ß-actin ratio: 0.34/*BLD*	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]

OCTN1 (*bovine*) *SLC22A4*	mRNA in bovine mammary gland	Present	Quantitative data not provided	[57]
Octn1 (*mouse*) *Slc22a4*	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary epithelial cell line	➢ 30-fold decrease ➢ present	during lactation, i.e. virtually disappears during lactation	[57]
	protein expression (by IHC) in mammary glands of C3H mice	increased	compared to expression in virgins	[86]
Octn1 (*rat*) *Slc22a4*	mRNA in Sprague-Dawley rat mammary gland	~3-fold increase	During early lactation as compared to late gestation	[81]
	mRNA and protein in Sprague-Dawley rat mammary gland	stable expression	between day 4 and day 10	[87]
OCTN2 (*human*) *SLC22A5*	mRNA in MEC in lactating versus non-lactating state	0.62/2.5	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Octn2 (*mouse*) *Slc22a5*	protein expression (by IHC) in mammary glands of C3H mice	increased	compared to expression in virgins	[86]
Octn2 (*rat*) *Slc22a5*	mRNA in Sprague-Dawley rat mammary gland	~10-fold reduction	By the end of lactation as compared to late gestation	[81]
	mRNA and protein in Sprague-Dawley	~2-fold	Between day 4 and day 10	[87]

	rat mammary gland	reduction		
Octn3 (rat) ***Slc22a6***	mRNA in Sprague-Dawley rat mammary gland	~2-fold reduction	By the end of lactation as compared to late gestation	[81]
	mRNA and protein in Sprague-Dawley rat mammary gland	~2-fold reduction	Between day 4 and day 10; much lower expression than Octn1/2	[87]
Octn3 (*mouse*) *Slc22a6*	protein expression (by IHC) in mammary glands of C3H mice	increased	compared to expression in virgins	[86]
OAT (*bovine*) ***SLC22A***	ES transport across the BME-UV immortalized cell line	Transport polarity factor [BL->AP/AP->BL)]: 3.33	Polarized transport towards secretory direction [BL->AP]	[38]
OAT1-4 (*bovine*) *SLC22A6-9*	➢ mRNA expression in BME-UV immortalized cells ➢ ES transport polarity across the BME-UV immortalized cell line	➢ < 3% ➢ BL->AP/AP->BL: ~ 2.4	➢ Relative to kidney levels; OAT4 >> OAT1-3 ➢ Polarized transport towards secretory direction [BL->AP]; no transport polarity for PAH or salicylic acid	[37]
SVCT1 (*human*) ***SLC23A1***	mRNA in MEC in lactating versus non-lactating state	0.96/0.44	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Svct1 (*rat*) *Slc23a1*	mRNA in Sprague-Dawley rat mammary gland	~3.5-fold increase	By the end of lactation as compared to late gestation	[81]
Svct2 (*rat*)	mRNA in Sprague-Dawley rat	~7-fold	By the end of lactation as compared to late	[81]

Slc23a2	mammary gland	reduction	gestation	
CNT1 (*human*) ***SLC28A1***	mRNA in MEC in lactating versus non-lactating state	0.18/BLD	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Cnt1 (*rat*) *Slc28a1*	mRNA in Sprague-Dawley rat mammary gland	~2-fold peak	During mid lactation as compared to late gestation	[81]
Cnt2 (*rat*) ***Slc28a1***	mRNA in Sprague-Dawley rat mammary gland	~2.5-fold peak	During early lactation as compared to late gestation; 3-fold reduction towards late lactation	[81]
CNT3 (*human*) ***SLC28A2***	mRNA in MEC in lactating versus non-lactating state	0.33/0.05	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Cnt3 (*rat*) *Slc28a2*	mRNA in Sprague-Dawley rat mammary gland	~3.3-fold peak	During early lactation as compared to late gestation	[81]
ENT1 (*human*) ***SLC29A1***	mRNA in MEC in lactating versus non-lactating state	0.49/0.66	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
Ent1 (*rat*) *Slc29a1*	mRNA in Sprague-Dawley rat mammary gland	~2.7-fold peak	During early lactation as compared to late gestation; 2-3-fold reduction towards late lactation	[81]
Ent2 (*rat*) ***Slc29a2***	mRNA in Sprague-Dawley rat mammary gland	~7-fold reduction	By the end of lactation as compared to late gestation	[81]
ENT3 (*human*)	mRNA in MEC in lactating versus non-	0.08/BLD	Values represent transporter gene to ß-actin	[80]

