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ConcePTION

WP3 – Determination of drug milk transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.1 Report on scope and limitations of *in vivo* and *in vitro* non-clinical and computational models for drug milk excretion and breastfed infant exposure; Selection of a panel of at least 10 model compounds for initial evaluation of non-clinical models

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Abbreviations

3R	Refine, Reduce & Replace
ADME	Absorption, Distribution, Metabolism & Excretion
AUC	Area Under The Curve
BCRP	Breast Cancer Resistance Protein
BMAA	beta-N-methylamino-alanine
B:P ratio	Blood-plasma ratio
C	Chronic
CaCo-2	Cancer Colon 2
EGF	Epidermal Growth Factor
EMA	European Medicines Agency
FDA	Food and Drug Administration
f_m	Percent of enzyme contribution to the metabolism
f_u	Fraction unbound (in plasma)
$f_{u,\ matrix}$	Fraction unbound *(In vitro* test matrix)
GA-1000	Gentamicin sulfate amphotericin
GIVIMP	Guidance Document on Good *In Vitro* Method Practices
HMEC	Human Mammary Epithelial Cells
I	Incidental
IMI	Innovative Medicines Initiative
IGF-1	Insulin like growth factor 1
IVIVE	*In vitro / in vivo* extrapolation
MATE	Multidrug And Toxin Extrusion protein
MEBM	Mammary Epithelial cell Basal Medium
M/P ratio	Milk-to-plasma ratio
MRP	Multidrug Resistance-associated Protein
OAT	Organic Anion Transporter
OATP	Organic Anion Transporting Polypeptide
OCT	Organic Cation Transporter
PBPK modelling	Physiologically Based Pharmacokinetic modelling
PEPT	Peptide Transporter
pgMECs	Primary goat mammary epithelial cells
P-gp	P-glycoprotein
PK	Pharmacokinetic
PopPK	Population PK
rH-TGF-alfa	Recombinant human transforming growth factor alpha
SmPC	Summary of Product Characteristics
T	Term
TEA	Tetraethylammonium
Vd	Distribution volume
VP	Very Preterm
WP3	Work Package 3
WP4	Work Package 4

Content

Abstract

Breastfeeding plays a major role in the health and wellbeing of mother and infant. However, information on the safety of maternal medication during breastfeeding is lacking for most medicines. This leads to discontinuation of either breastfeeding or maternal therapy, although many medicines are likely to be safe during breastfeeding. Human lactation studies to document the pharmacokinetics (PK) and –dynamics (PD) are costly and challenging. To improve the efficacy and feasibility of this research field, non-clinical methods would be an asset. *In vitro* cell culture models are a first approach that can be used to investigate the transfer of drugs from the maternal blood circulation into the human breast milk. Several *in vitro* models are available, but characterization and quantitative drug transport data remain rather limited. Furthermore, animal *in vivo* models have been used successfully in the past to predict safety of maternal medication during breastfeeding. However, caution is required due to species differences (e.g. in transporters, enzymes and milk composition). In addition, *in vivo* animal models are more costly then *in vitro* models. The choice of both an *in vitro* or *in vivo* animal model is critical as it should be representative for the human mammary epithelial barrier. Also, the use of animals rightfully gives rise to ethical issues. Consistent with the 3R (Refine, Reduce & Replace) principle, the use of animals should be limited. Several efforts have been made to predict drug transfer into the milk only based on physicochemical characteristics of the drugs and milk. However, these methods are not adequate for all medicines, as they do not take transporter-mediated and other physiological processes into account. A more mechanistic and biorelevant strategy is taken by Physiologically Based Pharmacokinetic (PBPK) modelling. Currently, PBPK modelling in the view of lactation has mostly been applied for pollutants, human toxicology, epidemiology or in the dairy industry. To date, lactation PBPK models were reported for 10 drugs (escitalopram, efavirenz, isoniazid, codeine, ethambutol, rifampicin, alprazolam, caffeine, tramadol, clonidine and lamotrigine). Although there are still some hurdles to overcome, these PBPK models show that PBPK modelling is a feasible and valuable approach to predict transfer of medicines into human milk, along with neonatal systemic exposure. The main disadvantage of the current PBPK models is that the milk-to-plasma (M/P) ratio is derived from human *in vivo* data. However, human *in vivo* data are lacking for most drugs. This was illustrated with rifampicin, where an algorithm had to be used to estimate the M/P ratio as the available data were not conclusive Therefore, an iterative development of *in vitro,* animal *in vivo* and PBPK modelling methods seems to be a promising approach to predict the transfer of maternal medication into the human breast milk, and subsequent neonatal systemic exposure. On the one hand, human *in vitro* models will deliver essential data on the transepithelial transport of drugs for implementation in the PBPK model. Animal *in vitro* models in combination with animal *in vivo* studies on the other hand will deliver essential information for accurate *in vitro / in vivo* extrapolation (IVIVE) factors of the transport data and mechanistic insights for development of the PBPK model, while limiting the use of animals. This non-clinical platform will be developed within Work Package 3 of the Innovative Medicines Initiative project ConcePTION. A thorough evaluation of the non-clinical platform will be done using pre-selected model compounds for which the main criterion was the availability of human PK data for mother and infant, to allow thorough verification of the PBPK-based predictions.

Introduction

In April 2019, ConcePTION was launched. ConcePTION is a private public partnership that aims to generate information about the use of medication during pregnancy and breastfeeding. Work Package 3 (WP3) of ConcePTION aims to generate a non-clinical testing platform to determine drug transfer into the human breast milk, and subsequent neonatal exposure. This deliverable aims to provide an overview of the state-of-the art of non-clinical (*in vitro, in vivo* and *in silico*) methods to determine transfer of medicines during lactation. The specific aims of this deliverable were:

(i) To select a set of model compounds for evaluation of the predictive performance of the non-clinical testing platform that will be developed by WP3.
(ii) To provide an overview of the state-of-the art of the *in vitro* cell models to study transfer of medicines across the mammary epithelial barrier.
(iii) To provide an overview of the state-of-the art of the *in vivo* animal models to study transfer of medicines during lactation.
(iv) To provide an overview of the state-of-the art of the empirical and semi-mechanistic models to predict transfer of medicines into the breast milk.
(v) To provide an overview of the available Physiologically-based pharmacokinetic (PBPK) models to determine transfer of medicines into the human breast milk, and subsequent neonatal exposure.

This deliverable will reveal the advantages and limitations of the current state-of-the art, which will serve as a starting point for the development of a non-clinical testing platform to study transfer of medicines during lactation.

Methods

1 Model compounds

The aim was to select at least ten model compounds. These model compounds will be used for the evaluation of the non-clinical platform that will be developed to predict the transfer of medicines into the human breast milk, and subsequent neonatal exposure. The selected model compounds will be used for the development and evaluation of an *in vitro* model for the blood milk epithelial barrier and in a later stage, for the evaluation of Physiologically Based Pharmacokinetic (PBPK) models. Additionally, some of these model compounds will also be used for the *in vivo* animal studies.

First, an extensive list of possible model compounds was made with input from WP3 participants, primarily focusing on clinical relevance of the compounds in the lactating population. An Excel file was made to collect the following information for each compound:

(i) compound name;
(ii) indication;
(iii) class;
(iv) chronic or short term use;
(v) route of administration;
(vi) availability of bioanalytical assay;
(vii) metabolites;
(viii) milk-to-plasma ratio (M/P ratio: concentration-based or AUC-based);

(ix) LogP;
(x) distribution volume;
(xi) unbound fraction in plasma;
(xii) *in vivo* milk/systemic concentrations in mother and neonate via breastfeeding;
(xiii) *in vivo* neonatal systemic concentrations after direct administration;
(xiv) population pharmacokinetic modelling (popPK);
(xv) *in vivo* animal data;
(xvi) Cancer Colon 2 (caco-2) permeability;
(xvii) Human Mammary Epithelial Cells (HMEC) permeability;
(xviii) substrate for Breast Cancer Resistance Protein (BCRP);
(xix) neonatal PBPK models; (xx) clinical relevance;
(xx) Hale classification;
(xxi) LactMed advice on use during lactation; and
(xxii) SmPC information on use of during lactation.

Information was searched for in:

(i) PubMed;
(ii) PubChem;
(iii) the Summary of Product Characteristics (SmPC);
(iv) LactMed;
(v) Drugbank; and
(vi) CYBELE.

Secondly, the set of model compounds was selected from this initial list of possible model compounds. Selection criteria for the model compounds were:

(i) different chemical structures and physicochemical properties;
(ii) different modalities (both low molecular weight and biologicals);
(iii) clinical relevance in the lactating population;
(iv) quality and resolution of available reference data
(v) availability of popPK for breast milk exposure
(vi) availability of PK data.

In addition, the model drugs from the clinical studies of WP4 were considered, as *in vivo* data will become available for these drugs in the near future. The criteria used by WP4 for the selection of model drugs were:

(i) availability of human data;
(ii) diverse therapeutic areas;
(iii) diverse physicochemical properties (including a biologic medicine);
(iv) societal impact of data generation;
(v) ability to assess PK;
(vi) existence of networks to raise awareness for patients to participate in research and to develop ethical standards via empirical qualitative studies (i.e. patient groups);
(vii) patient compliance; and
(viii) feasibility to recruit a sufficient sample size for analysis at appropriate population levels for popPK
(ix) analytical feasibility (1).

2 *In vitro* models

The aim of this literature search was to explore *in vitro* models for the blood milk epithelial barrier as a tool to predict drug transfer into the breast milk. Available human or animal *in vitro* models will serve as starting point for the development of an *in vitro* model by WP3 of ConcepTION.

A literature review was performed searching PubMED and Embase using following search words:
(“epithelial”[tiab] OR ”epithelium”[tiab])
AND
(“cell-culture”[tiab] OR “monolayer”[tiab] OR “cell-cultures”[tiab] OR “monolayers”[tiab])
AND
("transport"[tiab] OR "transported"[tiab] OR "transfer"[tiab] OR "transferred"[tiab] OR "excretion"[tiab] OR "excreted"[tiab] OR "secretion"[tiab] OR "secreted"[tiab] OR "exposure"[tiab] OR "exposed"[tiab] OR "migration"[tiab] OR "migrate"[tiab])
AND
(“mammary”[tiab])

The selection of the articles was performed using Rayyan (2). Articles about *in vitro* models for the blood milk epithelial barrier were included. Both *in vitro* models using cell lines and primary cells were included. The focus was on (i) the culture technique; (ii) the differentiation technique; (iii) the characterization; and/or (iv) the transfer of medicines across the mammary epithelial barrier. Articles were excluded if no full text was available or if they were not written in English.

An additional search has been performed for starting from the reference list of the retrieved articles and utilizing free key words, including: (i) “*in vitro*”; (ii) “mammary epithelial cells”; (iii) “rodent”; and (iv) “animal”.

3 *In vivo* Animal Models

The aim of this literature search was to explore *in vivo* models for the blood milk epithelial barrier as a tool to predict drug transfer into the breast milk. Available animal models will be explored before the development of a specific *in vivo* model within the WP3 of ConcepTION project.

A literature review was performed searching PubMED and Embase using the following search words:
(“animal model” OR “animal models”)
AND
("breastfeeding"[tiab] OR "breast-feed"[tiab] OR "breastfed"[tiab] OR "breast-fed"[tiab] OR "breastfeed"[tiab] OR "breast-feeding"[tiab] OR "lactation"[tiab] OR "breast feeding"[MeSH Terms] OR "breast feeding/adverse effects"[Mesh Terms] OR "lactation/metabolism"[Mesh Terms] OR milk, human/metabolism[Mesh Terms] OR "breast-milk"[tiab])
AND
("drug"[tiab] OR "drugs"[tiab] OR "medicine"[tiab] OR "medicines"[tiab] OR "medication"[tiab] OR "medications"[tiab] OR "pharmaceutical-agent"[tiab] OR "pharmaceutical-agents"[tiab]) AND ("transfer"[tiab] OR "transferred"[tiab] OR "excretion"[tiab] OR "excreted"[tiab] OR "excreted"[tiab] OR "secretion"[tiab] OR "secreted"[tiab] OR "exposure"[tiab] OR "exposed"[tiab] OR "migration"[tiab] OR

"migrate"[tiab])

The search was not wide enough to retrieve all the different existing animal models. Additional searches were performed starting from the reference list of the retrieved articles or with a free search.

