Drug Monitoring in Breast Milk: Novel Insights on Pharmacokinetics and Infant Exposure Estimates

by

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Abstract

The health benefits of breastfeeding are numerous and well described. It has been reported that ~70% of women take medication in the postpartum period; however, little is known about drug excretion into breast milk and infant exposure estimates are often unreliable. This gap in knowledge is due to the fact that women are excluded from clinical trials if they are lactating and carrying out a formal pharmacokinetic study is both ethically and practically challenging. The lack of knowledge and uncertainty on drug safety during lactation causes women to discontinue breastfeeding prematurely in order to take a medication or decide not to initiate breastfeeding altogether. While most drugs are considered to be compatible with breastfeeding, inaccurate infant exposure predictions of drugs in breast milk have led to infant adverse events and even fatalities. The overall aims of this thesis were to investigate the safety of three drugs and their use during lactation: methotrexate, escitalopram, and infliximab. Using a combination of newly developed analytical techniques, *in silico* methods, and animal lactation models, this thesis helps provide an updated framework for studying the safety of drugs during breastfeeding.

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Most importantly, I would like to *dedicate* this work to my Mom, the model of perseverance and resilience. Thank you for your endless love and support.

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List of Abbreviations

MTX	Methotrexate
ESC	Escitalopram
IFX	Infliximab
PPD	Postpartum Depression
sIgA	Secretory Immunoglobulin A
DHA	Docosohexaenoic Acid
HMO	Human Milk Oligosaccharides
FMN	Flavin Mononucleotide
FAD	Flavin Adenine Dinucleotide
BCRP	Breast Cancer Resistance Protein
ABCG2	2nd member of the G subfamily of the ABC transporter superfamily (BCRP)
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL-6	Interleukin-6
IL-8	Interleukin-8
IFNγ	Interferon Gamma
TNFα	Tumer Necrosis Factor Alpha
IBD	Inflammatory Bowel Disease
MHC	Major Histocompatibility Complex
MDR1	Multidrug Resistance Protein 1/P-Glycoprotein
ABCB1	1st member of the B subfamily of the ABC transporter superfamily (MDR1/P-gp)
MRP1	Multidrug Resistance-Associated Protein 1
ABCC1	1st member of the C subfamily of the ABC transporter superfamily (MRP1)
MRP2	Multidrug Resistance-Associated Protein 2

ABCC2	2nd member of the C subfamily of the ABC transporter superfamily (MRP2)
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
AUC_{milk}	Area Under the Milk Concentration-Time Curve
$C_{av, milk}$	Average Milk Concentration
V_{milk}	Milk Volume
SSRI	Selective Serotonin Reuptake Inhibitor
RID	Relative Infant Dose
M/P	Milk-to-Maternal Plasma Ratio
PBPK	Physiologically-Based Pharmacokinetic
RA	Rheumatoid Arthritis
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
DMARDs	Disease-Modifying Anti-Rheumatic Agents
7OH-MTX	7-Hydroxymethotrexate
SLC	Solute Carrier Family Proteins
ABC	ATP-Binding Cassette Proteins
RFC	Reduced Folate Carrier
PCFT	Proton-Coupled Folate Transporter
OATP1B1	Organic Anion Transporting Polypeptide 1B1
AAP	American Academy of Pediatrics
DSM-V	Diagnostic and Statistical Manual of Mental Disorders 5^{th} Edition
D-ESC	S-demethylcitalopram
NEC	Necrotizing Enterocolitis
CD	Crohn's Disease
UC	Ulcerative Colitis
mAb	Monoclonal Antibody
FcRn	Neonatal Fc Receptor

DLAC	Drugs in Lactation Analysis Consortium Study
MTX-d3	Deuterated Methotrexate
MS	Mass Spectrometry
HPLC	High-Performance Liquid Chormatography
QC	Quality Control
DP	Declustering Potential
CE	Collision Energy
EP	Entrance Potential
CxP	Collision Cell Exit Potential
CLSI	Clinical & Laboratory Standards Institute
LoQ	Limit of Quantification
WT	Wild-Type
Bcrp-/-	Bcrp Knockout
V_d	Volume of Distribution
$C_{av,ss\ plasma}$	Steady-State Average Plasma Concentration
CV	Coefficient of Variation
AMR	Analytical Measuring Range
TIC	Total Ion Chromatogram
SC	Subcutaneous

List of Appendices

Chapter 1
Introduction

1 Introduction

1.1 Statement of the Problem

The infant and maternal benefits to breastfeeding are numerous and well described. Because of this, more women are initiating breastfeeding and continue breastfeeding for longer. It has been reported that as high as 80% of women take some sort of medication, either over-the-counter or prescribed, within the first six months postpartum; however, information on drug secretion into milk is limited. This is because women who are pregnant or lactating are often excluded from clinical trials, and carrying out a formal pharmacokinetic study is both ethically and practically challenging[1]. Together, these create a knowledge gap on drug safety during lactation and leads to uncertainty in infant exposure estimates. Further contributing to this issue is the fact that global rates of breastfeeding continue to rise due to an increase in promotional efforts endorsing the nutritional, immunological and economic benefits to breastfeeding[2]. This improvement in breastfeeding rates may increase the likelihood of infants being exposed to drugs via breast milk. This is problematic, as there have been reports of infant adverse events and even fatalities with improper medication use during lactation[3,4]. Due to the lack of knowledge and uncertainty on drug safety during lactation, women are often encouraged to discontinue breastfeeding prematurely because of the lack of risk assessment data. Even when a drug is considered to be compatible with breastfeeding, this cause issues of maternal non-adherence to medication [5].

The mechanisms involved in transporting drugs into milk are complex and poorly understood. The way that drugs are excreted into milk – whether through passive diffusion or carrier-mediated transport – can affect the amount of drug that the nursing infant is exposed to. Further complicating this, milk is a challenging and dynamic matrix requiring meticulous sample processing in order to accurately analyze drug concentrations[6]. Information on infant blood levels following drug exposure through breast milk is an ideal objective measure to better understand the risk of infant adverse effects. However, obtaining this data and assessing infant outcomes is unethical and largely unachievable due to parental hesitancy. The overarching aim of this thesis is to better understand drug exposure to the nursing infant via breast milk. Using an

interdisciplinary approach, with a combination of analytical and *in silico* techniques, and an animal model, the goal of these investigations is to further contribute to the body of knowledge on drug safety during lactation.

1.2 Thesis Structure

This thesis is divided into the following: one section on background (chapter 2), one materials and methods section (chapter 3), three results sections (chapters 4-6), and one section discussing the thesis findings (chapter 7). Specifically, the second chapter describes the current state of knowledge and provides a review of the literature on breastfeeding and drugs in lactation. The third chapter describes the methods from each different approach used for the three projects: methotrexate (MTX), escitalopram (ESC), and infliximab (IFX). Chapters 4 - 6 discuss different approaches to address the safety of the three different drugs during lactation. Chapter 4 focuses on MTX, which examines the mechanism of drug entry to milk and also investigated the potential of using benign nutritional substrates to reduce drug excretion into milk. Chapter 5 used physiologically-based pharmacokinetic modeling to explore ESC plasma exposure in breastfeeding infants. Chapter 6 explores the extent of IFX excretion into milk and the subsequent extent of exposure to the nursing infant. Detailed objectives for each project is discussed at the end of chapter 2. Chapter 7, discussion, focuses on study conclusions, limitations and future directions.

Chapter 2
Background

2 Review of Literature

2.1 Breastfeeding

2.1.1 Breastfeeding Recommendations

The American Academy of Pediatrics and World Health Organization endorse that breastfeeding and the use of human milk are superior for infant feeding and affords numerous nutritional benefits[7,8]. It is recommended that infants are exclusively breastfed the first six months of life, with the introduction of complimentary foods and the continuation of breastfeeding up to two years thereafter [9]. Over the last several decades, global breastfeeding rates have been steadily increasing, largely due to The Baby Friendly Hospital Initiative, which was designed to support, promote, and protect breastfeeding [10]. The breastfeeding initiation rate is as high as 90% in Ontario, with approximately 23% of women continuing to breastfeed at 6 months [11–13]. In the United States, the Centre for Disease Control Breastfeeding Report Card stated that only 79% of newborns commence breastfeeding, while 49% were breastfeeding at 6 months and 27% at 12 months[14]. In order to further increase the rates of breastfeeding by the year 2020, The Office of Disease Prevention and Health Promotion launched the Healthy People 2020 campaign, as shown in **table 2-1**[2]. Their campaign targets seek to have breastfeeding initiation rates rise to 81.9% and are sustained at 60.6% and 31.4% at 6 months and 12 months, respectively[2].

Table 2-1. Healthy people 2020 targets for breastfeeding initiation and intervals (with permission from the American Academy of Pediatrics[15]).