SLC29A3	lactating state		ratio; in lactating / non-lactating state	
Ent3 (*rat*) *Slc29a3*	mRNA in Sprague-Dawley rat mammary gland	~40% reduction	By the end of lactation as compared to late gestation	[81]
RFVT2 (human) ***SLC52A2***	mRNA in human milk fat	2.75	Relative to ß-actin	[88]
Rfvt2 (mouse) *SLC52A2*	mRNA expression in FVB mouse mammary gland	Peaks 15-fold	At 1 week lactation as compared to virgin levels	[88]
RFVT3 (human) ***SLC52A3***	mRNA in human milk fat	0.04	Relative to ß-actin	[88]
Rfvt3 (mouse) *SLC52A3*	mRNA expression in FVB mouse mammary gland	Peaks 4-fold	At 1 week lactation as compared to virgin levels	[88]
SLCO family				
OATP1A2 (*human*) ***SLCO1A2***	mRNA in MEC in lactating versus non-lactating state	0.08/0.05	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
OATP1A2 (*bovine*) *SLCO1A2*	mRNA in bovine mammary gland	present	Quantitative data not provided	[57]
Oatp1a5 (*mouse*) ***Slco1a5***	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary	➢ < 1% ➢ ~2-fold	➢ compared to virgin levels ➢ differentiation results in increase	[57]

	epithelial cell line	increase		
OATP2B1 (*human*) ***SLCO2B1***	mRNA in MEC in lactating versus non-lactating state	0.95/0.64	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
OATP3A1 (*human*) ***SLCO3A1***	mRNA in MEC in lactating versus non-lactating state	3.6/6.6	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
OATP4A1 (*human*) ***SLCO4A1***	mRNA in MEC in lactating versus non-lactating state	0.14/0.37	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
ABC family				
MDR1 (*human*) ***ABCB1***	mRNA in MEC in lactating versus non-lactating state	0.03/1.33	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
MDR1 (*bovine*) *ABCB1*	➢ mRNA in bovine mammary gland ➢ mRNA in BME-UV cells	➢ present ➢ present	➢ quantitative data not provided ➢ not affected by prolactin treatment	[57]
Mdr1 (*mouse*) *Abcb1*	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary epithelial cell line	➢ ~10-fold reduction ➢ ~2-fold reduction	➢ During lactation; stays low early after weaning ➢ differentiation to 'lactation state' results in reduced expression	[57]
	Protein expression in murine HC11	60% reduction	Compared to undifferentiated HC11 cells	[89]

	mammary epithelial cell line			
MDR1 (porcine) *ABCB1*	mRNA expression in non-lactating mammary gland	present	Relatively low expression compared to other tissues (~5-fold lower than liver)	[60]
Mdr1a/b (*rat*) *Abcb1*	mRNA in Sprague-Dawley rat mammary gland	~10-fold reduction	By the end of lactation as compared to late gestation	[81]
MRP1 (*human*) ***ABCC1***	mRNA in MEC in lactating versus non-lactating state	0.36/0.92	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
MRP1 (*bovine*) *ABCC1*	➢ mRNA in bovine mammary gland ➢ mRNA in BME-UV cells	➢ present ➢ present	quantitative data not provided	[57]
Mrp1 (*mouse*) *Abcc1*	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary epithelial cell line	➢ ~10-fold reduction ➢ present	➢ during lactation; stays low early after weaning ➢ differentiation to 'lactation state' does not affect expression	[57]
Mrp1 (*rat*) *Abcc1*	mRNA in Sprague-Dawley rat mammary gland	~6-fold reduction	By the end of lactation as compared to late gestation	[81]
MRP2 (*human*) ***ABCC2***	mRNA in MEC in lactating versus non-lactating state	0.05/0.06	Values represent transporter gene to ß-actin ratio; in lactating / non-lactating state	[80]
MRP5 (*human*)	mRNA in MEC in lactating versus non-	0.07/0.04	Values represent transporter gene to ß-actin	[80]