Articles on animal models to predict human breast milk exposure or human neonatal systemic exposure via breastfeeding of pharmaceutical agents were included. Articles were excluded if no full text was available or if they were not written in English.

4 Empirical and semi-mechanistic models (human)

The aim of this literature search was to explore empirical and semi-mechanistic models for the prediction of drug transfer into the human breast milk during lactation. Purely empirical models are models that describe the correlation between data, without accounting for the underlying physiological processes as mechanistic models, including PBPK models, do. Semi-mechanistic models are models that lay between the empirical models and the mechanistic models. For some aspects, they rely on physiologically relevant mechanisms, whereas other aspects of the model are not physiologically relevant.

5 Physiologically Based Pharmacokinetic (PBPK) models

The aim of this literature search was to explore Physiologically Based Pharmacokinetic modelling as a tool to predict drug transfer into the human breast milk and subsequent neonatal exposure to drugs via breastfeeding. Reviews on the use of PBPK modelling regarding lactation have been done previously (3). However, at that time PBPK models were only available for chemical substances or for the dairy industry. This review aims to identify PBPK models for medicines used in humans while breastfeeding.

A literature review was performed in PubMed using following search words:
((((("infant"[tiab] OR "neonate"[tiab] OR "infants"[tiab] OR "neonates"[tiab] OR "neonatal"[tiab] OR "newborn"[tiab] OR "newborns"[tiab] OR "infant, newborn"[MeSH Terms])
AND
("exposure"[tiab] OR "poisoning"[tiab] OR "accumulation"[tiab] OR "systemic-concentration"[tiab] OR "plasma-concentration"[tiab] OR "plasma-concentrations"[tiab] OR "systemic-concentrations"[tiab]))
OR (("drug"[tiab] OR "drugs"[tiab] OR "medicine"[tiab] OR "medicines"[tiab] OR "medication"[tiab] OR "medications"[tiab] OR "pharmaceutical-agent"[tiab] OR "pharmaceutical-agents"[tiab]) AND ("transfer"[tiab] OR "transferred"[tiab] OR "excretion"[tiab] OR "excreted"[tiab] OR "excreted"[tiab] OR "secretion"[tiab] OR "secreted"[tiab] OR "exposure"[tiab] OR "exposed"[tiab] OR "migration"[tiab] OR "migrate"[tiab])))
AND
((pbpk model[tiab] OR pbpk modeled[tiab] OR pbpk modeling[tiab] OR pbpk modelling[tiab] OR pbpk models[tiab]) OR "PBPK"[tiab] OR (physiologically based pharmacokinetic model[tiab] OR physiologically based pharmacokinetic modeling[tiab] OR physiologically based pharmacokinetic modelling[tiab] OR physiologically based pharmacokinetic models[tiab]) OR "modeling"[tiab] OR

"modelling"[tiab] OR "mathematical-modeling"[tiab] OR "mathematical-modelling"[tiab] OR "Computer-Simulation"[tiab] OR "pharmacokinetic-modelling"[tiab] OR "pharmacokinetic-modeling"[tiab] OR "computer simulation"[MeSH Terms] OR "models, biological"[MeSH])) AND
("breastfeeding"[tiab] OR "nourish"[tiab] OR "lactate"[tiab] OR "nurse"[tiab] OR "nurture"[tiab] OR "breast-feed"[tiab] OR "breastfed"[tiab] OR "breast-fed"[tiab] OR "breastfeed"[tiab] OR "nursed"[tiab] OR "nourished"[tiab] OR "nurtured"[tiab] OR "breast-feeding"[tiab] OR "lactation"[tiab] OR "lactated"[tiab] OR "breast feeding"[MeSH Terms] OR "breast feeding/adverse effects"[Mesh Terms] OR "lactation/metabolism"[Mesh Terms] OR "milk, human/metabolism"[Mesh Terms] OR "breast-milk"[tiab] OR "lactate"[tiab])

The selection of the articles was performed using Rayyan (2). Articles about PBPK models to predict human breast milk exposure or neonatal systemic exposure to maternal medication via breastfeeding were included. Articles were excluded if no full text was available or if they were not written in English. An additional search has been performed for starting from the reference and citation list of the included articles.

Data extracted from the selected articles included: (i) information about the medicine (e.g. compound name, indication, administration route and simulated dose); (ii) information about the model development (e.g. PBPK software platform used, genotype specific simulations, source of input parameters and method to implement breastfeeding); and (iii) information about the model verification (e.g. method to determine model performance, acceptance criteria and sensitivity analysis)

In addition, a literature search was performed to investigate the effect of maternal conditions on the macro-nutrient composition of breast milk. A literature search in PubMed was performed using “human milk composition” as search term. The articles were screened for maternal specific characteristics that may have an influence on the macro-nutrient composition of the milk and may be of relevance during the modeling (like for instance diabetes, coeliakie, but also mastitis, obesity or specific diets). An overview of the overall findings for each condition was made.

Results

1 Model compounds

An excel file with the initial list of possible model compounds contained more than 100 rows, including the model drugs selected in WP4 (*Table 1: Model compounds selected by work package 4*). Considering these WP4 compounds, WP3 selected a first set of 10 model compounds from this list (*Table 2: First set of model compounds selected by work package 3*).

Table 1: Model compounds selected by work package 4

Drug **Indication** **C/I** (4)	**Route of administration** (4)	**M/P ratio** (5)	**pKa** **logP** **Vd (L/kg)** **Fu** **$T_{1/2}$ (h)** **CL** (5,6)	**Transporters and enzymes** (7)(8)	**Metabolites** **Active metabolites in bold**(6)	**Available *in vivo* milk/plasma concentrations** (9)
Venlafaxine Depression, general anxiety C	Oral	2.5 – 4.1 (10)	8.91[c], 14.42[c]; 2.69 - 2.74[c]; 7.5; 0.73; 5; 0.585 L/kg/h [a]	Induction of BCRP expression(11) P-gp CYP2C19 CYP3A4 CYP2D6	**O-desmethylvenlafaxine** N-desmethylvenlafaxine N,O-didesmethylvenlafaxine N,O-didesmethylvenlafaxine glucuronide N,N,O-tridesmethylvenlafaxine O-desmethylvenlafacine glucuronide	Maternal milk and plasma concentrations available Neonatal concentrations via breastfeeding available Rat data available (12)
Amoxicillin Antibiotic I	Oral Intravenous	0.013 – 0.043	3.23[c], 7.43[c]; 0.87; 0.3; 0.8; 61 min;	PEPT1/2 Not a BCRP substrate (14)	amoxicilloic acid amoxicillin diketopiperazine-2'5'-dione (15)	Limited maternal milk and plasma concentrations available (6 women) No neonatal concentrations via breastfeeding found

			21.3 L/h; (13)			Neonatal data after direct administration available Cow, mink and data available Neonatal popPK available (16–20)
Clavulanic acid Antibiotic (combination with amoxicillin) I	Oral Intravenous	N/A	2.7; -2.3; 12 L; 0.75; 45 – 90 min; 12.6 L/h (21)	N/A	2,5-dihydro-4-(2-hydroxyethyl)-5-oxo-1H-pyrrole-3-carboxylic acid 1-amino-4-hydroxy-butan-2-one	N/A
Metformin Antidiabetic C	Oral	0.35 – 0.63	12.4; -2.6; 4; 1; (22) Plasma: 6.2 Blood: 17.6; 27.54 L/h (23)	OCT1/2/3 MATE1/2K PMAT	Not metabolized	Maternal milk and plasma concentrations available Limited neonatal concentrations available (undetectable) via breastfeeding Rat and mice data available (24)(25)
Cetirizine (seasonal rhinitis) I/C	Oral	N/A	1.52, 2.92, 8.27; 2.8; 0.44 -0.56; 0.07; 8.3; Cl/F = 3.18 L/h[b]	MATE1/2K inhibitor OCT2 inhibitor P-gp	oxidative O-dealkylation metabolite	N/A
Levo-cetirizine	Oral	N/A	3.59 [c]; 7.42 [c] ; 0.87 - 2.98[c];	P-gp	Levocetirizine Dihydrodiol Metabolite (M2)	N/A

Seasonal rhinitis I/C			0.33 -0.4; 0.039 - 0.081; 7.05; Cl/F = 34.2 L/h/kg[b]		Levocetirizine Hydroxymethoxy Metabolite (M4) Levocetirizine Hydroxy Metabolite (M5) Levocetirizine N-oxide Metabolite (M3) Levocetirizine O-glucuronidated Metabolite (M1) Levocetirizine O-dealkylated Metabolite (M6) Levo etirizine Taurine Conjugated Metabolite (M8) Levocetirizine N-dealkylated and Aromatic Hydroxylated Metabolite (M9) Levocetirizine 4-chloro-4'-hydroxybenzhydryl Mercapturate Metabolites (M10a and M10b)	
Infliximab Crohn's disease, rheumatoid arthritis Psoriatic arthritis Ankylosing spondylitis C	Intravenous	N/A	N/A; N/A; 0.065 – 0.081; N/A; 7.7-9.5 days; 0.0099 L/h (26)	N/A	N/A	Maternal milk and plasma concentrations available Limited neonatal concentrations via breastfeeding available

C: chronic; I: incidental; M/P ratio: milk-to-plasma ratio; Vd: distribution volume; $t_{1/2}$: half-life; Cl: clearance; BCRP: breast cancer resistance protein;

PEPT: peptide transporter; MATE: multidrug and toxin extrusion protein; OCT: organic cation transporter; a: calculated from bioavailability and apparent clearance; b: only apparent clearance found; CYP: cytochrome P450; PMAT: plasma membrane monoamine transporter; a: calculated from bioavailability and apparent clearance; b: only apparent clearance found; c: predicted values by ChemAxon and ALOGPS

Work package 4 selected the following model compounds:

(i) venlafaxine; Venlafaxine is an antidepressant commonly used in the postpartum. Some human data is available, but no popPK data so far.

(ii) amoxicillin (alone or in combination with clavulanic acid); Amoxicillin is a commonly used antibiotic in women in the 1st week postpartum. Guidelines differ between countries regarding its use alone or combined to clavulanic acid; therefore both amoxicillin alone and amoxicillin in combination with clavulanic acid will be included in the human lactation studies.

(iii) metformin; Metformin is included in WP4 because of the raising prevalence of diabetes type 2 in young women.

(iv) (levo)cetirizine; Anti-allergic drugs like (levo)cetirizine are commonly used during breastfeeding as seasonal allergies or other allergies are highly prevalent.

(v) infliximab; Infliximab was chosen in WP4 as a biological used to treat for instance rheumatoid arthritis.