	2007[a]	2010 Target	2020 Target
Any breastfeeding			
Ever	75.0	75	81.9
6 mo	43.8	50	60.5
1 y	22.4	25	34.1
Exclusive breastfeeding			
To 3 mo	33.5	40	44.3
To 6 mo	13.8	17	23.7
Worksite lactation support	25	—	38.0
Formula use in first 2 d	25.6	—	15.6

[a] 2007 data reported in 2011.[10]

2.1.2 Infant Benefits

Epidemiological evidence supports that breastfeeding is associated with a number of infant health benefits[16]. As shown in **table 2-2,** exclusively breastfed infants have a reduced risk of developing health problems such as acute otitis media[16], lower respiratory tract infections[16,17], gastrointestinal tract infections[16,18], and type 1 diabetes[19]. Exclusive breastfeeding within the first 28 days of life is associated with improved cognitive outcomes in preterm infants[20]. An interventional trial examining the effects of breastfeeding promotion found that prolonged and exclusive breastfeeding improves infant cognitive function [21]. Breast milk also provides allergen-mediated protection from allergic diseases such as asthma and atopic dermatitis[16,22]. Another recent study found that there is an interaction between breast milk and the infant's saliva can enhance the infants microbiome and boost innate immunity [23]. Overall, the described medical benefits are thought to be directly associated with amount and duration of breastfeeding[24].

Table 2-2. Dose-response benefits to breastfeeding (with permission from American Academy of Pediatrics[15]).

Condition	% Lower Risk[b]	Breastfeeding	Comments	OR[c]	95% CI
Otitis media[13]	23	Any	—	0.77	0.64–0.91
Otitis media[13]	50	≥3 or 6 mo	Exclusive BF	0.50	0.36–0.70
Recurrent otitis media[15]	77	Exclusive BF ≥6 mo[d]	Compared with BF 4 to <6 mo[d]	1.95	1.06–3.59
Upper respiratory tract infection[17]	63	>6 mo	Exclusive BF	0.30	0.18–0.74
Lower respiratory tract infection[13]	72	≥4 mo	Exclusive BF	0.28	0.14–0.54
Lower respiratory tract infection[15]	77	Exclusive BF ≥6 mo[d]	Compared with BF 4 to <6 mo[d]	4.27	1.27–14.35
Asthma[13]	40	≥3 mo	Atopic family history	0.60	0.43–0.82
Asthma[13]	26	≥3 mo	No atopic family history	0.74	0.6–0.92
RSV bronchiolitis[16]	74	>4 mo	—	0.26	0.074–0.9
NEC[19]	77	NICU stay	Preterm infants Exclusive HM	0.23	0.51–0.94
Atopic dermatitis[27]	27	>3 mo	Exclusive BFnegative family history	0.84	0.59–1.19
Atopic dermatitis[27]	42	>3 mo	Exclusive BFpositive family history	0.58	0.41–0.92
Gastroenteritis[13,14]	64	Any	—	0.36	0.32–0.40
Inflammatory bowel disease[32]	31	Any	—	0.69	0.51–0.94
Obesity[13]	24	Any	—	0.76	0.67–0.86
Celiac disease[31]	52	>2 mo	Gluten exposure when BF	0.48	0.40–0.89
Type 1 diabetes[13,42]	30	>3 mo	Exclusive BF	0.71	0.54–0.93
Type 2 diabetes[13,43]	40	Any	—	0.61	0.44–0.85
Leukemia (ALL)[13,46]	20	>6 mo	—	0.80	0.71–0.91
Leukemia (AML)[13,45]	15	>6 mo	—	0.85	0.73–0.98
SIDS[13]	36	Any >1 mo	—	0.64	0.57–0.81

ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; BF, breastfeeding; HM, human milk; RSV, respiratory syncytial virus.

[a] Pooled data.

[b] % lower risk refers to lower risk while BF compared with feeding commercial infant formula or referent group specified.

[c] OR expressed as increase risk for commercial formula feeding.

[d] Referent group is exclusive BF ≥6 months.

2.1.3 Maternal Benefits

Aside from the numerous infant benefits, breastfeeding has also been associated with a reduced risk of developing maternal diseases. Breastfeeding has been shown to regulate glucose levels,

and reduce the risk of hyperlipidemia and cardiovascular disease[25]. Breastfeeding can also reduce blood loss in postpartum haemmorhage[26], improves weight loss during the first year postpartum[27], and is associated with a lower risk of developing reproductive cancers [28]. Furthermore, the act of breastfeeding can promote bonding and create a sense of emotional well-being in both mother and infant[29]. Women who breastfed have a hormone-mediated reduction in maternal stress[30], which is accompanied by an increase in oxytocin and prolactin levels and leads to feelings of relaxation and general well-being[31]. These hormonal influences may be especially important in mediating postpartum depression (PPD), which will be discussed later in this chapter.

2.2 Breast Milk

2.2.1 Mammary Gland Anatomy

Figure 2-1 displays a diagram of the lactating breast (left) and mammary gland (right). The lactating mammary gland is comprised of a single layer of polarized milk-producing epithelial cells, which come together to form the alveolus and milk ducts[32,33]. During pregnancy, lactogenesis I is initiated by the differentiation of these secretory epithelial cells, which begin to produce small amounts of milk[33,34] (the different stages of lactation are discussed in the following section). These alveolar epithelial cells are bordered by basal myoepithelial cells, which are stimulated by oxytocin to contract to aid in milk ejection from the apical side of the alveoli, into the lumen, down the lactiferous sinuses and out through the nipple[32,33].

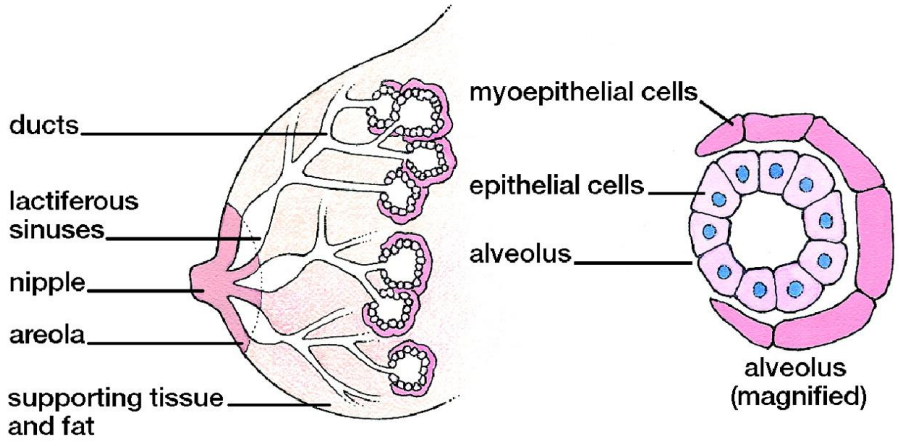


Figure 2-1. Lactating breast (left) and mammary gland (right) anatomy (with permission from the American Academy of Pediatrics[35]).

2.2.2 Stages of Lactation

As the mammary epithelial cells become differentiated during mid-pregnancy, lactogenesis I begins and small volumes of milk begin to be secreted [34]. The secretion product from this phase of lactation postpartum is called the colostrum, which contains high concentrations of sodium, chloride, lactoferrin, and immunologic components. The colostrum is ejected from the nipple starting in the latter stages of pregnancy and during the first several days postpartum[34,36,37]. The main function of colostrum is to provide the infant with maternally-derived immunoglobulins[38]. During this phase of lactation, the tight junctions between mammary epithelial cells have yet to form, allowing for easy passage of substances (lipids, proteins, immunoglobulins) from maternal circulation into milk[37]. The volume of milk produced during the first day postpartum is ~10% of that produced after one week postpartum[39].

After the first few days postpartum, lactogenesis II is initiated and "transitional" milk is secreted, which is produced in higher volumes to meet the increasing nutritional demands for the developing infant[38]. This is accompanied by the closure of tight junctions between the mammary epithelial cells, a decrease in the ratio of sodium to potassium, and an increase in the concentration of lactose [40]. Breast milk is defined as "mature" after 4-6 weeks postpartum when the concentrations of bioactive and nutritional components essentially stabilize until involution (end of lactation)[38].

Milk composition varies across the first 2 months postpartum, but also changes across an individual feed. Specifically, the lipid content is the most variable component in breast milk, and differs between the hindmilk and foremilk[38]. The hindmilk is the milk from the latter part of a feed and contains 2-3-fold higher lipid content than the milk from the beginning of a feed (foremilk)[41]. Milk fat content is also found to be significantly higher in the afternoon and evening compared to night and morning feeds[42]. Implications on variable milk composition will be discussed in parts of section 2.3: Maternal Medication use in the Postpartum.