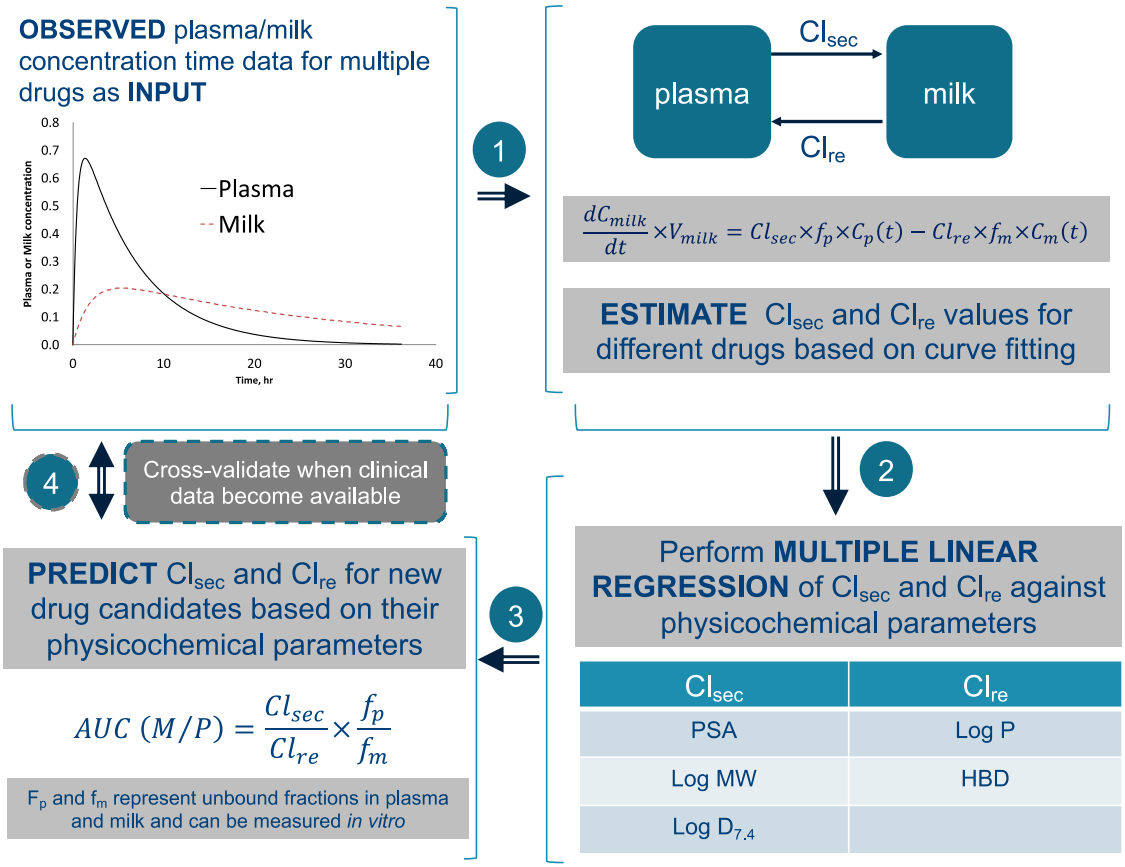
ABCC5	lactating state		ratio; in lactating / non-lactating state	
BCRP (*human*) ***ABCG2***	Very strong protein induction in mammary gland	IHC detection		[90]
BCRP (*bovine*) *ABCG2*	➢ Very strong protein induction in mammary gland ➢ mRNA in bovine mammary gland ➢ mRNA in bovine mammary gland	➢ IHC detection ➢ Present ➢ ~6-fold increase	➢ very strong induction during lactation ➢ no quantitative data ➢ compared to non-lactating state	[90] [57] [91]
BCRP (porcine) *ABCG2*	mRNA expression in non-lactating mammary gland	present	mRNA levels 40% higher than in liver	[60]
Bcrp (*mouse*) *Abcg2*	Very strong protein induction in mammary gland (by Western and ICH)	present	detected in apical membrane of alveolar epithelial cells	[90]
	➢ mRNA in murine mammary tissue (NMRI mice) ➢ mRNA in murine HC11 mammary epithelial cell line	➢ ~30-fold increase ➢ Present	➢ during lactation as compared to non-lactating ➢ No quantitative data available	[57]
	➢ mRNA in murine mammary tissue (FVB mice) ➢ activity as reflected by M/P ratio of	➢ ß-actin ratio: 5-18 ➢ > 2-fold	➢ Peaks at lactation day 10 ➢ Reduced M/P ratio in Bcrp knockout mice	[84]

	cimetidine, acyclovir	reduction		
	Activity as reflected by M/P ratio of ciprofloxacin	➢ ~2-fold reduction	Reduced M/P ratio in Bcrp knockout mice	[92]
	Activity as reflected by M/P ratio of nitrofurantoin	➢ ~80-fold reduction	Reduced M/P ratio in Bcrp knockout mice	[93]
	Protein expression in murine HC11 mammary epithelial cell line	➢ 50% increase	Compared to undifferentiated HC11 cells	[89]

Table notes and abbreviations: ABC = ATP binding cassette (transporter family); BCRP/Bcrp = breast cancer resistance protein; BLD = below detection limit; BME-UV = bovine mammary epithelial cell line; CNT/Cnt = concentrative nucleoside transporter; ENT/Ent = equilibrative nucleoside transporter; ES = estrone sulfate; HMEC = human mammary epithelial cell line; IHC = immunohistochemistry; Mct = monocarboxylate transporter; MEC = mammary epithelial cells; M/P= milk/plasma ratio; MDR/Mdr = multidrug resistance protein (family); MRP/Mrp = multidrug resistance-associated protein (family); OAT/Oat = organic anion transporter (family); OATP = organic anion transporting polypeptide (family); OCT/Oct = organic cation transporter (family); OCTN/Octn = organic cation/carnitine transporter; PAH = p-aminohippuric acid; PEPT/Pept = peptide transporter; RFVT/Rfvt = riboflavin transporter; SLC = solute carrier (transporter family); SLCO = solute carrier organic (transporter family); SVCT/Svct = sodium (ascorbate) vitamin C transporter; TEA = triethylammonium

FIGURE LEGENDS

Figure 1: Approach used by Koshimichi et al. [29] to predict the M/P concentration ratio of new drug candidates based on the physicochemical properties. Clinical data regarding milk and plasma concentrations of about 50 marketed drugs were used to calculate compound specific values for milk secretion (Cl_{sec}) and reuptake (Cl_{re}) clearance. Via multiple linear regression, empirical equations were derived to describe these clearance values based on physicochemical properties of the compounds. These equations can then be used to predict Cl values of new drug candidates for which no clinical data are available. The M/P concentration ratio can thus be obtained at an early stage of drug development.



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