Table 2: First set of model compounds selected by work package 3

Drug **Indication** **C/I** (4)	**Route of administration** (4)	**M/P ratio** (5)	**Pka** **logP** **Vd (L/kg)** **Fu** **$T_{1/2}$** **Cl** (5,6)	**Transporters and enzymes**(7)(8)	**Metabolites** (6) **Active metabolites in bold**	**Available *in vivo* milk/plasma concentrations** (9)
Venlafaxine Depression C	Oral	2.5 – 4.1 (10)	8.91[c], 14.42[c]; 2.69 - 2.74[c]; 7.5; 0.73; 5; 0.585 L/h/kg[a]	Induction of BCRP expression(11) P-gp CYP2C19 CYP3A4 CYP2D6	**O-desmethylvenlafaxine** N-desmethylvenlafaxine N,O-didesmethylvenlafaxine N,O-didesmethylvenlafaxine glucuronide N,N,O-tridesmethylvenlafaxine O-desmethylvenlafaxine glucuronide	Maternal milk and plasma concentrations available Neonatal concentrations via breastfeeding available Rat data available, (12)
Amoxicillin Antibiotic	Oral Intravenous	0.013 –	3.23[c], 7.43[c] 0.87;	PEPT1/2 Not a BCRP	amoxicilloic acid amoxicillin diketopiperazine-	Limited maternal milk and plasma concentrations

I		0.043	0.3; 0.8; 61.3 min; 21.3 L/h (13)	substrate (14)	2'5'-dione (15)	available (6 women) No neonatal concentrations via breastfeeding found Neonatal data after direct administration available Cow, mink and data available Neonatal popPK available (16)(17)(18)(19)(20)
Metformin Antidiabetic C	Oral	0.35 – 0.63	12.4; -2.6; 4; 1; (22) Plasma: 6.2 Blood: 17.6; 27.54 L/h (23)	OCT1/2/3 MATE1/2K PMAT	Not metabolized	Maternal milk and plasma concentrations available Limited neonatal concentrations available (undetectable) via breastfeeding Rat and mice data available (24)(25)
Valproic acid Epilepsy C	Oral	0.42	4.8; 2.75; 0.1-1.4; 0.9 – 0.815; 13 – 19; 0.504 L/h/m^{2} [a]	Monocarboxylate transporter Not a BCRP substrate (27) CYP2A6 CYP2C9 CYP2B6 UGT1A4 UGT1A10 UGT1A3 UGT1A9 UGT1A8	**2-ene valproic acid ,** **Toxic: 4-ene valproic acid, 2,4 diene valproic acid CoA, 3-oxo valproic acid CoA** 3-hydroxy valproic acid 5-hydroxy valproic acid 4-hydroxy valproic acid Valproate glucuronide 4-ene valproic acid CoA Valproic acid CoA Valproic acid dephosphoCoA 2-ene valproic acid CoA Thiol conjugates	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding Monkey and rat data available PBPK model for adults and children for direct administration of valproic acid (19)(28)(29)(30)

				UGT1A6 UGT2B7 UGTB15 2-methyl-branched chain acyl-CoA dehydrogenase medium-chain acyl-CoA synthase Isovaleryl-CoA dehydrogenase enoyl-CoA hydratase, crotonase 2-methyl-3-hydroxybutyryl-CoA dehydrogenase 3-keto-valproyl-CoA thiolases	3-oxo valproic acid CoA 3-ene valproic acid CoA 2,3-diene valproic acid CoA C3 CoA C5 CoA	
Tacrolimus Allogenic organ transplant C	Oral	0.54	-2.9 [c], 9.96 [c]; 3.3; 1.07-3.9; 0.01; 35;	OATP2 inhibitor P-gp (31) CYP3A5 CYP3A4	**31-O-demethyl tacrolimus** 13-O-Desmethyltacrolimus 15-O-Desmethyltacrolimus	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding

			0.040 L/h/kg			
Tenofovir HIV, PrEP C	Oral	0.03-0.07 (32)	3.8, 6.7; -1.6; 0.813 – 1.3; 0.93; 32; Cl/F = 145.63-163.93 L/h[b] (33,34)	MRP2/4, OAT1/3 Not a BCRP substrate (35) Adenylate kinases Nucleotide diphosphate kinases	**Tenofovir biphosphate** Tenofovir monophosphate	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding or direct administration Macaques data available(36) Neonatal popPK model (37)
Zidovudine HIV C	Oral Intraveneous	3.21	-3 [c], 9.96 [c]; 0.05; 1.6; 0.62 – 0.70; 1.1; 1.3715-1.405 ; L/h/kg[a] (38)	OAT1/2/3/4 MDR1 MRP4/5 BCRP CNT1/3 Weak BCRP inhibition (35) UGT2B7 Thymidine kinase Thymidylate kinase Nucleoside diphosphate kinase CYP2C9 CYP2A6 CYP2E1 CYP3A4	**Zidovudine triphosphate** **Toxic: 3'-amino-3'-deoxythimidine** 3'-azido-3'-deoxy-5'- O-beta-D-glucopyranuronosylthymidine 3'-amino-3'-deoxythimidine glucuronide 5'glucuronyl zidovudine	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding or direct administration Rat data available (39)(40)

Nevirapine HIV C	Oral	0.95(41)	5.06, 10.37; 2.5; 1.21- 1.4; 0.40; 45; 0.0181-0.0228 L/kg/h[a] (42)	MRP7 Weak BCRP inhibition (35) Strong P-gp inhibitor CYP2B6 CYP2D6 CYP3A4 Cyp3A5 CYP2C9 UGT	2-Hydroxynevirapine 12-hydroxynevirapine glucuronide 4-carboxynevirapine 2-Hydroxynevirapine 2-hydroxynevirapine glucuronide 8-Hydroxynevirapine 8-hydroxynevirapine glucuronide 3-Hydroxynevirapine 3-hydroxynevirapine glucuronide 2-OH-nevirapine 8-OH-nevirapine 3-OH-nevirapine 12-OH-nevirapine	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding or direct administration (43)
Sertraline Depression C	Oral	0.89	9.16; 5.1; 20; 0.02; 26; Cl/F = 1.09-1.41 L/h/kg[a] (44)	Weak P-gp substrate CYP2B6 CYP2C9 CYP2C19 CYP3A4 CYP2D6 CYP2E1 Monoamine oxidase A/B UGT1A6 UGT1A3 UGT2B4	**N-desmethylsertraline** Alfa-hydroxy sertraline ketone Sertraline carbamoyl-O-glucuronide Alfa-hydroxy sertraline ketone glucuronide	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding Rat data available (12)

				UGT2B7		
Leve-tiracetam Epilepsy C	Oral Intravenous	1.0	-1.6 [c]; 16.09 [c]; -0.6; 05 - 0.7; 0.9; 6-8; Cl/F=0.0576 L/h/kg[b] (45)	Not a BCRP substrate (46) B-esterase	levetiracetam carboxylic acid metabolite	Maternal milk and plasma concentrations available Neonatal concentrations available via breastfeeding or direct administration Neonatal popPK (47)

C: chronic; I: incidental; M/P ratio: milk-to-plasma ratio; Vd: distribution volume; $t_{1/2}$: half-life; Cl: clearance; BCRP: breast cancer resistance protein; PEPT: peptide transporter; MATE: multidrug and toxin extrusion protein; OCT: organic cation transporter; OAT: organic anion transporter; OATP: organic anion-transporting peptide; MRP: multidrug resistance-associated protein; P-gp: P-glycoprotein*; CYP: cytochrome P450; PMAT: plasma membrane monoamine transporter*; UGT: UDP-glucuronosyltransferase;CNT: concentrative nucleoside transporter; *a: calculated from bioavailability and apparent clearance; b: only apparent clearance found; c: predicted values by ChemAxon and ALOGPS*

The additional model compounds selected by WP3 are:

(i) valproic acid;
(ii) tacrolimus;
(iii) tenofovir;
(iv) zidovudine;
(v) nevirapine;
(vi) sertraline; and
(vii) levetiracetam.

This selection was made from the initial list of possible model compounds. All compounds included in this initial list were either clinically relevant for breastfeeding women, and/or with another strong reason for including a compound in this list (e.g. compounds that could help to obtain mechanistic insights in the PBPK models that will be developed). The initial list was reduced by excluding all compounds for which limited clinical data (maternal milk concentrations and neonatal systemic concentrations) were available, since this is important to evaluate the non-clinical tools that will be developed. The list of possible WP3 model compounds was reduced to ten model compounds (model compounds from WP4 not

included) in this 1st step. Therefore, it was not possible to account for all the other pre-specified criteria in this first set of model compounds. The final model compounds were selected from this list based on differences in physicochemical properties, M/P ratios and the availability of PK data. PopPK was available for some of the model compounds, but was not used as a critical criterion for the first set of model compounds, although the availability of clinical data and differences in physicochemical properties are essential for the development of robust *in vitro* and PBPK models. No biologicals were included in the first set of model compounds to reduce complexity and because extensive clinical data (milk concentrations and neonatal systemic concentrations) were not available for any biological drug.

2 *In vitro* models

2.1 Available *in vitro* models for the mammary epithelium

In vitro cell culture models for the mammary epithelium have been established to predict drug partitioning into the breast milk, based on the assumption that the mammary epithelium is the main barrier between the systemic circulation and the milk. Many cell culture models have been established based on human or animal mammary epithelial cell cultures, including both primary cells and cell lines. In 2006, the first human model to predict drug transfer into the human breast milk was developed by Kimura et al. (48). They used the method from Schmidhauser et al. (49) to obtain trypsin-resistant cells, which have the ability to differentiate into the lactating state. More recently, Andersson et al. (50) developed a model to evaluate the transfer of the neurotoxic amino acid beta-N-methylamino-alanine (BMAA) into the breast milk based on the human mammary MCF7 cell line. Other cell lines have been used to investigate the mammary gland (e.g. PMC42-LA (51)) and in the field of breast cancer (e.g. R5, MCF7, MDA-MB-231-LUC, MCF10A and primary epithelial cells (52)). In addition, MDCK II cells transfected with human BCRP have also been used to optimize predictions of M/P ratios for drugs (53). Many *in vitro* models relying on mammary epithelial cells have been established based on cells obtained from animal tissue. Two main categories can be distinguished for the animal *in vitro* models: rodent epithelial cells (*Table 3: rodent epithelial cell culture models)* and non-rodent *in vitro* models (*Table 4: Non-rodent animal cell culture models*).

Table 3: Rodent epithelial cell culture models

Cell culture model	Species	References
HC11	Mouse	(50)
CIT3	Mouse	(54–56)
RME cells	Rat	(57)

Table 4: Non-rodent animal cell culture models

Cell culture model	Species	References
Primary culture of Porcine Mammary Epithelial Cells	Porcine	(58–61)
BME-UV Immortalized bovine mammary epithelial cells	Bovine	(62–65)
Primary goat epithelial cells pgMECs	Goat	(66)

2.2 Culture conditions

Mammary epithelial cells are either obtained via isolation from normal breast tissue (67), tumor breast tissue (67) or breast milk (68) or are obtained from commercial cell suppliers (e.g. ATCC, Lonza, Promocell or Sigma-Aldrich). Mammary epithelial cell basal medium (MEBM) (*Table 5 Reported basal cell culture media for in vitro cell models)*, with addition of several supplements is recommended by all suppliers for the growth of primary human mammary epithelial cells. Different supplements are recommended by the suppliers and published articles on *in vitro* models (*Table 6 Reported cell culture media supplements for in vitro cell models)* MEBM has also been used by Kimura et al. for their cell model for drug transfer across the mammary epithelium (48). RPMI 1640 has been used by Andersson et al. for the culture of the MCF7 cell line (50). RPMI 1640 and DMEM:F12 (50:50) have been used for human mammary epithelial cell lines in other applications. Both basal media are also used regarding the growth of cells from animal origin. Basal RPMI 1640 and DMEM:F12 (50:50) are not specific for epithelial cells. However, the addition of particular supplements *(Table 6 Reported cell*

culture media supplements for in vitro cell models) makes them suitable for the growth of mammary epithelial cells. Different supplements and concentrations are recommended by the suppliers and previously mentioned *in vitro* culture models. The choice of supplements and in particular of prolactin is strategic to work with a model of secreting cells or not. Freestone et al. (51) were even able to make a model for the resting, lactating and suckled mammary epithelium, by adding no, 200 ng/mL, or 800 ng/mL prolactin respectively to the PMC42-LA cell line.