2.2.3 Milk Secretory Pathways

Breast milk is a complex biological matrix that is uniquely adapted to meet the biological needs of the nursing infant, in order to promote optimal growth and development[43]. As shown in **figure 2-2**, the nutritional components in milk are either produced by the mammary alveolar epithelial cells directly or can originate from nutritional sources or from maternal stores[38]. Milk components such as lactose, oligosaccharides, phosphate and calcium are all produced within the cytoplasm of the lactating alveolar cells, and enter the milk via exocytosis on the apical side of the membrane (pathway I)[33]. Lipid components found in milk are also exocytosed in a mechanism that is considered unique for the mammary gland[44]. Cytoplasmic lipid droplets are synthesized, then migrate to the apical side of the cell to enter the milk as a membrane-coated milk fat globule (pathway II)[33,44]. Maternal-derived immunoglobulins, transferrin and hormones, and immune factors such as cytokines are transported into breast milk via a transcellular transport mechanism (pathway III)[33,45–48]. Ions and small molecules such as glucose amino acids move directly across the basal side of the mammary epithelial cell into the alveolar lumen on the apical membrane. The transport of these substances is facilitated by transporters that are solute-specific and can require transporters or channels on both sides of the plasma membranes (pathway IV)[49]. Lastly, substances such as low molecular weight compounds and large biomolecules can enter the milk directly from maternal circulation between the mammary epithelial cells during pregnancy, involution, or during periods of mammary inflammation, prior to the formation of tight junctions between the cells (pathway V)[33,50].

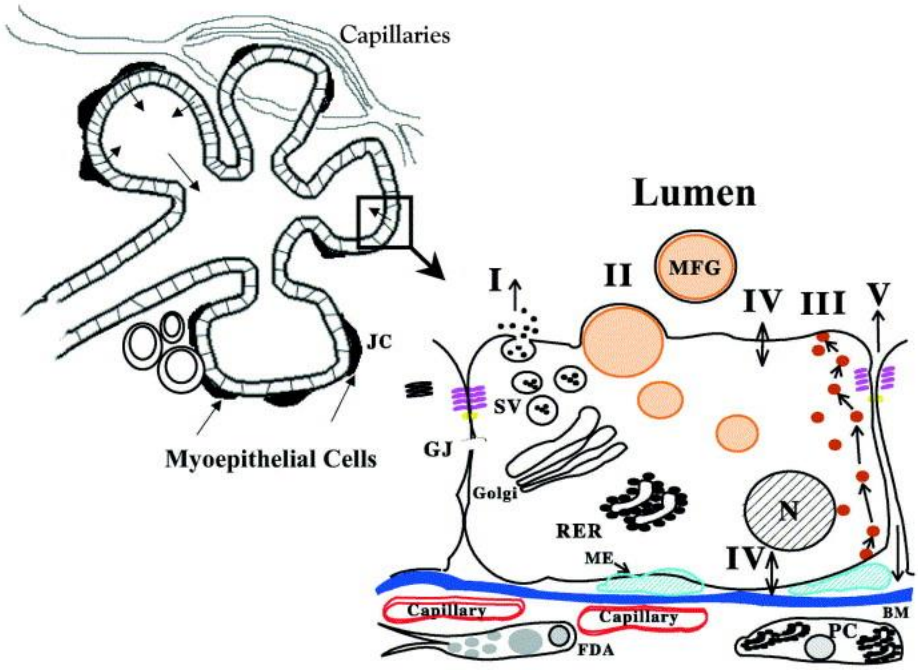


Figure 2-2. Mammary alveolus (left, top) and mammary alveolar epithelial cell (right, bottom). Milk secretion pathways are depicted in the mammary alveolar epithelial cell: pathway I shows exocytosis; pathway II depicts milk fat secretion; pathway III demonstrates vesicular transcytosis; pathway IV shows the transporters for direct movement of solutes across the cell; pathway V depicts paracellular transport (with permission from McManaman and Neville[33]).

2.3 Components of Breast Milk

2.3.1 Macronutrient Content

As introduced previously, secretion rates and amounts of macronutrients vary throughout the stages of lactation and can be influenced by maternal intakes[51]. The mean concentration of protein, lipids and lactose in mature term milk is: 0.9-1.2 g/dL, 2.6-3.2 g/dL and 6.7-7.8 g/dL, respectively, which provide the infant with approximately 65-70 kcal/dL energy[38]. Interestingly, there is a statistically significant difference in macronutrient content (protein, carbohydrates, fat and energy) found in the milk of mothers who deliver preterm versus those who carry until full term[52]. The way in which the macronutrient composition can influence drug concentrations in breast milk will be discussed later in this chapter.

Over 400 proteins contributing to the unique nutritive and immunological properties of human breast milk have been described[53]. The types of proteins found in milk are: whey proteins,

caseins, and mucin proteins[54]. The whey proteins exist in solution and consist of α-lactalbumin, secretory immunoglobulin A (sIgA), lactoferrin, lysozymes and serum albumin[55]. Caseins and mucin are not found in solution, but rather are present in suspended micelles and in the milk fat globule membrane, respectively[55,56]. The protein concentration in human milk also decrease steadily until the first 6 weeks postpartum and appear to remain constant until involution[52].

The most variable macronutrient in breast milk, lipids, are found in abundance in human breast milk and provide 40-55% of the energy source to the nursing infant. Nearly 98% of lipids found in breast milk exist as triacyglycerides, which exist in the core of the milk fat globule[53,57]. The primary fatty acid found in human milk is oleic acid; however, fatty acids only make up a small portion of the lipid fraction and are found in low concentrations[38,53,58]. Essential omega-3 fatty acids, such as docosahexaenoic acid (DHA), are important for infant neurodevelopment and maintenance of the immune system[59]. The concentration and availability of these essential fatty acids are largely influenced by maternal consumption; therefore, it is recommended that women who consume diets low in omega-3s should consider supplementation[38].

A number of complex carbohydrates are present in human breast milk. Lactose, a disaccharide consisting of glucose and galactose, is present in the highest concentration[60]. Synthesis of lactose in the mammary epithelial cells is also dynamic across different stages of lactation - mean lactose concentrations on lactation day 4 is approximately 5.6 g/dL and increases to 6.9 g/dL at 4 months[60]. Interestingly, preterm milk contains lower concentrations of lactose than term milk, likely lowering osmolality and contributing positively to nourishing the infant [61]. Human milk oligosaccharides (HMO) also comprise a significant portion of carbohydrates in milk and function as prebiotics to support growth of beneficial gastrointestinal bacteria in the infant gut[62]. While these HMO are found in concentrations of 1g/dL in milk, they are not able to be digested by the infant and thus do not contribute to the total energy provided by carbohydrates in milk[38,53].

2.3.2 Micronutrient Content

Consistent with the macronutrient composition, the status of various micronutrients (vitamins and minerals) in human milk is largely affected by maternal intake[51]. The secretion of selenium,

iodine, thiamin (vitamin B_1), riboflavin (vitamin B_2), vitamin B_6, vitamin B_{12}, choline, and vitamin A, C and D all vary based on maternal ingestion and can be reduced by depletion of maternal stores [38,51,63]. In contrast, certain vitamins and minerals such as folate, calcium, iron, zinc and copper remain relatively constant in milk and are not influenced by maternal diet or stores [63]. However, depletion of maternal status can occur when diet does not meet the demands of milk secretion, potentially causing maternal deficiencies[63].

2.3.3 Riboflavin (Vitamin B_2)

Riboflavin is a member of the B-vitamin complex and plays an essential role in the metabolism of carbohydrates, nucleic acids and amino acids [64]. It is an essential vitamin that must be obtained from the diet, primarily from dairy and dark leafy green vegetables[64]. Riboflavin is converted to its cofactors, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which act as electron carriers in various biological processes [64]. Riboflavin is found in high concentrations in breast milk, playing an essential role in supporting optimal growth and development for the neonate[65]. Recently, the transport mechanisms of riboflavin excretion into milk have been characterized. The multidrug efflux transporter, breast cancer resistance protein (BCRP/ *ABCG2*), is highly upregulated on the apical membrane of the mammary epithelium during lactation (will be discussed in more detail in section 2.5)[66]. The most notable physiological function that BCRP plays during lactation is to preferentially secrete riboflavin and its cofactor, FMN, into milk[67]. Further, riboflavin transporters *Slc52a2 and Slc52a3* are expressed in the milk fat globule in human milk and in the mouse mammary tissue during the first week of lactation[68], suggesting that these riboflavin transporters also help facilitate riboflavin excretion into milk.

2.3.4 Immunological Components

The immunological constituents in breast milk afford the infant with unique protective anti-inflammatory and anti-infective properties[69]. During the early stages of lactation, the colostrum contains approximately 4×10^6 cells/mL maternal leukocytes[70]. Interestingly, a number of cell subsets have been identified; neutrophils, myeloid precursors, non-cytotoxic T-cells, and

immature granulocyes all contributing to the leukocyte content in breast milk[71]. The colostrum is comprised of 80% macrophages, derived from maternal-derived peripheral blood monocytes. These monocytes contribute to infant immunoprotection by helping stimulate the infant immune system development [38,72].