Table 5: Reported basal cell culture media for in vitro cell models

Medium	References
(DMEM) with Ham's F12 (50:50)	(55,58–62,69)
RPMI 1640	(50,63–66)
MEBM	(48)

Table 6: Reported cell culture media supplements for in vitro cell models

Supplement	Typical concentration range	Function	References
Insulin	1-10 µg/ml	Cell health and productivity in serum free medium Growth factor helping cells in utilization of glucose and amino acids	(48,50,55–59,65,66,69–73)
IGF-1 Insulin like growth factor 1	0-100 ng/mL	Cell proliferation and survival, similar function as insulin, allows to use a lower concentration of insulin	(57,60,61,74)
(Apo-) transferrin	5-10 µg/ml	Cell health and productivity in serum free medium Iron carrier, providing iron, regulation of iron uptake for maintenance of homeostasis	(57,70,71)
Insulin-transferrin-selenium	5 µg/ml	Cell health and productivity in serum free medium Selenium is sometimes added when working in serum free medium	(60,61,70)
Epinephrine	1 µM	Stimulation of cell proliferation	(71,75)
Epidermal growth factor (EGF)	1-10 ng/ml	EGF has a role in the development of mammary tissue. EGF stimulates cell proliferation	(48,50,55–57,59–61,65,69,72,73,76)
rH-TGF-alfa (recombinant human, transforming growth factor alfa)	5 ng/ml	Induction of epithelial development, similar biological function as EGF	(71,77)
Fetal bovine	2 % -10 %	Cell growth	(50,55,56,58–

serum			62,65,66,69)
Bovine pituitary extract	0.4 %	Hormones, cytokines, mitogens and growth factors for growth in serum free medium	(48,55,69,71–73,78)
Iron supplemented bovine calf serum	2 %	Cell growth	(62)
Newborn bovine serum	3 %	Cell growth	(62)
Bovine serum albumin fraction V	2.5 mg/ml	Cell growth	(57)
Hydrocortisone (hemi succinate)	100 ng/ml – 0.5 µg/ml	Growth and differentiation	(48,55,57–61,65,66,69,71–73,79)
Prolactin	1-3 µg/ml	differentiation	(48,55,57,65,66,69)
Gentamicin sulfate-amphotericin (GA-1000)	0.5 ml/500 mL	Antibiotics	(48,72)
Amphotericin B	2.5 µg/ml	Antibiotic	(57)
Gentamicin sulfate	50 µg/ml	Antibiotic Induction of fibroblast cell death (for isolation of mammary epithelial cells)	(57,59,65)
Antibiotic/antimycotic solution	1 %	Antibiotic/antimycotic	(58)
Penicillin	1000 U/ml	Antibiotics	(50,55,56,60–62,66,69)
Streptomycin	100 µg/ml	Antibiotics	(50,55,56,60–62,66,69)
Penicillin Streptomycin Neomycin	1x PSN Sigma 50 U/ml 50 µg/ml 100 µg/ml	Antibiotics	(59)
MEM non-essential amino acids	1 %	Amino acids, growth	(50,80)
L-Glutamine	2-6 mM	Amino acid, energy source	(66,71,81)
L-methionine	0.1 mM	Amino acid, energy source	(66,81)
L-lysine	0.4 mM	Amino acid, energy source	(66,81)
Trace element	1 nM MnCl2, 10	Cell growth	(57,82)

cocktail	nM H2SeO3, 1 nM (HN4)6MO7O24, 5 nM NH4VO3, 0.5 nM NiCl2, 0.5 nM SnCl2		
Estradiol	0.5 ng/mL	Cell growth	(57,83)
Cholera toxin	0.1 µg/ml	Stimulates growth	(57,84)
Progesterone	0.05 µg/ml	Growth promotion	(57)

Characterization of the *in vitro* models is important to ensure that the model is applicable to the *in vivo* situation for a given compound. An overview of all available information regarding transporters has recently been given by Ventrella et al. (85).

2.3 Human *in vitro* models to predict drug transfer into the breast milk

Two human models have currently been reported for the prediction of drug transfer into the breast milk: one using primary cells and one using a cell line. Primary cells are known to mimic the *in vivo* physiology closer than cell lines (86). The major advantage of cell lines is that they are easier to handle and have an infinite life span (86). Kimura et al. (48) used primary human mammary epithelial cells. They were able to obtain a monolayer with a transepithelial electrical resistance of 227 +/- 11 Ohm cm^2 after three trypsin treatments. They detected beta-casein mRNA, indicating that the monolayer is differentiated into the lactating state. Shipman et al. (87) showed that beta-casein is only expressed in the lactating state. Besides beta-casein, Kimura et al. (48) also detected mRNA of organic cation transporter (OCT) 1 and OCT3. Interestingly, they found that OCT1 mRNA increased, whereas OCT3 mRNA decreased with increasing the number of trypsin treatment. This observation was consistent with the observations of Alcorn et al. (88), who observed that OCT1 RNA levels are higher, while OCT3 RNA levels are lower *in vivo* in the lactating state compared to the non-lactating state. Kimura et al. (48) also investigated the function of OCT and organic anion transporter (OAT) with the substrates tetraethylammonium (TEA) and p-aminohippurate respectively. A clear directionality was observed for TEA, indicating a functional OCT transporter is present. However, no directionality was observed for p-aminohippurate, although OAT mRNA is present *in vivo*. Other transporters, for instance BCRP, that have not been investigated in this study play an important role *in vivo*. Therefore, further characterization of this *in vitro* model is required in order to conclude whether this is a good model to evaluate drug transfer into the human breast milk.

Andersson et al. (50) developed a human *in vitro* model to investigate the transfer of D-BMAA and L-BMAA into the human breast milk based on the MCF7 cell line. MCF7 is a cell line derived from a metastatic site of an adenocarcinoma, via pleural effusion (89). MCF7 expresses estrogen and progesterone receptors and is known to have some characteristics of the differentiated mammary epithelium (89). Andersson et al. (50) found that uptake was higher in the differentiated model. Furthermore, via inhibition studies with natural amino acids, they concluded that several amino acid transporters might be involved in the uptake. Additionally, they found some differences in mRNA levels of orthologous transporters in the MCF7 cell line compared to the mouse HC11 cell line. Finally, they compared mRNA expression in undifferentiated and differentiated HC11 cells and found that mRNA increased for some transporters, whereas mRNA decreased for others.

Besides MCF7, many other human cell lines have been used to investigate the mammary gland. For

example, MCF10A has been used commonly as a model to investigate normal breast cells. MCF10A is an immortalized, non-tumorigenic cell line obtained from benign proliferative breast tissue (90). MCF10A does not express estrogen or progesterone receptors. Furthermore, no beta-casein or alfa-lactalbumin were detected (90). Ying Qu et al. (90) questioned whether MCF10A cells are a good model for normal breast cells. They conclude that further investigations are required. Another frequently used cell line is the MDA-MB-231 cell line. This cell line was obtained via pleural effusion of a patient with metastatic mammary adenocarcinoma (91). The MDA-MB-231 cell line does not express an estrogen or progesterone receptor. MDA-MB-231 might not be suitable as a model for normal lactating mammary epithelial cells, as it is a highly aggressive, invasive and poorly differentiated cell line that is mainly used to investigate triple-negative breast cancer. The PMC42-LA cell line, although this cell line has not been used as frequently as the previously mentioned cell lines, might be a good model for the lactating mammary gland. PMC42-LA is a mesenchymal breast carcinoma cell line that has been obtained from a pleural effusion. Freestone et al. (51) were able to develop a resting, lactating and suckling *in vitro* model with this cell line by using different concentrations of prolactin. They indicate that the capacity to differentiate is a major advantage of this cell line compared to many other cell lines. It has also been shown that PMC42-LA cells express beta-casein after stimulation with lactation hormones(51).

Animal *in vitro* models to predict drug transfer into the breast milk

Prediction of drug transfer into the human breast milk based on animal *in vitro* models might be difficult due to species differences (e.g. differences in enzyme and transporter expression). Animal *in vitro* models can play a key role for the *in vitro / in vivo extrapolation* of human data. Working with animal cells offers the possibility of having different models. In fact, the cells can be isolated from glands in all the different physiological phases, including the lactating gland.

RME cells are derived from normal mammary glands of 50-60-day-old virgin female Lewis rats (57), while both mouse cell lines, HC11 and CIT3 cells are derived from COMMA-1D cells, obtained from mammary tissue of BALB/c mice in the middle of pregnancy. CIT3 are selected for resistance to triple trypsinization while HC11 have been immortalized. Both cell lines have been used for active transport studies of drugs such as nitrofurantoin. Porcine mammary epithelial cells can be derived from non-pregnant and non-lactating gilt (59). These primary cells can be maintained in culture for at least 15 passages and could represent a good model for studying molecular regulation and synthesis of milk. Alternatively, porcine mammary epithelial cells can be obtained from mammary gland after parturition (60); from these primary cells arose a cellular line spontaneously immortalized with secreting features.

BME-UV is a clonal cell line established from primary epithelial cells from a lactating Holstein cow. This cell line expresses functional markers such as microvilli and desmosomes and secreting properties (62), and expresses functional organic anion and cation transporters (63,64). Primary goat mammary epithelial cells (pgMECs) derived both from mammary tissue of lactating or non-lactating juvenile goats grow *in vitro* for several passages and remain hormone and immune responsive with a secreting phenotype (55).

3 *In vivo* Animal Models

Different aspects need to be considered when selecting an animal species for modelling the lactational transfer of xenobiotics in human (92). The main parameters related to lactation (anatomy of the udder, amount of milk production, composition of milk, duration of lactation) are variable in the

different animal species as well as the drug metabolism and transporters (enzymatic tools and pathways) (65,93–103).

Rodents are usually considered an excellent model in many fields of research, but their metabolic and digestive patterns and milk composition are quite different from humans with significant differences in drug levels reached in blood and the potential lactational transfer (104–106). Indeed, in such species, the general aspects of reproduction (age of sexual maturation, hormone sensitivity, reproductive lifespan, litter size) are very different when compared to humans (99). Nevertheless, most of studies clarifying the development of mammary cancer have been performed in rodents due to their relatively easy manipulation and housing requirements (107,108). An additional issue working with rodents relates to the body dimension that allow only for a low milk sampling limiting the possibility of end point analysis.

The drug transfer from blood to milk has been extensively studied in ruminants (96,109), mainly for human safety reasons, due to their role as food producing animals (in particular milk and dairy products). Nevertheless, these animals differ significantly from human from an anatomical point of view and mainly, from metabolic point of view due to their peculiar gastrointestinal physiology.

Swine offer a generally accepted model in translational medicine mainly based on anatomical and physiological similarity with human. Its use as a model for nutritional physiology, drug testing and metabolism has been generally acknowledged (93,102,110–113). Mammary gland anatomy shows macroscopic differences but, at molecular level, the presence of drug transporters similar to human has been reported ((*See Table 2 from Ventrella et al.* (85)*).*The lactation duration is shorter, compared to humans. These points have to be considered when studying the lactational transfer in the different phases of lactation, especially for colostrum composition (95). The litter size allows for an easy milk collection without interfering with the lactation process and piglet's growth; piglets can be analyzed individually and have been already utilized as animal model for pediatric PK/PD (114). In recent years, the minipig, and in particular Göttingen Minipigs, has been proposed as a more relevant animal model in translational medicine in particular for toxicological studies (115,116). They show, together with all the positive characteristics described for domestic swine breeds, a reduced growth rate that makes them easier to use. Moreover, a much more precise control of genetical, microbiological and dramatipical standardization, allows a significant reduction in the number of animals needed for experimental trials (117,118). The fact that Göttingen Minipigs are available in a constant and uniform quality worldwide forms the basis of their recognition by regulatory bodies as a suitable non-rodent species for toxicology studies. However, data regarding qualitative and quantitative composition of the milk, as for the domestic pig, are lacking.

The most relevant papers are reported in *Table 7: Animal models of lactational transfer* with the indication of the relevant aspects and the species studied.

Table 7 Animal models of lactational transfer

Main aspect of model discussed	Species	Reference
Animal models in evaluation of the safety of drugs used during lactation	Multiple	(92)
Biomarkers	Swine	(111)
	Swine	(112)
Colostrum	Swine	(95)

Comparative pathology of tumors	Mice	(107)
Digestive system	Swine	(102)
Drug development	Multiple	(113)
Drug metabolism	Multiple	(97)
	Rodents	(104)
Drug milk transfer	Sheep	(109)
	Rat	(106)
Efflux Transporter	Multiple	(100)
	Multiple	(101)
	Multiple	(65)
	Cow	(96)
	Swine	(103)
General	Mice	(99)
Hormonal sensitivity	Mouse	(108)
Metabolism	Rodents	(105)
Metformin treatment during pregnancy	Swine	(110)
Nutritional aspects	Swine	(93)
Non-clinical models of Lactational transfer	Multiple	(85)
Pharmacokinetic	Swine (minipig)	(116)
Pharmacokinetic and pharmacodynamic	Swine	(114)
Placentation	Multiple	(94)
Sexual maturation	Swine (minipig)	(117)
Toxicology	Swine (minipig)	(118)
	Swine (minipig)	(115)

4 Empirical and semi-mechanistic models (human)

Several attempts have been made to predict the drug milk transfer. In 1959, Rasmussen et al. (119) first assumed pH-dependent diffusion of drugs. Notarianni et al. (120) developed an equilibrium dialysis model to test the partitioning of drugs between freeze dried plasma and baby formula powder over a dialysis membrane. Other diffusion models have been developed by Atkinson et al (121) and Fleishaker et al. (122). Meskin and Lien (123) developed an *in silico* model based on the relation between physicochemical properties (the molecular weight, partition coefficient and degree of dissociation) and the transfer of drugs into the milk. This model was later extended with an artificial neural network by Agatonovic-Kustrin (124). Quantitative structure-property relationship/activity relationship tools have been explored as well (125). Many others have tried to predict milk transfer using similar methods. However, the major issue with all of these methods was that they do not take transporter-mediated processes into account.

In 2011, a semi-mechanistic model was developed by Koshimichi et al. (126) (*See Fig (1) from Ventrella et al.* (85).) to predict drug transfer into the milk (126). Milk secretion and reuptake clearance values were estimated by curve fitting against observed milk and plasma concentration-time profiles. Next, the fraction of unbound drug in the milk was compared to the fraction of unbound drug in the plasma for each drug to determine whether passive diffusion or transporter-mediated transfer is the most likely route for drug transfer into the human breast milk. For the drugs with passive diffusion as main pathway, an equation describing the relation between the physicochemical properties and the secretion and reuptake clearance values was determined by multiple linear regression. This semi-

mechanistic model might be applied to determine M/P area under the curve ratios for new drugs. Koshimichi et al. predicted the M/P area under the curve for several drugs, including metformin and tacrolimus which are two of the model compounds selected for the development of the non-clinical platform in this Innovative Medicines Initiative (IMI) project (*Table 8: Predicted and observed milk-to-plasma (M/P) area under the curve ratios for metformin and tacrolimus by Koshimichi et al.*) (126).

Table 8: Predicted and observed milk-to-plasma (M/P) area under the curve ratios for metformin and tacrolimus by Koshimichi et al. (126)

Compound	Observed M/P ratio	Predicted M/P ratio
Metformin	0.48 +/- 0.12	0.25
Tacrolimus	2.20	22.7

Koshimichi et al. (126) were able to predict M/P area under the curve values for 71.9% of the drugs, be it within a 3-fold error. However, they also mention some limitations. First, they did not take the dynamic volume of the milk compartment into account. Milk containing the drug can be eliminated by nursing. Furthermore, the volume of the milk compartment is variable during lactation due to milk production.
Secondly, there might be a prediction error on the unbound fraction of drugs in the milk. The unbound fraction in the milk was experimentally determined for some drugs and predicted using the equation from Atkinson and Begg (127) for other compounds. The prediction error might be especially important for the drugs for which no experimentally derived unbound fraction in the milk is available.
Thirdly, the lipid content in the milk is variable during a feed and over time in the postpartum period. Drug partitioning into the human breast milk can be affected by the lipid content.
Lastly, drugs reaching rapid equilibrium (net reuptake clearance over 5000 mL/h) were not considered for the multiple linear regression. However, the predictions for these drugs seem to be equally well as for other drugs. Moreover, predicted net reuptake clearance seemed to be less than 5000 mL/h for these drugs. Koshimichi et al. assumed that the net reuptake clearances over 5000 mL/h were obtained by mistake due to fluctuations in the concentrations. They assume that the model can be applied for most drugs, but caution is required when predicted net reuptake clearance values are above 5000 mL/h.

5 Physiologically Based Pharmacokinetic (PBPK) models

5.1 Physiologically based pharmacokinetic (PBPK) modelling

PBPK modelling is a bottom-up approach, whereas population popPK is a top-down approach. PopPK aims to analyze *in vivo* data to understand the underlying parameters leading to variability in the observed PK profile. A PBPK model is defined by the European Medicines Agency as “a mathematical model that simulates the concentration of a drug over time in tissue(s) and blood, by taking into account the rate of the drug’s absorption into the body, distribution in tissues, metabolism and excretion (ADME) on the basis of interplay between physiological, physicochemical and biochemical determinants” (128)

PBPK modelling is for instance often applied to predict drug-drug interactions and to select an initial dose for pediatrics and first-in-human trials (128). Furthermore, PBPK modelling can be used to predict transfer of compounds into the breast milk and subsequent neonatal exposure. Research in this field has focused on persistent, bio-accumulative substances (e.g. trichloroethylene, tetrachloroethylene, organic solvents and p,p’,-dichloro-2,2-bis(p-chlorophenyl)ethylene), milk

transfer in animals providing milk for human consumption (3), and PBPK in human milk setting in the field of toxicology. More recently, some PBPK models for the prediction of transfer of medicines into the human breast milk and subsequent neonatal exposure, further referred to as lactation PBPK models, have been reported (*Table 9: PBPK models for transfer of medicines into the human breast milk and subsequent neonatal exposure*). Five articles and four conference abstracts about lactation PBPK models were retrieved. The reported lactation PBPK models consist of a maternal PBPK model coupled to a neonatal PBPK model, allowing them to predict milk transfer and neonatal exposure via breastfeeding. The PBPK models for alprazolam, caffeine and tramadol only consist of a maternal PBPK model, and can thus only predict milk transfer(129,130). The model for escitalopram is a combination of popPK to analyze human breast milk data and PBPK modelling to predict infant exposure (131). Only the five articles will be discussed in the next sections

Table 9: PBPK models for transfer of medicines into the human breast milk and subsequent neonatal exposure

Compound (indication)	Dose	Administration route	Software	Reference
Escitalopram (depression, including postpartum)	20 mg/day	Oral	PK-SIM version 6.3 MATLAB	(131)
Isoniazid (Mycobacterium tuberculosis infection)	300 or 900 mg/3days	Oral	R version 3.4.1. Packages: deSolve ggplot2 zoo	(132)
Codein (post-labor pain)	2.5 mg/kg/day (twice a day administration to 60 kg female)	Oral	PK-Sim version 4.0 MoBi version 2.0 MATLAB version 7 MoBi Toolbox for Matlab version 2.0	(133)
Ethambutol (Mycobacterium tuberculosis infection)	25.4 mg/kg	Oral	MATLAB version 8.0	(134)
Rifampicin (Mycobacterium tuberculosis infection)	10.9 mg/kg	Oral	MATLAB version 8.0	(134)
Efavirenz (HIV)	400 mg/day or 600 mg/day	Intramuscular	SimBiology version 5.1 MATLAB 2014b	(135)
Alprazolam (Anxiety disorder)	0.5 mg single dose	Oral	SimCyp Simulator V16	Abstract (129)
Caffeine	200 mg single dose	Oral	SimCYP Simulator V16	Abstract (129)
Clonidine	150 µg twice a day	Oral	ADAPT II Software	Abstract (136)'
Lamotrigine (antiepileptic)	200 mg/day		ADAPT II Software PK Sim	Abstract (137)
Tramadol	100 mg twice a	Oral	SimCYP Simulator	Abstract

	day		V16	(130)

The goal of the lactation PBPK models was to predict the exposure of neonates to maternal medicines via breastfeeding. In the case of codeine, the focus was on differences in exposure due to maternal and neonatal differences in CYP2D6 genotype and morphine clearance (133). The PBPK model for isoniazid also aimed to investigate the impact of the polymorphic N-acetyltransferase 2 (132). No genotype specific simulation was performed for escitalopram, rifampicin or ethambutol (131,134). In the case of efavirenz, CYP2B6 polymorphism is known to have an impact on the metabolism. Olagunju et al. did not perform genotype specific simulations, but used reported data for pediatric CYP2B6 protein expression in which they induced variability.

5.2 Physiologically based pharmacokinetic (PBPK) model structure

Transfer of drugs into the human breast milk can be modelled in several ways (132):

(i) direct transfer of the medicines from the blood into the human breast milk (138);
(ii) via uptake into the breast adipose tissue (139); or
(iii) a combination of both routes (140).

All three approaches have been used for chemical substances. The currently available lactation PBPK models for medicines all used the first approach.

The lactation PBPK models used a whole-body PBPK modelling approach. They combine a maternal PBPK model including a breast compartment with a neonatal PBPK model. The model for escitalopram combines popPK on maternal monitoring data with a neonatal PBPK model (131). Willmann et al. (133) combined four PBPK models: maternal PBPK models for codeine and its main active metabolite morphine and neonatal PBPK models for codeine and morphine. Different approaches are taken for coupling the maternal and neonatal models. Delaney et al. (131) first analyzed the escitalopram concentrations in the breast milk using popPK. In a next step, they calculate daily infant doses using a random combination of the predicted milk escitalopram concentrations, milk volumes per feed and frequencies of feeding. The calculated daily infant doses are then administered to the neonatal PBPK model as a single dose. Willmann et al. (133) use a similar approach, but they administered the drugs to the neonatal PBPK models as multiple doses. They assumed breastfeeding, and thus dosing the neonate to take place each 3 hours. The doses were calculated using the drug concentrations at the time of breastfeeding predicted by their maternal PBPK model and the breast milk volume. Willmann et al. assumed that the absorption of the drugs in the neonatal model is fast and complete. Olagunju et al (135) calculated the milk concentration by multiplying the M/P ratio with the simulated plasma concentration. Subsequently, they calculated the infant dose per breastfeeding session by multiplying the milk volume with the milk concentration. Garessus et al. (132) used another approach, assuming that the breast is completely emptied during each feed. This means that the dose that is given to the absorption compartment of the neonatal PBPK model is equal to the amount of isoniazid in the breast milk at the time of breastfeeding. Dosing of the neonatal model is repeated every two hours. Partosch et al. (134) developed a PBPK model for ethambutol and a PBPK model for rifampicin. The PBPK models for both drugs have a similar structure. They included the breast compartment in the maternal PBPK model as a reservoir. Excretion into the reservoir can be calculated by multiplying the milk volume with the milk concentration. The milk concentration is calculated by multiplying the plasma concentration with the M/P ratio. Every 4 hours, the reservoir is opened for 30 min, allowing the drug to transfer to the neonate via a milk dose compartment. *Table 10: Breastfeeding parameters used in the lactation PBPK models* gives an overview of the different breastfeeding parameters that have been used in the lactation PBPK models.