Immunoglobulins are also found in high abundance in milk, functioning as highly targeted secretory antibodies to help stimulate the infant's immune development and protect the infant from environmental agents [38,70]. The primary immunoglobulin found in human milk is sIgA; however, immunoglobulin M (IgM) and immunoglobulin G (IgGs) are also present in low abundance[53,73]. Consistent with other immunological factors, the colostrum contains the highest concentration of sIgA (12 mg/mL), which decreases to approximately 1 mg/mL in mature milk[74]. IgG concentrations also increase in mature milk, suggesting the infant immunological needs might also be dynamic across the different stages of lactation[75]. While most macromolecules undergo degradation, sIgA is able to resist proteolysis and exert its protective effects against pathogens in infant's gastrointestinal tract[76].

2.3.5 Cytokines and Chemokines

A number of cytokines and chemokines have been identified in human milk. Cytokines are polypeptides that function in an autocrine/paracrine manner to influence the function of the immune system[77]. The cytokines found in breast milk can cross the infants intestinal barrier to either boost inflammation or protect against infection or reduce inflammatory processes[38]. Three TGF-β isoforms are found in milk, and play a role in controlling inflammation, enhancing oral tolerance to dietary antigens and preventing allergic diseases[38,78]. Interleukin-6 and 8 (IL-6 and IL-8), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are pro-inflammatory cytokines that are found in milk[79]. TNFα is produced by epithelial and immune cells in the mammary gland and is found in abundance in breast milk[80]. TNFα concentrations in healthy human milk range from 34-151 pg/mL, depending on lactation stage, with concentrations reaching as high as 2933 pg/mL[81]. In contrast to these high TNFα levels, serum of healthy individuals is 0.02 pg/mL, up to several thousand-fold lower compared to milk[82]. Patients suffering from inflammatory bowel disease (IBD) have an average TNFα serum concentration of 11.5 pg/mL even in remission[82]. Given these findings, TNFα in breast milk from women with

IBD may be even higher than healthy individuals. It has also been postulated that the TNFα in milk could potentially affect the maturation of the immune system in the infant by affecting parts of the proximal gastrointestinal tract that is devoid of proteolytic enzymes[83]. This could, in turn, affect the development of monocytes, and the production of various major histocompatibility complex (MHC) class I and II antigens and cytokines in the infant[83].

2.4 Drugs in Lactation

2.4.1 Medication Use During the Postpartum Period

Breastfeeding initiation rates have been reported to be as high as 90% in Canada[12]; however, it has been reported that 66% to 80% of women are on some type of medication during the postpartum period[1,84,85]. Consequently, with more infants initiating breastfeeding and women breastfeeding their infants for longer, there is an increase in the likelihood of infant drug exposure through breast milk. Although the infant dose of drug through breast milk could be much less than the mothers, even after body weight adjustments, there have been reports of adverse events, including fatalities, in infants exposed to drugs through breast milk [3,4,86–88]. For example, a case by Neuman et al. reported an incidence of a bupropion-induced seizure in an exclusively breastfed infant whose mother was receiving bupropion therapy for postpartum depression[3]. Another report described a morphine-overdose fatality in a neonate whose mother was prescribed codeine therapy[4]. These cases highlight the importance of understanding drug excretion into milk. This section will explore the various pathways or transporters responsible for excreting drugs into breast milk.

2.5 Mechanisms of Drug Transport

2.5.1 Passive Diffusion

The most common way that drugs, and potentially drug metabolites, cross the mammary epithelium and into breast milk is down a concentration gradient via passive diffusion[89]. The possibility of a drug being excreted into breast milk can also be determined by the molecular weight of the drug, pKa, extent of plasma protein binding, lipophilicty and maternal plasma

levels[37]. Ionized, water-soluble drugs and drugs with molecular mass higher than 200 kDa are not able to cross the mammary epithelial membrane[89,90]. Breast milk is slightly acidic (pH 7.2); therefore, drugs that are slightly basic can become ion trapped and concentrated in the milk[90]. Highly protein-bound medications are less likely to pass into milk or simply get excreted in low concentrations[91]. Further, drugs with longer half-lives tend to accumulate in the breast milk more readily[92]. Drugs that are highly lipid-soluble are able to dissolve into the milk fat and can become concentrated by binding to the protein fraction[89]. The lipid content is lower in the foremilk (beginning of feed) and higher in the hindmilk (end of feed), suggesting that drug concentration can also vary throughout an individual feed, depending on the physicochemical properties of the drug[37,93]. In addition, neonatal drug exposure may be increased during the colostrum period due to increased porosity of the mammary alveolar epithelium caused by immature tight junction formation between cells[37].

2.5.2 Active Transport

It has been discussed how passive diffusion accounts for a large proportion of drug transport across the mammary gland. However, certain drugs such as cimetidine and nitrofurantoin are accumulated in milk at much higher concentrations than predicted by passive diffusion[94]. This suggests that carrier-mediated transport could contribute to drug transfer into milk. The most noteworthy drug transporter BCRP, encoded by *ABCG2*, which is highly upregulated in the lactating alveolar epithelial cells [66]. In contrast, p-glycoprotein (MDR1; *ABCB1*) and the multi-drug resistance proteins, MRP1 (*ABCC1*) and MRP2 (*ABCC2*), are all functionally down-regulated during lactation[95]. It remains unclear how these drug transporters contribute to drug disposition into milk.

2.5.3 The Breast Cancer Resistance Protein (BCRP)

As described above, there has been emerging evidence suggesting xenobiotic and nutrient drug transporting proteins such as BCRP play a role in facilitating drug excretion into milk [66,67,96]. BCRP is an ATP-binding cassette transmembrane protein that actively effluxes numerous compounds from cell, most commonly recognized for its contribution to multidrug resistance of

tumor cells [97]. Interestingly, BCRP is highly expressed in the apical membrane of the alveolar mammary epithelium in the human, mouse and cow, starting in the third trimester of pregnancy and throughout lactation[66]. BCRP is a multi-substrate transporter that has been shown to actively transport dietary carcinogens (e.g. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)) and medications (e.g. cimetidine, ciprofloxacin) across the mammary gland and into milk [66,98]. As mentioned in section 2.3, the most notable physiological function of BCRP during lactation is to preferentially secrete riboflavin (vitamin B_2) and its cofactor, FMN, into milk[67]. Other dietary-derived compounds such as genistein, an isoflavone found abundantly in soybeans, has been described to have potent BCRP-inhibitory effects *in vitro* and has also demonstrated the ability to alter the pharmacokinetics of other BCRP substrates in mice [98–100]. The ability of these compounds to modulate drug disposition and clearance may have potential implications for the transport of drugs into milk. This will be further explored in this thesis.

2.6 Measuring Drugs in Milk: Analytical Considerations

The majority of drug measurement methods used clinically are optimized for measurement in blood or urine. Due to dynamic differences in matrix, measuring drugs in breast milk is complex and requires specific considerations for analysis and validation. As with plasma, breast milk drug concentrations are subject to high inter-individual variability, depending on plasma concentrations, genetic variability in drug metabolizing enzymes, milk phase (foremilk vs. hindmilk), stage (colostrum vs. mature milk) or time of day (morning vs. night)[6,37]. Both lipid and protein content have wide inter- and intra-day variability, which are also factors that could contribute to sampling bias[6,101]. Infant exposure estimates can be significantly affected by a failure to consider macromolecule partitioning. Drug concentrations in milk are often largely dependent on plasma concentration and mechanism of entry, requiring additional sensitivity for measurement of drugs that may be excreted in milk in lower concentrations. For those drugs with low levels of excretion, it may seem trivial to determine true measurements; however, this information is essential to assessing infant exposure and potential risk of toxicity. Another consideration that is often overlooked is the extent of drug metabolite excretion into milk. In certain cases, such as the morphine-related neonatal fatality reported by Koren et al.[4], the metabolite can be responsible for infant adverse events.

In order to better understand exposure of a drug and/or metabolite through milk and define the magnitude of infant risk, it is essential to develop analytical methods optimized specifically for milk measurement. Furthermore, validation parameters such as assay reproducibility (precision), accuracy (bias), linearity, limit of detection/quantification, and ion suppression in LC-MS/M must meet specific criteria in order to accurately establish infant exposure estimates.