Table 10: Breastfeeding parameters used in the lactation PBPK models

Reference	Infant weight	Milk intake	Frequency of feeds	Duration of breastfeeding
(131)	5.43 kg SD: 1.3	76.0 mL/feed SD: 12.6 150 mL/kg/day	11 feeds/day SD: 3	N/A
(132)	4 kg	0.1134 L/feed	Every 2h	N/A
(133)	N/A	13 g/kg/day (d1) 40 g/kg/day (d2) 98 g/kg/day (d3) 140 g/kg/day (d4) 155 g/kg/day (d5)	Every 3h	N/A
(134)	3.5 kg	0.185 L/kg/day (8d – 4 months)	Every 4h	30 min
(135)	N/A	Milk volume controlled by infant suckling rates from literature	Every 2h	N/A

Abbreviations: SD: standard deviation

PBPK modelling aims to predict *in vivo* concentration-time profiles based on:

(i) drug-specific parameters; and

(ii) physiological parameters.

The drug-specific data required for the development a PBPK model using SimCYP are listed in *Table 11: Drug-specific input parameters for a basic PBPK model using SimCyp* (141). Several sources of input data can be used. Delaney et al. (131) measured *in vivo* escitalopram concentrations in breast milk from 18 lactating women. They used clearance values from literature, which had been determined *in vitro.* Age-dependent algorithms were used to scale the parameters for the neonatal PBPK model. Garessus et al. (132) used *in vivo* data, including an AUC based M/P ratio, obtained from literature. AUC based M/P ratios are preferred over single M/P ratios, since single M/P ratios vary over time. Some of the *in vivo* data were re-calculated to match their population. Partition coefficients were calculated according to an algorithm from Schmitt et al. (142), which was also used by Partosch et al. (134). Both Garessus et al. (132) and Willmann et al. (133) used adult clearance values fitted from *in vivo data*, whereas the neonatal clearances were *in vivo* values obtained from literature. Willmann et al. (133) used a range of M/P ratios based on several *in vivo* values reported in literature for both morphine and codeine. Partosch et al. (134) used physiological data from literature. *In vivo* clearance values were obtained from literature. For ethambutol, the M/P ratio was based on two *in vivo* data pairs from literature. For rifampicin, an algorithm to estimate the M/P ratio was used, because it was not clear how the measurements were done for the sparse available *in vivo* data (134). Olagunju et al. (135) used an *in vivo* M/P_{AUC} ratio. They used anthropometric values to predict organ weight and blood flows based on a HIV positive cohort of breastfeeding women. For the maternal PBPK model, CYP450 abundances were taken from *in vivo* data. For the neonatal PBPK model, data from human liver microsomal samples were used.

Table 11: Drug-specific input parameters for a basic PBPK model using SimCyp

Parameter	*In vitro* test system

Molecular weight (g/mol)	Physicochemistry property measurement (or *in silico* prediction)
LogP	Octanol:water partition coefficient
pK_a (s)	Physicochemistry property measurement (or *in silico* prediction)
Compound type (base/acid/neutral)	Based on chemical structure or pH-dependent solubility test
pH-dependent solubility (µg/mL)	Measured in buffer with different pH
Plasma protein binding (f_u)	*In vitro* in human plasma (adapted for pregnancy, breastfeeding and/or neonates)
Milk protein binding	*In vitro* in milk (adapted for pregnancy, breastfeeding and/or neonates)
Blood-plasma partitioning (B:P)	*In vitro* in human blood
Apparent permeability (10^{-6} cm/s)	Caco-2, MDCK
Intrinsic clearance in microsomes (µL/min/mg)**, or S9** (µL/min/mg)**, or hepatocytes** (µL/min/million cell)**, or rhCYP** (µL/min/pmol)	*In vitro* assay (or *in vivo* clearance)
Protein concentration in *in vitro* test (mg/mL)	*in vitro* assay for intrinsic clearance
***In vitro* test matrix binding** ($f_{u,\ matrix}$)	Measure the free fraction using the same protein concentration in the *in vitro* test system
V_{max} (pmol/min/mg) **and K_m** (µmol/L) **(in case of saturable PK)**	The same *in vitro* system where intrinsic clearance was determined
Percent of enzyme contribution to the metabolism (f_m)	*In vitro* reaction phenotyping
Reversible inhibition, IC_{50} (µmol/L)	Human liver microsomes or suitable *in vitro* system
Mechanism-based CYP inhibition, k_{inact} (h^{-1})**, K_i (**µmol/L)	
CYP Induction, J_{max} (fold induction)**, EC_{50}** (µmol/L)	Human hepatocytes with positive controls in 3 donors
Milk to plasma ratio	*In vitro* model for the human mammary epithelial barrier (or *in vivo* measurement or *in silico* prediction)

Adapted from Zhuang et al. 2016 (141)

5.3 Evaluation of the Physiologically based pharmacokinetic (PBPK) models

PBPK models should be evaluated for their ability to predict *in vivo* pharmacokinetic data. All lactation PBPK models exist as a maternal PBPK model coupled to a neonatal PBPK model (131–135). The lactation PBPK models, except for codeine (133) and escitalopram (131), used a separate evaluation for the maternal PBPK models and the neonatal PBPK models. The evaluation for the maternal PBPK models was done by comparing *in vivo* plasma concentration profiles from literature with predicted

plasma concentration profiles. Garessus et al. (132) used matched dosing regimens and also compared breast milk concentrations. For escitalopram, drug milk concentration data was coupled to a neonatal PBPK model. They first evaluated an adult PBPK model against *in vivo* data and then extrapolated it to neonates and again verified this with *in vivo* data (131). Delaney et al. (131) used population matched predictions. Additionally, a bootstrapping technique was used by Delaney et al. to evaluate the adult PBPK model, for which they pre-specified that the PBPK model would be accepted if the mean plasma AUC_{∞} of the observed data fell within a 95% confidence interval of the mean of the predicted data. Olagunju et al. (135) mentioned an acceptance criterium of a 2-fold difference against observed data.

Different approaches were taken to evaluate the neonatal PBPK models. On the one hand, Delaney et al. (131) and Olagunju et al. (135) compared predicted neonatal plasma concentrations to *in vivo* plasma concentrations obtained after exposure via breastfeeding. On the other hand, Garessus et al. (132) and Partosch et al. (134) compared their predicted neonatal plasma concentrations with *in vivo* concentrations after direct oral or intravenous dosing of the neonates. Partosch et al. (134) only evaluated the neonatal model for rifampicin. Delaney et al. (131) also compared four age groups within the first year of life and concluded that the variation was limited for escitalopram. They concluded that there was a significant difference, but it was not clinically relevant for escitalopram. Olangunju et al. (135) also predicted the infant exposure for four age groups. Garessus et al. (132) also simulated a worst-case scenario by implementing breastfeeding at the time of maximal breast milk concentration and using the highest reported individual M/P ratio.

Willmann et al. (133) simulated a situation comparable to a reported fatal case of codeine use during breastfeeding and compared the simulated breast milk and plasma concentrations with the values observed in this case. Willmann et al. (132), Garessus et al. (132), and Partosch et al. (134) all performed a sensitivity analysis. Willmann et al. (132) investigated the effect of different values for maternal and neonatal morphine clearances in the sensitivity analysis. They found that the concentration in the neonate was mainly dependend on the the morphine clearance of the neonate and the maternal daily dose of codeine. Garessus et al. (132) found that the maternal PBPK model for the fast metabolizers was most sensitive to the clearance, the partition of the liver, the dose, the liver organ blood flow and the breast milk volume. Partosch et al. (134) found that the maternal model was most sensitive to the dose, the clearance and the partition coefficient of the liver, whereas the neonatal model was most sensitive to the M/P ratio and the bioavailability in the infant.

One of the assumptions made by the PBPK models is that a general, ‘mean’ milk composition exists for macronutrients. However, milk composition changes, especially during the first days after delivery, but with composition changes also during the time course of each feed, differences related to either preterm or term delivery, and the duration of lactation. Delaney et al. (131) investigated the intra-feed alteration by comparing concentrations in foremilk with concentrations in hindmilk. In addition, maternal characteristics might have an impact on the milk composition. An overview of the effect of several maternal conditions on milk composition is given in *Table 12: Effect of specific maternal conditions on human milk composition for macro-nutrients*. Overall, the differences in macro-nutrient composition are rather limited but can be considered during modelling for condition specific setting, like e.g. diabetes.

Table 12: Effect of specific maternal conditions on human milk composition for macro-nutrients

Maternal condition	Overall findings	Reference
Overweight or	Protein: higher (4.2 to 3.9 g/dl) in term colostrum, no	(143,144)

obesity compared to term normal weight, colostrum	differences from 2nd week onwards Fat: increased in colostrum (craematocrit, 5.6 to 3.3 %) Carbohydrates: similar or higher (3.2 to 1.9 mmol/l) Caloric content: higher(688 to 538 kcal/l)	
Systematic search on maternal conditions (diabetes, hypertension and overweight)	Qualitative, not quantitative reporting. ***Diabetes studies (n=9)*** Lactose: lower concentration (n=3) Fat: lower concentration (n=4) Protein: lower (n=1) Energy value: higher (n=1) No differences (n=2) ***Hypertensive mothers (n=1)*** Protein: higher (n=1) ***Overweight (n=4)*** No differences (n=2) Fat: higher (n=2) Energy content: higher (n=2)	(145)
Maternal nutrition and perinatal factors	367 milk samples from 81 mothers after preterm delivery Carbohydrates: 6.8 (4.4-7.3) g/100 ml Lipids: 3.4 (1.3-6.4) g/100 ml Proteins: 1.3 (0.1-3.1) g/100 ml There was a (weak) relationship between mothers' carbohydrates intake (r =0.164; p < 0.01) and milk composition [lipids, r2=0.087; protein 0.299; calories 0.101]. Postnatal age was the most relevant covariate for protein (r=-0.505) and carbohydrates (r=0.202).	(146)
Celiac disease	Protein: decrease during first three months n-6 long chain polyunsaturated fatty acids: decrease during the first three months of lactation No relevant effect of celiac disease	(147)
Preterm delivery	Protein: decline from 4.1±2.1 g/dL on the 3rd postpartum day to 2.2±0.6 g/dL by the 28th day postpartum Lactose: increase from day 3 to day 28 (from 2.2±0.7 g/dL to 3.0±0.9 g/dL), Fat: increase from day 3 to day 28 (1.9±1.8 g/dL to 3.4±2.1 g/dL) and Energy: increase from day 3 to day 28 (42.3±18.8 Kcal/dL to 51.9±21.5 Kcal/dL)	(148)
Maternal fat mass	Protein: Higher with higher maternal % fat mass (difference 0.16, SD 0.07 g/L, p = 0.028) Limited effect as the mean concentrations were 12.94, 11.7, 10.83, 12.83 and 11.96 g/L in the 2nd, 5th, 9th and 12th month of lactation.	(149)
Maternal pre-pregnancy BMI, colostrum	Macro-nutrient: not affected Energy content: not affected Protein: positively related related to pre-pregnancy BMI	(150)

composition	(normal weight vs obese, 4.23 instead of 3.9 g/dl)	
Preterm delivery	Systematic review and meta-analysis, including 13 papers. Protein: decreases massively and significantly (r2=0.93) from day 1 to 3 to reach 50 % of the initial value at week 10-12 Lactose: Significant linear increase (r2=0.80) Fat: Significant linear increase (r2=0.94) Energy: Significant linear increase (r2=0.81)	(151)
Across 9 different countries, protein content in mature human milk	Total protein: steady decline from 30 to 151 days of lactation, significantly higher in the second month of lactation compared with the following 4 months $y=23.251x^{-0.1554}$ (g/L), where x are the lactation days True protein: steady decline from 30 to 151 days of lactation, significantly higher in the second month of lactation compared with the following 4 months $y=18.86x^{-0.1705}$ (g/L), where x are the lactation days Individual amino acid: steady decline from 30 to 151 days of lactation, significantly higher in the second month of lactation compared with the following 4 months There is a high level of consistency in the protein content and amino acid composition of human milk across geographic locations, with Chile as an outlier. Stage of lactation explained 22.9 and 16.9 % of the variation in total protein and total amino acid concentration.	(152)
Systematic review on human milk composition after preterm delivery	Based on 24 studies, comparing lactation week 1 to lactations weeks 2-8, and in mean values. Protein: 1.9 to 1.27 g/100 ml Lipid: 2.59 to 3.46 g/100 ml Carbohydrate: 6.55 to 6.15 g/100 ml Energy content: 57.11 to 65.6 kcal/100 ml	(153)
Lactating adolescents	Protein: significant reduction ($P<0.05$) during the postpartum weeks studied (6th: 16.6 ± 1.1; 10th: 13.7 ± 1.0; 14th: 12.3 ± 1.1 g/day) Lactose: unaffected (6th: 60.2 ± 1.9; 10th: 60.4 ± 2.6; 14th; 65.1 ± 4.0 g/day) Fat: unaffected (6th: 41.6 ± 3.3; 10th: 36.2 ± 3.4; 14th 31.5 ± 9.0 g/day)	(154)
preterm to term delivery	No significant differences between preterm and full-term milk ($p>0.05$). The lowest creamatocrit, calories and fat concentration was in the preterm milk obtained in the morning (4.86 %, 663.8 kcal/L and 33.6 g/L, respectively). The highest milk parameters were observed in the night samples of full-term milk (9.6 %, 919.7 kcal/L, and 60.7 g/L, respectively).	(155)
preterm to term	Carbohydrate: higher ($p<0.05$) in preterm milk (6.3 to 8.5	(156)

delivery	and 5 to 7.4 g/dl, week 1 to 8, preterm versus term) Fat: higher (p<0.05) in preterm milk (2.9 to 6.8 and 2.9 to 4.9 g/dl) Energy higher (p<0.05) in preterm milk Protein: both preterm (2.6 to 1.9 g/dl) and term milk (2.2 to 1.1 g/dl) decreased with lactation duration with significantly higher values in extremely preterm milk (<28 weeks) than in moderately preterm and term milk (p<0.0001).	
very preterm (VP) to preterm (P) to term (T) delivery	Fat: colostrum, transitional and mature milk was 4.05, 4.76 and 4.67 (VP), 2.58, 3.75, 2.98 (P) and 2.6, 3.11, 3.06 g/100 ml (T). Creamatocrit: 6.3, 7.1, 7 (VP), 4.2, 5.8, 5 (P) and 4, 5.1, 5 (T) %.	(157)
donor milk, compared to literature	Protein: Banked donor milk mean values (g/100 ml) were found to be 1.16, SD 0.25 Fat: 3.22, SD 1.00 Lactose: 7.80, SD 0.88 Energy: 65+/-11 kcal/dL Macronutrient: differs from the values reported in the literature for mature human milk.	(158)
preterm (<33, or 33-36 weeks) to term delivery	Human milk samples were collected from 86 mothers on days 3, 7, 14 and 28 of lactation. Day 3 to 28, <33, 33-36, or term: Protein: (g/dl): 4.1, 4 and 1.9 to 1.6, 0.9 and 1.1 Higher in preterm samples, post-delivery decrease Lactose: (g/dl): 3.8, 4.74 and 5.18 to 7, 7.5 and 7.7 Lower in preterm samples, post-deliver increase Fat: 1.2, 1.3 and 2 to 3.1, 3.6 and 3.11 g/dl Lower in preterm samples, post-delivery increase	(159)
Maternal diet	Macronutrient: (fat, protein, and lactose) not affected by maternal diet Fatty acid profile: affected by maternal diet	(160)
Maternal Body Mass Index	Carbohydrate: 7.0 g Protein: 1.1 g Fat: 3.5 g (IQR 3-4.1) Energy content: 66 (62-72.5) kcal. Maternal BMI related to lipid (r=0.37) and energy (r=0.39) to milk content (p<0.05).	(161)
Maternal diet and Body Mass Index	Not diet, but rather the maternal body composition (BMI) associated with human milk composition. Milk fat content related (r=0.33) to BMI, and between protein content and body composition (% fat mass (r=0.60), fat-free mass/kg (r = 0.63; p = 0.001), and muscle mass (r = 0.47; p = 0.027). However, postnatal age is a relevant driver (1th, 3th and 6th month).	(162)

Vegetarian versus omnivore diet	Fat: lower in women with a vegan (3.0), compared to vegetarian (4.0) or omnivore (4.0) g/dl diet, with qualitative differences in (un)saturated fats	(163)
Pre-eclampsia	Macro-nutrient: no quantitative differences Free fatty acids: qualitative differences	(164)
smoking	Nicotine: 3-fold higher for smoking women than in maternal plasma. Fat: (3.47 vs. 4.34 g/dL) lower in smokers (Hopkinson et al, 1992)	(165)
Maternal HIV infection	Protein: HIV-infected women contained higher (1.95 to 1.78 g/100 g) Fat: higher (4.42 to 3.49 g/100 g) Copper: higher (0.64 to 0.56 mg/L) Carbohydrate: lower (5.37 to 6.67 g/100 g) Zinc: lower (5.26 to 5.78 mg/L)	(166)
Lactational mastitis	Lactational mastitis (n=15) to controls (n=15): Carbohydrates: different 5.1 to 6.9 g/dl Fat: different 2.1 vs 3.6 g/dl Energy: different 54 to 67 kcal/dl Protein: not different 1.8 vs 1.4 g/dl	(167)
Case report, hemodialysis	Creatinine: different Urea: different Sodium: different Chloride: different Phosphate: different Otherwise high similarity	(168)
a high-altitude adapted population (Tibet)	Fat: averaged 5.2 ±2.0 g/100 mL Sugar: 7.37 ± 0.49 g/100 mL Protein: 1.26 ± 0.35 g/100 mL Energy density: 81.4 ± 17.4 kcal/100 mL No associations between altitude of residence and milk composition	(169)
manually expressed milk	Paired study in 21 women, 48-72 h after delivery. Fat: higher (2.3 to 1.84 g/100 ml) in breastmilk expressed manually	(170)
after bariatric surgery	Fat: higher on day 4 after delivery3.0 ± 0.7 versus 2.2 ± 0.9 g/100 ml Carbohydrate: slightly higher on day 4 and 6.6 ± 0.6 versus 6.3 ± 0.4 g/100 ml Energy: higher on day 4 61.0 ± 7.2 versus 51.7 ± 9 kcal/100 ml The nutritional value of breast milk after bariatric surgery appears to be at least as high as in non-surgical controls.	(171)
Passive smoking	Lipids: affected (-28 % and -35 % in triglycerides at baseline and at 4 months)	(172)
small-for-gestational-age	Crematocrite: similar (SGA to AGA) on day 3 (7.8 to 6.8), 7 (11.9 to 9.7) and 14 (9.6 to 10.3) %	(173)

(SGA) to appropriate (AGA) infant		
Maternal age, < or ≥ 35 years	Fat: colostrum and mothers with advanced age are elevated Carbohydrate: mature milk mothers with advanced age are elevated, there is also a positive correlation between maternal age and carbohydrate content in mature milk.	(174)
Feeding over 24 h time interval	Fat: significantly differed over 24 h (P = .01) Lactose: remained the same, positively (P=.03) related to the number of feeds per day Protein: remained the same, the mean 24-hour total protein, whey, and casein inversely (P<.01) related to the number of feeds per day Pre-feed samples differ from post-feed samples.	(175)
Active smoking	Lipid: lower (-26%, 31.1 vs 42.4 mg/ml) Protein: lower (-12%, 13.1 vs 14.9 mg/ml)	(176)
+24 h of fasting	Immediately after fast, mean Sodium: increase Calcium: increase Protein: increase Phosphorus: decrease Lactose: decrease Triglycerides: unchanged 24 hours after fast, parameters are no longer significantly different from baseline except for mean protein levels and lactose	(177)
Ramadan fasting	Macronutrient: no significant effect Zinc: decreased Magnesium: decreased Potassium: decreased	(178)
Cystic fibrosis	Milk secreted by 2 women with CF appears to be physiologically normal, including sodium.	(179)
Cystic fibrosis	Single case report, confirming the data of Shiffman et al.	(180)
vegetarian and non-vegetarian women	Precursors of arachidonic acid: higher (n = 12) Fat: no differences	(181)
Homogenous familial hypo-betalipoproteinemia	Lipid: lower, with another profile, based on 2 cases	(182,183)

Animal PBPK models

Lactation PBPK models have also been developed for animals. These animal lactation PBPK models address different types of research questions. Animal PBPK lactation models have been used to addresses risk assessment questions in food producing animals about residual drugs in edible tissues and milk for human consumption (e.g. (184–186)). Other animal lactation PBPK models, typically for rodents, aim to get insight into (human) toxicology (e.g. (187–189)). Animal PBPK lactation models

have also been used as the basis for the development of human lactation PBPK models (138). In this case, the animal PBPK lactation model is first developed and validated for animals and thereafter extrapolated to humans by interspecies scaling of the physiological factors.

Discussion

1 Model compounds

All compounds selected by WP4 were considered as model compounds for WP3, as *in vivo* data will become available for these drugs through the human lactation studies of WP4. *In vivo* data is critical to verify the relevance of the non-clinical platform.

High quality and high resolution clinical data are critical for the evaluation of the non-clinical tools that will be developed to study the breast milk exposure and subsequent neonatal systemic exposure to maternal medication. As clinical data are very limited for most drugs, the compounds used in the human lactation studies of WP4 are of high interest for WP3. However, the results from these human lactation studies will only become available after quite some time. Therefore, WP3 decided to include in the first set of model compounds only those for which some human data existed:

(i) venlafaxine;
(ii) amoxicillin; and
(iii) metformin.

This will allow exploration of the PBPK models earlier in the project. Afterwards, the newly collected WP4 data will be used for further evaluation of the PBPK models. The other WP4 compounds will be included in WP3 as soon as clinical data becomes available.

The first set of ten model compounds selected in WP3 are:

(i) venlafaxine;
(ii) amoxicillin;
(iii) metformin;
(iv) valproic acid;
(v) tacrolimus;
(vi) tenofovir;
(vii) zidovudine;
(viii) nevirapine;
(ix) sertraline; and
(x) levetiracetam

This set of model compounds will be used for the development of an *in vitro* model for the blood milk epithelial barrier and PBPK models for lactation. Most likely, more than ten model compounds will be included, and additional compounds will be selected in the future. First, the model compounds of WP4 should be included as soon as clinical data becomes available. Furthermore, biologicals (e.g. infliximab) should be included as well in a next set of model compounds. The other compounds for which extensive clinical data was available (lopinavir/ritonavir, lamivudine and lamotrigine) can also be included. Other compounds should be selected based on the other pre-specified criteria. In addition, a typical BCRP substrate (e.g. cimetidine or nitrofurantoin (190)) could be included, as BCRP has been shown to be the most important transporter at the mammary epithelium. Model compounds representing the ion trapping phenomena should also be considered. Also drugs that impact milk production could be explored.

The drugs for the animal *in vivo* experiments that will be performed by WP3 will be selected from this initial set of model compounds. Among other, existing knowledge on PK and PD characteristics of the drug in the selected species will be considered.