2.7 Infant Exposure Estimates

Accurate analytical measurement is one of many essential components in generating reliable infant exposure estimates of drugs through breast milk. Due to ethical challenges, measurement of drugs in milk must be completed when a lactating woman is receiving a medication therapeutically. The most common approach is to collect serial milk samples either following a single administration or across a dosing interval when a woman has steady-state plasma concentrations[90]. Collection of 5-6 milk samples during one dosing interval is considered to be sufficient to calculate the area under the milk concentration-time curve (AUC_{milk})[6]. The average milk concentration ($C_{av,\ milk}$) can be calculated by dividing the AUC by the collection time interval[90]. Once the average drug concentration is determined, the infant dose through breast milk can be assessed. Despite obvious variability in milk intake volumes (V_{milk}), it is still well-accepted that infant drug exposure estimates can be based on a daily milk intake of 150 mL/kg/day[102]. Average infant drug dosage can be assessed by the equation below[90]:

$$Infant\ daily\ dose = C_{av,\ milk} \times V_{milk}$$

2.7.1 Relative Infant Dose

Many drugs used to treat common diseases and disorders in the postpartum period do not have any corresponding data for infant drug doses or safety information (e.g. infants would not be prescribed a selective serotonin reuptake inhibitor (SSRI) such as escitalopram). The Relative Infant Dose (RID) was established to provide insight on the amount of the weight-adjusted

maternal dose the infant would be ingesting[103]. The RID is expressed as a percentage and is calculated using the following equation[103]:

$$RID = [Infant\ dose\ (mg/kg/day) / Maternal\ dose\ (mg/kg/day)] \times 100$$

It is widely accepted that drugs with an RID of 10% or less are considered "safe" and compatible for use while breastfeeding; although, updated guidelines suggest that the cut-off should be 5% for drugs with psychotropic effects [103–105]. A study examining over 200 drugs found that approximately 50% had RIDs of less than 1% and 87% of drugs had RIDs lower than10%[90]. Despite being a well-accepted safety standard, there are no experimental data that contributed to establishing these cut-offs, questioning the reliability of this measure in assessing infant risk estimates[104]. Furthermore, there are cases, such as the bupropion-induced seizure seen in an infant[3], where the RID was less than 10%, yet an adverse event was still reported. There are also a number of other limitations with the use of the RID. Factors such as variability in dosage, volume of milk ingested, and immaturity of infant metabolism and clearance are not considered, possibly contributing to inaccurate risk assessment[104].

2.7.2 Milk-to-Plasma Ratio

The milk-to-maternal plasma ratio (M/P ratio) is used to determine the extent of drug distribution from maternal plasma to milk[6]. The M/P ratio can be variable across the dosing interval; therefore, it is described as a time-averaged value[86]. The M/P ratio can be predicted by the physicochemical properties of the drug and can also provide insight into the potential mechanism of drug transport into milk[6,106]. Approximately 80% of all drugs have M/P ratios less than 1 (less than 0.1 – 0.9)[106]. While an M/P ratio greater than 1 implies the drug is accumulated in the milk, these observations may not necessarily be clinically significant. As with the RID, the M/P ratio has a number of limitations that could potentially contribute to misleading interpretation of data on the safety of a drug during breastfeeding. Drug clearance, which is not fully mature during the neonatal period, must be considered when assessing the M/P ratio. Drugs that are not readily

cleared by the infant may result in significant accumulation, even if the drug is believed to have a low M/P ratio[106]. In contrast, if an infant were to consume a drug with a high M/P ratio (e.g. M/P of 10) but with relatively high clearance, it would not necessarily result in a clinically or toxicologically significant plasma concentrations[106]. Sole emphasis on the M/P ratio in predicting the safety of a drug during breastfeeding may mislead risk assessment; therefore, consideration of other parameters should be used to generate infant exposure estimates.

2.7.3 Mouse Lactation Models

Animal models are a useful surrogate to understanding drug transport and disposition into milk. Specifically, the mouse mammary gland has been well-characterized and has been described as a suitable model due to its anatomical similarities to the human mammary gland[107,108]. Furthermore, mice and humans share similarities within their active transport systems in the mammary epithelial cells. Bcrp is highly induced in the apical side of the alveolar cells, allowing for the exploration of Bcrp-mediated xenobiotic transport into milk[66]. Despite its utility to better understand the role of active transporters in drug excretion into milk, making inferences to human applications must be done cautiously.

2.7.4 Physiologically-Based Pharmacokinetic (PBPK) Models

The most clinically valuable way to examine infant exposure of a drug through breast milk is to measure steady-state plasma concentrations in an infant whose mother also has achieved steady-state[109]. There are obvious ethical constraints to this approach, especially if the safety of a drug in breastfeeding is not well-known or has never been described. *In silico* techniques may be useful to help predict infant plasma exposure to a drug through breast milk. One *in silico* approach used to better understand the pharmacokinetics of a drug is physiologically-based pharmacokinetic (PBPK) modeling. PBPK models are mathematical representations of a biological system that helps to predict drug pharmacokinetic parameters using a series of differential equations[110]. These models integrate the physicochemical properties of the drug of interest, along with the anatomical and physiological characteristics of the organism of interest. The generic structure of a PBPK model is shown in **figure 2-3**. Each compartment is represented

by a specific organ, and the compartments are interconnected by respective blood flows. The drug transfer across each representative organ is expressed as a mass-balance equation, allowing for the amount of drug to be calculated for any given time[111]. Coupling drug monitoring data in milk to PBPK modeling techniques has the potential to better understand infant plasma exposure, while eliminating invasive infant blood sampling. This approach will be described in Chapter 5: Escitalopram.

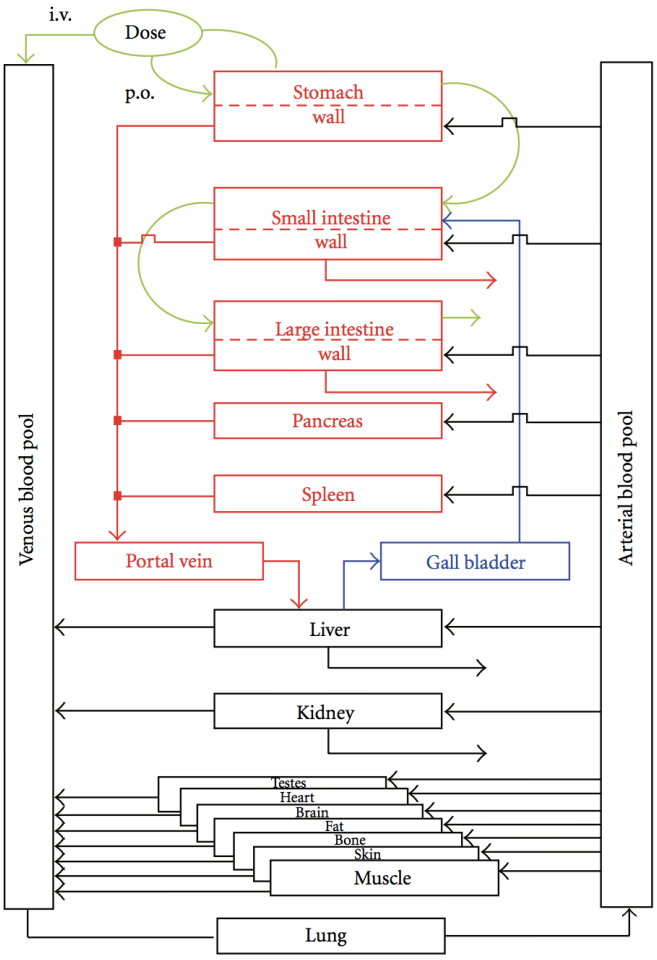


Figure 2-3. Whole body PBPK model from [112] (open access distributed under the Creative Commons Attribution License)

2.8 Chronic Disease During the Postpartum Period

Chronic immune diseases commonly affect women during peak reproductive years. Fortunately, with advancements in therapeutic strategies, remission rates are common and women are able to

conceive and have successful pregnancies[113]. Deciding on the most effective drug therapy for use during lactation is a complicated decision, and often mothers with chronic diseases are often discouraged from breastfeeding due to the perceived risk of infant drug exposure and the fear of adverse events. In the United States, the Food and Drug Administration has removed its pregnancy and lactation lettered risk categories – A, B, C, D, X – and has recently updated their labeling guidelines to provide more detailed information on the use of a medication during lactation[114]. The new labeling requirements require a risk summary on the presence of a drug and/or its metabolites in milk, the effects on the nursing infant, and the effects on milk production[114]. Clinical considerations on options for minimizing exposure, monitoring adverse effects, and, when available, provide data on the scientific basis of the risks described[114]. Still, maternal adherence to medication during breastfeeding and establishment of breastfeeding are impacted due to lack of sufficient drug safety data. It was the goal of this thesis to describe novel approaches to help fill in the gaps of knowledge and allow for nursing women with chronic diseases to be informed on the risk-benefit of using a medication while breastfeeding.

2.9 Rheumatoid Arthritis (RA)

Rheumatoid Arthritis (RA) is a chronic immune-mediated inflammatory disease affecting approximately 1% of the population[115]. Women are at a 2 to 3- fold higher risk of developing RA than men, with disease onset typically occurring during the reproductive years[116]. It has been postulated that women who breastfeed have a higher risk of developing RA, likely due to changes in hormonal status and increased pro-inflammatory prolactin levels[117] . While many women with RA experience disease remission during pregnancy, there is an increased risk for developing a disease flare in the immediate postpartum period [118]. The postpartum period is especially challenging for woman suffering from RA, as the combination of increased physical demands and sleep deprivation could worsen symptoms of the disease[119]. This emphasizes the need to use medication to control or induce remission during this time. There are significant maternal and infant benefits associated with breastfeeding; however, there are little safety data surrounding the use of anti-rheumatic drugs during lactation. Women and healthcare practitioners are faced with effectively managing symptoms while making the decision to breastfeed.