2 *In vitro* models

Several *in vitro* models have been developed to investigate the transfer of drugs into breast milk. The *in vitro* model needs to be 'biorelevant', i.e. representative for the *in vivo* physiology. Therefore, human primary mammary epithelial cells will be used as a model for the human blood milk epithelial barrier. HMEC have previously been used by Kimura et al. (48), but further characterization is required before concluding whether this is a good model for drug partitioning over the mammary epithelium. The most suitable culture medium for HMEC seems to be MEBM, with addition of several supplements. However, other media commonly used for cell lines and animal cells might be explored with HMEC as well. Several supplements should be added to the media. A first group of supplements are insulin and other compounds with a similar function for cell health and proliferation. Furthermore, EGF is almost always added to stimulate growth. Glutamine is also a supplement commonly added in cell cultures to provide energy. Hydrocortisone is added both for growth and differentiation. Fetal bovine serum is frequently added. However, we will pursue to perform cell cultures according to the Guidance Document on Good In Vitro Method Practices (GIVIMP) guideline (191), which recommends to work serum-free. Bovine pituitary extract is commonly used in the culture of HMEC when working serum free. The guideline further advises minimize the use of antibiotics. Consistent with this guideline, we will add several supplements to the MEBM to proliferate the cells. Next, as we want to develop a differentiated model to reflect the drug transfer into the breast milk, EGF will be removed and prolactin will be added to the medium to induce differentiation of the cells. Several other supplements could be added to cell culture models, but did not seem to be critical.

As an alternative for the HMEC, we might also explore some human cell lines. The advantage is that cell lines are easier to culture and have a longer life span. However, the cell lines that will be used should still be representative for the *in vivo* physiology. Therefore, MCF7 or PMC42-LA might be good options. In addition, also animal cells and/or cell lines will be explored. This might especially be useful for *in vitro / in vivo* extrapolation (IVIVE), which is required to implement the *in vitro* data into a PBPK model. Even if rodent mammary epithelial cells are the most widely studied and provided many biological insights, it is evident that the rodent mammary gland is not fully representative of the human setting. Therefore, other *in vitro* animal models have been explored, including bovine, goat and porcine. From a physiological anatomical and metabolic point of view, ruminants provide a model very far from humans, whereas the porcine species is recognized as an excellent model for translational purposes. Among the different *in vitro* models of mammary epithelial cells available, primary cell cultures offer the opportunity to study the factors that regulate physiologically relevant development of normal mammary epithelial cells under defined conditions.

The most relevant *in vitro* cell culture models developed in WP3 will be used to perform transport studies for the previously mentioned model compounds. For these transport studies, buffers will be used as transport medium. However, the pH of milk is slightly more acidic than plasma, thus, evaluating the effect of pH on the transfer might be useful. According to the pH partition theory, it is expected that weak bases will be trapped in the milk. Furthermore, additional physiologically relevant media (e.g. diluted or undiluted plasma and milk) as transport media should be explored as well.

3 *In vivo* Animal Models

Drug excretion in milk during lactation can be successfully investigated utilizing *in vivo* studies in lactating animals (85,92). The principal benefit of *in vivo* animal studies in this field are:

(i) the possibility to clarify also the mechanistic aspect of milk/blood barrier;
(ii) the possibility to evaluate the influence of various parameters on the rate of drug excretion in milk (milk composition, timing of milking, drug-drug interaction, and different models of excretion even at molecular level); and
(iii) the possibility to evaluate the effects of excreted drugs or metabolites on pups.

The combination of the animal model with an *in vitro*-based preliminary screening phase may reduce the number of animals needed and the ethical concerns issues.

4 Empirical and semi-mechanistic models (human)

Koshimichi et al. (126) showed that semi-mechanistic models can be used to predict the transfer of drugs in the human breast milk. The main advantage of this semi-mechanistic model over other reported methods to predict drug transfer into the human milk is that the model of Koshimichi did consider that milk and plasma concentration-time profiles do not change in parallel. Koshimichi et al. found that secretion and reuptake values are similar for most drugs, suggesting mainly passive diffusion. However, for some drugs, transporter-mediated secretion or reuptake plays an important role. The model developed by Koshimichi et al. thus allows to distinguish between drugs that undergo passive diffusion into the milk and drugs that undergo transporter-mediated partitioning.

The model of Koshimichi has been applied for several drugs, including metformin and tacrolimus. However, as shown in *Table 8: Predicted and observed milk-to-plasma (M/P) area under the curve ratios for metformin and tacrolimus by Koshimichi*, the prediction for tacrolimus is not within the 3-fold error range. One reason for this discrepancy may be the exceptionally high distribution of tacrolimus in blood cells, as reflected by a high B/P ratio (± 15). This high extent of blood cell distribution is not taken into account in the model of Koshimichi et al., potentially explaining the significant overprediction of the M/P ratio. In the IMI project ConcePTION, PBPK models will be developed. The main advantage of PBPK models over empirical and semi-mechanistic models is that PBPK models are based on the underlying *in vivo* physiological mechanisms. This will allow inclusion of transporter-mediated milk secretion or reuptake in drug-specific PBPK models. Therefore, we expect to get a more reliable prediction using PBPK modelling. Additionally, the model of Koshimichi might be applied to all model compounds. Predictions using the model of Koshimichi can then be compared with predictions using the newly developed PBPK models.

5 Physiologically Based Pharmacokinetic (PBPK) models

5.1 Human lactation PBPK models

Recently, some PBPK models became available for the prediction of breast milk exposure and neonatal systemic exposure to maternal medication via breastfeeding (131–135) The available models, despite some limitations, show the value of PBPK modelling in this research field. The models illustrate that PBPK modelling can be used to handle several research questions, including breast milk exposure and neonatal exposure via breastfeeding. A major advantage of PBPK modelling is that non-clinical data can be used to predict *in vivo* PK behavior of medicines. This is especially important, given that clinical studies in a vulnerable population like lactating women and their

neonates gives rise to ethical and practical issues.

One of the main challenges for PBPK modelling is the need for high quality input data. The knowledge regarding the physiology of lactating women and neonates is growing, but research in this field is still required to optimize PBPK models. Furthermore, an immense information gap exists regarding the excretion of drugs into the human breast milk and subsequent neonatal gastrointestinal absorption. However, information will become available within the course of ConcePTION. First, an *in vitro* model will be developed to predict transfer of drugs into the human breast milk. The *in vitro* data can be used as input for a lactation PBPK model. The quality of this input data is critical for the quality of the final PBPK model. Secondly, clinical studies will be performed in WP4. Clinical data is critical for the evaluation of the predictive performance of the PBPK models. Lastly, combination of the *in vitro* animal and *in vivo* animal studies will provide both essential information for *in vitro* / *in vivo* extrapolation (IVIVE) of the drug transfer data and mechanistic insights that can be implemented in the PBPK models, while at the same time limiting the number of animals used.

One of the goals of WP3 is to develop PBPK models for the prediction of drug exposure in the human breast milk, along with subsequent systemic exposure in breastfed neonates. The model structure will be similar to the model structure of the available lactation PBPK models. In a first step, maternal whole-body PBPK models to predict breast milk concentrations of the model drugs will be developed, using data for the milk/plasma partitioning from the *in vitro* model as input. The need for the *in vitro* model is illustrated by the hurdles that some of the articles had for obtaining the M/P ratio for the respective model drugs. Furthermore, AUC-based M/P ratios, which are more reliable than single M/P ratios, are not always available. In the absence of (high quality) clinical data, some of the M/P ratios were estimated using the Schmitt et al. algorithm (142). However, a major limitation of this algorithm is that it does not account for transporter involvement. An *in vitro* model will allow to predict transfer of drugs into the human breast milk while accounting for both passive and active transport pathways. In a second step, neonatal PBPK models will be coupled to the maternal models, allowing prediction of neonatal exposure. During the development of the PBPK model, the guideline on the reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation of European Medicines Agency (EMA) (128) and the Physiologically Based Pharmacokinetic Analyses – Format and Content Guidance for Industry from Food and Drug Administration (FDA) (192) will be followed closely to assure the quality of the developed PBPK models.

Several software platforms have been used for the development of the PBPK lactation models. SimCYP will be used for the development of our own PBPK lactation models, but alternative software platforms (e.g. PK-Sim®/MoBi®, or R-based packages such as PLETHEM (193)) will be explored.

Multiple aspects are important to consider during the development of the PBPK models, as indicated by the available models. For example, some of the lactation PBPK models did a genotype-specific simulation, whereas others did not take genotype into account. However, the example of codeine shows the importance of genotype specific simulation for certain drugs. Codeine has long been considered safe until the death of a neonate. The PBPK model of Willmann et al. (133) showed that this was due to the genotype of mother and infant. Neglecting the genotype might lead to the conclusion that a drug is safe, while this is not the case for all genotypes. Especially since the prevalence of polymorphisms in the infant are not disconnected from the mother.

Another important aspect is how the transfer of drugs into the breast milk and breastfeeding of the neonate is implemented in the PBPK models. The transfer of drugs has been modelled as either direct

transfer from the blood to the breast milk or as transfer via uptake into the breast tissue. Furthermore, there was some variation in the parameters that have been used to implement breastfeeding in the PBPK models (e.g. duration of breastfeeding, frequency of breastfeeding and daily milk consumption). Even though literature shows that the milk composition is relatively constant in several maternal conditions, possible effects of the specific disease population on the milk composition should be kept in mind. Also, Delaney et al. (131) showed that there was a significant difference in drug concentrations between foremilk and hindmilk. The composition of the milk might thus play a role in the exposure of infants to maternal medication via breastfeeding. It is therefore important to understand the factors that influence the milk composition. Delaney et al. also investigated the variation between different age groups within the first year of life. Although the conclusion was that the variation is limited between the age groups for escitalopram, the age might have an important effect on the exposure to some drugs, as it has been shown that clearance is dependent on the age of the neonate (194). Olugunja et al. (135) did take this into account by doing separate predictions for different age groups. All of these factors can also be used to simulate worst-case scenarios.

PBPK models should be evaluated for their ability to predict the *in vivo* exposure. Delaney et al. (131) and Olagunju et al. (135) were the only ones to use pre-specified acceptance criteria. There is no consensus on which criteria should be used for acceptance of PBPK models as this depends on the purpose, but a 2-fold deviation is often used as default in literature. The guideline for PBPK models (128) indicates that a comparison of the simulated and observed individual plasma concentration-time profiles should be presented as plots and tabulations. Matched predicted and *in vivo* data should be used. The human *in vivo* data that will become available from the clinical studies in WP4 will be key to evaluate the predictive performance of the PBPK models.

5.2 Animal lactation PBPK models

Besides human lactation PBPK models, several animal PBPK lactation models have been reported. These animal lactation PBPK models show the value of PBPK modelling in this research field. Typically, these animal lactation PBPK models have been developed for dairy animals or rodents. The goal can either be to gain information for the modelled animal species or to translate the information to humans. Translation of animal PBPK information to humans is especially valuable in case *in vivo* human data are lacking. However, species differences, for example in transporter expression, complicate the direct translation of data from animal PBPK models to humans. Nevertheless, lessons learned during IVIVE-PBPK modelling while relying on animal in vitro data for the blood-milk barrier, followed by comparison with corresponding in vivo data will be instrumental for improving human PBPK lactation models. At least initial estimates for scaling factors for the IVIVE step can be derived in this way. SimCYP V18 allows to build PBPK models in the rat, dog, mouse and monkey, while PK-Sim also supports mini-pig.

Conclusion

The iterative development of a non-clinical platform should allow to predict breast milk transfer, and subsequent neonatal systemic exposure to maternal medication via breastfeeding. First, a human-relevant *in vitro* cell culture model, representative for the *in vivo* physiology will be developed. Transport data will be generated with this model for strategically selected model compounds. This transport data will then be subjected to IVIVE followed by PBPK modelling. Essential scaling information for IVIVE will be generated by the paired interpretation of animal *in vitro* and *in vivo* models. Furthermore, the *in vivo* animal models will deliver key mechanistic insights to support the

physiological plausibility of the PBPK models. The non-clinical tools will be validated using the model compounds for which *in vivo* data are available. The iterative development of several non-clinical tools will ultimately lead to robust predictions of breast milk transfer and neonatal exposure to maternal medication, for which data are currently lacking, ultimately driving a paradigm shift in the domain of pharmacotherapy during lactation.

Repository for primary data[1]

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