2.9.1 Drugs Used to Treat RA During Lactation

As with most drugs, well-established literature on drugs used to treat RA during breastfeeding is largely lacking. Risk characterization data are often unreliable, due to the fact that guidelines are often based on theoretical risk. Furthermore, most RA drugs have inadequate milk concentrations data based on case reports, and have minimal or nonexistent infant serum data [119]. Short-term administration of short-acting non-steroidal anti-inflammatory drugs (NSAIDs) are considered to be safe during lactation[120]. While these NSAIDs are useful anti-inflammatory drugs, they are not effective as disease-modifying anti-rheumatic agents (DMARDs), which specifically inhibit disease progression and help induce remission[121]. In spite of limited data, the only DMARDs considered compatible with breastfeeding are hydroxychloroquine and sulfasalazine. Other more effective DMARDs, leflunomide and methotrexate, are considered contraindicated during lactation[122].

2.9.2 Methotrexate

Methotrexate (MTX) is an analogue of folic acid and is considered the "anchor drug" in the treatment and management of RA [123]. As the first line of treatment in RA, MTX is administered in nearly 70% of patients and is the most widely used DMARD [124]. Due to the increased risk of spontaneous abortion and significant teratogenicity, MTX is withdrawn from treatment in women trying to conceive [125]. As previously discussed, disease activity of RA often decreases during pregnancy, but tends to flare following delivery, necessitating the reestablishment of treatment[126]. To treat RA, MTX is commonly administered as a single weekly 15-25 mg intramuscular, subcutaneous or oral dose[127,128]. At physiological pH, MTX is ionized and hydrophilic, necessitating transport into the cell by various carrier proteins[128]. Approximately 50% of MTX is protein-bound and is primarily excreted in the urine unchanged[128]. The volume of distribution of MTX is 0.7L/kg, equivalent to that of the total body water volume[128]. The serum half-life of low-dose MTX is between 6-8 hours and is undetectable by 24 hours post-dose[127]. Approximately 10% of MTX is converted to its active metabolite, 7-hydroxymethotrexate (7OH-MTX) by aldehyde oxidase in the liver and both parent and metabolite are excreted in the urine[127]. Once taken up by cells, MTX and 7OH-MTX can be enzymatically modified to polyglutamate derivatives (MTX-diglutamate, -triglutamate, etc.),

which is stored for up to months post-dose [129]. It has been reported that the therapeutic efficacy of MTX is correlated with the concentration of MTX-polyglutamate derivatives within the red blood cells[130]. 7OH-MTX has limited aqueous solubility and its cytotoxicity is reported to contribute to renal damage following high dose MTX therapy[131]. The bioavalability of MTX is highly variable, but is on average around 70%[127]. Many factors can contribute to inter-individual variability and include: age, food, co-administered medication and drug transporter expression[132]. **Figure 2-4** outlines the transport and metabolism of MTX.

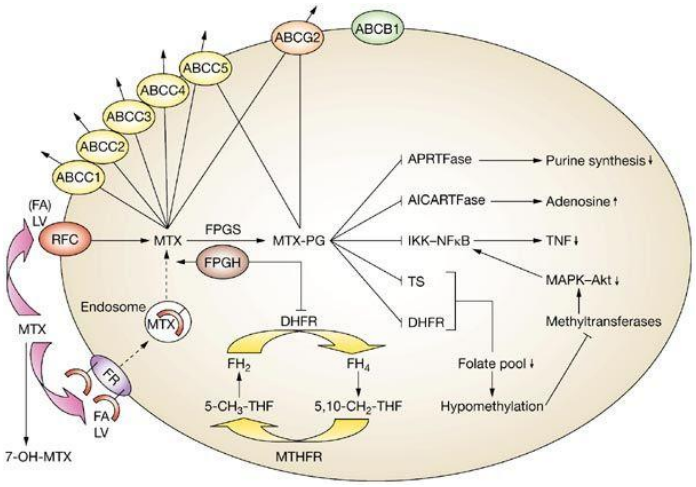


Figure 2-4. Methotrexate transporters and metabolism (with permission from van der Heijden et al. [133]).

2.9.3 Methotrexate Transport

Multiple solute carrier (SLC), ATP-binding cassette (ABC) and receptor-mediated transporters (e.g the folate transporter) contribute to the inter-individual variability in the pharmacokinetics of MTX. **Table 2-3** below summarizes the drug transporting proteins involved in MTX transport. MTX cellular influx is generally mediated by the reduced folate carrier (RFC), which is encoded by *SLC19A1*, and the proton-coupled folate transporter (PCFT), encoded by *SLC46A1*[134–136]. The presence of polymorphisms in transporting proteins responsible for MTX elimination are associated with clinical effectiveness and toxicity. The organic anion transporting polypeptide 1B1 (OATP1B1; *SLCO1B1*) is a key transporter involved in MTX and 7OH-MTX liver disposition and elimination[137]. Recently, a genome-wide association study identified a *SLCO1B1*

polymorphism could play a role in decreased MTX elimination and may be associated with toxicity in pediatric acute lymphoblastic leukemia patients[138,139]. ABC transporter expression also plays an important role in MTX efflux from cells. HEK293 cells expressing wildtype BCRP showed that BCRP has a capacity to transport MTX, MTX-di-glutamate and tri-glutamate; however, any BCRP variants are unable to transport MTX to any extent[140]. Similarly, Volk and Schneider used MCF7 cells to verify that wildtype BCRP transported MTX and its polyglutamated derivatives[141]. It has also been reported that the active metabolite of MTX, 7OH-MTX, is also a substrate for BCRP in membrane Sf9 vesicles[142].

Table 2-3. Summary of MTX drug transporters.

	Gene (transporter)			
Substrate	**ABC**	**SLC**	**SLCO**	**Other**
MTX	*ABCC1* (MRP1) *ABCC2* (MRP2) *ABCC3* (MRP3) *ABCC4* (MRP4) *ABCC5* (MRP5) *ABCB1* (MDR1) *ABCG2* (BCRP)	*SCL19A1* (RFC) *SLC46A1* (PCFT) *SLC22A6* (OAT1) *SLC22A8* (OAT3) *SLC22A11* (OAT4)	*SLCO1B* (OATP1B1) *SLCO1B3* (OATP1B3) *SLCO1A2* (OATP1A2) *SLCO4C1* (OATP4C1)	*FOLR1* (FR)

References: [136–138,140,141,143–152]

2.9.4 Methotrexate in Breast Milk

American Academy of Pediatrics (AAP) classifies MTX as a drug that "may interfere with cellular metabolism" of the nursing infant and is listed as contraindicated during breastfeeding[153,154]. Despite these recommendations, a recent survey reported that 8.5% of physicians and 13.7% of gastroenterologists would continue methotrexate therapy during breastfeeding[155]. However, the safety information on MTX in milk is limited to only three case reports in the literature[150,156,157]. Therefore, the true risk of toxicity and drug accumulation in the infant remains largely unknown. One case report stated that the peak MTX concentration in milk was 8% that of plasma levels following a 22.5 mg/day oral dose for the treatment of choriocarcinoma [156]. More recently, the first case report was published describing MTX levels at 2, 12 and 24 hours following a 25 mg weekly SQ dose for the treatment of RA[157]. This case

report was able to detect 22.7 ng/mL (0.05 μM) MTX in breast milk. The authors noted that this concentration was detectable but fell below the quantitation limits of their assay. This corresponded with a RID estimation of 3.4 μg/kg/day, indicating that the conservative estimate of the infant MTX dose in milk within the first 24 h of maternal dosing was as low as 1% of the weight-adjusted dose of the mother[157]. Similarly, Tanaka et al.[150] reported undetectable levels of MTX in milk (<22.7 ng/ml) 1h to 24 h after 65 mg intramuscular dose for ectopic pregnancy.

Due to the increased incidence of RA in the postpartum period and the well-established efficacy of low-dose MTX therapy in the management of RA, it is with great importance that the safety of MTX therapy during breastfeeding becomes fully defined. However, the publication by Johns et al. performed their analysis using a bioassay and the more recent publication by Thorne et al. used LC-MS/MS but did not describe their methods[157]. Bioassays are lacking in sensitivity and specificity and the limit of quantitation described by the case by Thorne et al. described a high limit of quantitation (>22.7 ng/mL)[157]. At present, there are no other reports on MTX secretion into human milk with a specific and sensitive mass-spectrometry method optimized to measure breast milk. Due to the lack of accurate information on milk concentrations of MTX in these patients, exposure-based risk assessments are not possible, compromising drug safety for those patients during lactation.

2.10 Postpartum Depression and Lactation

Postpartum depression (PPD) is a common disorder affecting between 10-15% of new mothers [158]. The Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) defines PPD as the current or most recent episode of major depressive disorder, if the onset is within the first four weeks postpartum[159]. If left untreated, it can lead to poor cognitive, behavioural and social outcomes for both the infant and mother [160,161]. Infants whose mothers suffered from chronic maternal depression throughout the first 12 months postpartum had poor psychomotor development at 15 months[162]. With the combined functional impairment in the mother and potentially delayed development in infants, treating PPD with pharmacological or non-pharmacological approaches is imperative[163].

2.10.1 Escitalopram

SSRIs are often considered to be a first-line therapy for the treatment of PPD, as they possess good safety profiles in women of childbearing age [164]. Escitalopram (ESC) oxalate is an SSRI antidepressant that is approved to treat major depressive disorder, generalized anxiety disorder and obsessive compulsive disorder[165,166]. Chemically, ESC is the S-enantiomer of citalopram, and follows linear pharmacokinetics after oral administration with a half-life of 27-33 hours, with steady-state plasma levels are reached approximately 7-10 days after initiating therapy[167]. ESC is 56% protein-bound and has a large volume of distribution of approximately 1100 L, corresponding to a wide tissue distribution [167,168]. Clearance is largely hepatic and involves CYP3A4, CYP2C19 and CYP2D6 enzymes [167]. The major metabolite is S-demethylcitalopram (D-ESC), which does not have clinically relevant antidepressant activity[167].

2.10.2 Escitalopram in Breast Milk

The data examining the safety of ESC during breastfeeding are limited to two small prospective cohort studies and three case reports[169]. The two prospective cohort studies investigated the transfer of ESC and D-ESC into breast milk. The first study measured milk concentrations in 5 women and found an RID equal to 4.5% of the maternal weight-adjusted dose, falling below the accepted RID cutoff of 5%[170]. Similarly, the second study investigated the excretion of ESC/D-ESC in 8 women and reported the mean RID to be 5.3%[171]. The case reports in the literature found consistent RIDs and all but one study found no major adverse outcomes in infants[172–174]. The last case reported by Potts et al. described a case of necrotizing enterocolitis (NEC) in a neonate who was exposed to ESC *in utero* and via breast milk[174]. The authors postulated that neonatal ESC exposure caused a decrease in platelet serotonin and thus led to platelet aggregation causing NEC[174]. Clearly, the available data surrounding ESC safety during lactation are limited and should be further explored.

2.11 Inflammatory Bowel Disease (IBD) and Lactation

The incidence of inflammatory diseases (IBD) – Crohn's disease (CD) and ulcerative colitis (UC)- are highest during the reproductive years[175]. An IBD diagnosis is not associated with an increased risk of an unsuccessful pregnancy; however, disease control before conception and maintenance of remission throughout pregnancy is imperative to optimize fetal and maternal health[176,177]. Ongoing active disease or flare during pregnancy is associated with poor neonatal outcomes, such as preterm birth and low birth weight[176]. It has been well-described that a disease flare during pregnancy has the potential for higher fetal risk than the use of medication *in utero*[178]. The concerns of the risking infant drug exposure while managing disease activity subsist during lactation, as it has been reported that women diagnosed with IBD have a higher risk of experiencing a disease flare during the postpartum period[5]. Similarly to RA, the increased risk of flare is thought to be associated with the rise in pro-inflammatory prolactin levels[179]. Despite the well-established benefits to breastfeeding, one study found that 56% of women with IBD chose not to initiate breastfeeding. The majority of women cited the reasons for not breastfeeding were due to concern about risks of infant drug exposure through milk or based their decision on physician counsel[5]. The remainder of women who chose to initiate breastfeeding decided to discontinue their IBD treatment, which evidently led to an increase in disease activity[5].

2.11.1 Drugs Used to Treat IBD During Lactation

A recent cross-sectional survey revealed that physician management of IBD therapy during pregnancy and lactation is highly variable, further highlighting the lack of established guidelines for medication use in the postpartum[155]. The drug categories used for the treatment and management of IBD are shown in **table 2-4**. The "pyramid of treatment" is often used to manage the disease, starting with 5-aminosalicylate and sulfasalazine as the first administered medications[155]. For more severe disease states, administration of immunosuppressants such as methotrexate or azathioprine are required. Other treatments for moderate to severe IBD include corticosteroids, such as prednisone, and anti-TNFα biologics monoclonal antibodies (mAb), such as infliximab.

Table 2-4. Drug categories used to treat IBD (Adapted from Triantadillidis et al.[180])

Category	Drugs
Anti-inflammatory	○ Mesalazine ○ Corticosteroids (prednisone, methylpredinisone, budesonide)
Immunosuppressants	○ Azathioprine, 6-mercaptopurine, methotrexate, cyclosporine, tacrolimus
Antibiotics	○ Metronidazole, ornidazole, clarithromycin, ciprofloxacin
Biologics	○ Infliximab, adalimumab, certolizumab pegol

It is widely accepted that the inflammatory cytokines, TNFα, plays a large role in the pathogenesis of IBD [181]. Evidently, TNFα-inhibition with anti- TNFα drugs has become a powerful tool in patients with moderate-to-severe CD, especially in those who are non-responsive to other treatment strategies[180].

As shown in **Figure 2-5,** the anti-TNFα drugs used to treat IBD include: infliximab (chimeric IgG1 mAb), Certolizumab pegol (human Fab' conjugated to PEG) Adalimumab and Golimumab (human IgG1 mAb)[182–184].

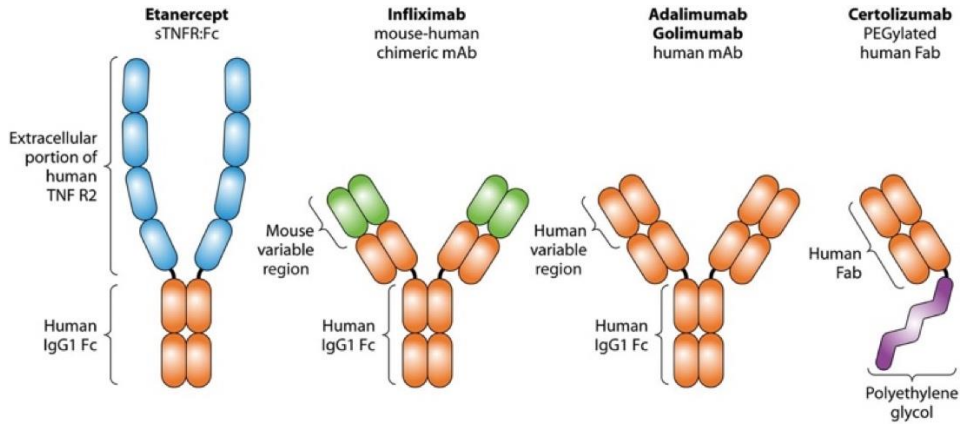


Figure 2-5. Structure of therapeutic antibodies used to treat inflammatory conditions; etanercept, infliximab, adalimumab/golimumab, and certolizumab (left to right) (with permission from Dele Davies[185]).

2.11.2 Infliximab

Infliximab (IFX) is a chimeric monoclonal antibody with a murine-derived variable region and a human constant region (**shown in Figure 2-5.**) that can bind and neutralize soluble and membrane-bound TNFα[186]. IFX is highly hydrophilic and has a molecular weight if 149.1 kDa. The volume of distribution is low, at 3-6 L, representing the intravascular space[187]. IFX is used in the treatment of IBD conditions, and has also been approved for management of RA, ankylosing spondylitis, psoriatic arthritis, and psoriasis[187]. To initiate treatment, multiple infusions of IFX are administered intravenously (3-5 mg/kg) and repeated after 2 weeks and 6 weeks. Once clinical remission is achieved, patients receive a maintenance dose is administered every 8 weeks[187]. The elimination half-life is 7-12 days due to very low renal clearance (~0.25 mL/min)[187,188]. IFX is systemically cleared following phagocyte internalization and subsequent proteolytic catabolism[189]. IFX can evade catabolism by neonatal Fc receptor (FcRn)-mediated recycling[188]. Antibodies bind with high affinity to the FcRn inside an acidified endosome, which allow for protection from proteolytic processes[189]. The FcRn-bound IFX within the endosome is returned to the cell surface and recirculated[189].

2.11.3 Infliximab in Breast Milk

Theoretically, due to the high molecular weight and low tissue distribution, excretion of IFX into milk should be low[122]. To date, there are limited and conflicting data on infliximab safety during lactation. Until recently, three case reports reported cases of IFX below detectable limits in breast milk[190–193]. In contrast, current publications describe conflicting data; concentrations in the range of 100-300 ng/mL have been measured in breast milk up to 8 days post-infusion[194,195]. Moreover, there are several limitations with the current literature contributing to largely unreliable infant exposure estimates. The majority of the assays used to measure IFX in breast milk were optimized for serum detection, not taking milk matrix differences into consideration nor validating their method for milk. Further, infant exposure to IFX via breast milk after day 8 post-infusion remains unknown.

Surprisingly, a case series reported an IFX serum concentration of 1700 ng/mL in a partially breastfed infant, contradicting the previous reports that demonstrated undetectable infant serum

infliximab levels [192,196]. Although no adverse events were noted in this infant, these findings warrant further exploration of infant exposure to IFX via breast milk, especially because the local mucosal effects remain unknown.

2.12 Thesis Objectives

The overarching objective of this thesis is to better understand drug safety during lactation. Using various analytical and *in silico* approaches, three drugs of particular interest for chronic disease will be explored; methotrexate, escitalopram and infliximab.

Project 1: Methotrexate

The primary goals of the methotrexate project were to elucidate the mechanism of transport of MTX into milk and determine the risk of MTX exposure to the nursing infant. The following are the specific aims:

Aim 1: Develop and validate a sensitive LC-MS/MS method to quantitate MTX and metabolite(s) in mouse and human milk.

Aim 2: Examine the pharmacokinetics of MTX and MTX-metabolites in human milk.

Aim 3: Using an animal lactation model, determine the role of the active transporter, Bcrp, in excreting MTX into mouse milk.

Aim 4: Explore the potential for Bcrp substrate competition to modulate MTX efflux across the lactating mammary epithelium.

Project 2: Escitalopram

The main objectives of the escitalopram project were to use an optimized analytical approach to determine infant drug exposure estimates and utilize *in silico* techniques to determine total systemic exposure of ESC in an infant.

Aim 1: Optimize an LC-MS/MS method to measure ESC and metabolite in human milk.

Aim 2: Determine the steady-state time-concentration profile of ESC and metabolite in the milk of women taking ESC in the postpartum.

Aim 3: Use the measured milk data from **Aim 2**, along with feeding-related parameters to simulate a total infant daily dose of ESC via breast milk.

Aim 4: Develop a PBPK model to simulate infant plasma concentrations using the "dose" an infant would receive through breast milk from **Aim 3**.

Project 3: Infliximab

The main objectives of this project were to develop and validate an analytical method to examine the concentration of infliximab in breast milk and assess the extent of exposure to the nursing infant.

Aim 1: Develop and validate an ELISA-based approach to measure infliximab concentrations in milk.

Aim 2: Determine the extent of drug exposure to the nursing infant.

Chapter 3
Materials and Methods

3 Materials and Methods

In this chapter the thesis methodology and materials are discussed. The overarching study is called the "Drugs in Lactation Analysis Consortium" (DLAC) Study, which served as the recruitment platform for all three sub-studies: methotrexate (MTX), escitalopram (ESC), and infliximab (IFX). The DLAC Study methodology and recruitment criteria are outlined at the beginning of this chapter, followed by an in-depth description of the three different scientific approaches for each project. The MTX section of this chapter describes methodology used to develop and validate the LC-MS/MS method used to measure the drug and metabolites in milk. It also describes the animal study used to determine the role of the drug transporting protein, Bcrp, in excreting MTX into milk. Next, the analytical and *in-silico* methods used to assess ESC infant exposure through breast milk are discussed. Finally, the analytical approach used to understand the IFX profile in milk is explained.

3.1 DLAC Study

Women were recruited through the DLAC Study framework via four main networks:

1. The Motherisk Pregnancy and Breastfeeding helpline
2. Searching for the DLAC Study via ClinicalTrials.gov
3. Advertisements via The Hospital for Sick Children poster boards and social media accounts
4. Physician referrals

Pregnant or breastfeeding women who contacted the Motherisk Helpline to receive counseling about MTX, ESC or IFX safety during pregnancy or lactation were first screened for eligibility by the Motherisk helpline counselors. The potential participants were asked if they would give permission to be contacted by a DLAC study member to learn more about what study participation entails. The name and phone number of interested individuals were provided to the study coordinator and contacted within a few days after their initial counseling call for oral

consent to be obtained. Patients referred by physicians were instructed to initially call the helpline and were recruited following the same procedure as above. Individuals who saw DLAC Study information on ClinicalTrials.gov or Hospital for Sick Children media contacted the study coordinator directly via phone or email.

The DLAC Study questionnaire and requisition (Appendix A) and consent forms (Appendix B) were approved in May 2014 by the Research Ethics Board (REB #1000036538) at the Hospital for Sick Children in Toronto, Canada.

3.2 Inclusion Criteria

1. Lactating women on MTX, ESC* or IFX
   (* steady-state)
2. Women who have provided signed consent to participate in the study

3.3 Exclusion Criteria

1. Unable to communicate in English
2. Colostrum phase (< 1 week postpartum)

3.4 Demographic Information and Sample Collection

Once the participants had agreed to participate and provided oral consent over the phone, women were sent study consent forms, questionnaire, requisition forms, 35 mL milk collection containers, a Styrofoam box, and ice packs to their homes. Demographic information such as age, height, weight and ethnicity were collected on the questionnaire (**Appendix A**). Information about concomitant medications, supplements or vitamins was also obtained. Participants asked to collect 5 mL of milk either by manually expressing by hand or via electrical or manual breast pumps at random times throughout their dosing interval (daily for ESC, weekly for MTX or q8 weeks for IFX). They indicated whether milk was collected pre-, post- or between infant feeds on the study requisition forms. Both foremilk and hindmilk were collected in separate milk

containers as part of the MTX and ESC sub-studies in order to determine whether the drugs preferentially accumulate in different milk phases. To examine the pharmacokinetics of the drugs in milk, women also indicated exactly when they were administered their dose on the requisition form. Once milk samples were collected at respective time points, women were instructed to immediately freeze the samples until all samples were collected and ready to ship to the lab at The Hospital for Sick Children. When all samples were obtained, each study participant packed their frozen samples and ice packs into the Styrofoam box provided, and shipped their samples using an expedited overnight courier service. For women taking MTX or ESC, samples were thawed and aliquoted upon arrival and frozen until analysis. For IFX samples, two approaches were used to ensure the number of freeze-thaw cycles were minimized: (1) if the sample arrived semi-thawed, the sample was allowed to thaw completely and was then aliquoted and frozen until analysis; or (2) if the samples arrived frozen, they were immediately placed back in the freezer and only thawed prior to analysis. Each IFX milk sample did not exceed more than two freeze-thaw cycles prior to analysis. IFX was found to be stable following up to 4 freeze-thaw cycles[197].

3.5 Methotrexate

3.5.1 Reagents

MTX, 7-hydroxymethotrexate (7OH-MTX) and methotrexate-d3 (MTX-d3; deuterated internal standard) were purchased from Toronto Research Chemicals (Toronto, Canada). Mass spectrometry (MS)- or high-performance liquid chromatography (HPLC)- grade solvents and all other chemicals used were obtained from Sigma-Aldrich. Ketamine was purchased from C.D.M.V Inc. with permission from Health Canada.

3.5.2 Study Participants

Two patients receiving MTX therapy for RA were recruited through the DLAC Study. The patients were instructed to collect milk once before taking their dose and then 5-7 subsequent milk samples throughout the MTX dosing interval. One patient was asked to collect foremilk and

hindmilk to determine whether MTX concentrations were influenced by milk composition. Both participants opted to discontinue breastfeeding their infants after initiating MTX therapy. Milk was collected either via electric pump or manual expression. Whole breast milk samples were aliquoted into 1.5 mL polypropylene tubes and stored at -80°C until sample preparation, extraction and analysis. Breast milk MTX and 7OH-MTX concentrations were calculated by using the ratio of the internal standard/AUC of the sample.

3.5.3 Sample Preparation and Extraction

Pooled, drug-free human milk was donated to the DLAC Study for the purpose of preparing milk standards and controls. Donated milk was received from healthy nursing mothers 2-18 months post-delivery and screened for possible drug contaminants. Only confirmed drug-free milk was used in method development.

Due to the complexity of the matrix, various drug extraction methods were attempted and optimized for methotrexate extraction from breast milk. Prior to extraction, calibrators, quality control (QC), samples and patient samples were allowed to thaw until reaching room temperature. Milk samples were prepared according to the protocol by Rezk et al[198]. with modifications as follows: 100 μL of milk (calibrator, QC, patient milk sample, mouse milk sample) was aliquoted into 1.5 mL polypropylene microcentrifuge tubes, to which 50 μL hexane, 100 μL acetonitrile and 100 μL methanol containing 10 ng/mL MTX-d3 (internal standard) were added. Samples were capped, vortexed for 10 seconds and centrifuged at 14,000 rpm for 5 minutes at room temperature. As per the method according to Rezk[198], the hexane mixed with the fat and formed a solid disc, allowing for easy removal of the remaining supernatant below the disc. 100 μL of the remaining supernatant was aliquoted into a new 1.5 mL polypropylene tube at which point 100 μL of methanol was added, capped, vortexed and centrifuged for another 5 minutes at 14,000 rpm. The liquid remaining was transferred into another clean 1.5 mL tube and left to sit at -20°C for 30 minutes to accelerate the formation of the remaining protein precipitate. Samples were removed from the freezer, centrifuged for 5 minutes at 14,000 rpm and the supernatant transferred to glass HPLC vials and 5 μL was injected onto LC-MS/MS.

3.5.4 Preparation of Standards

Stock solutions of MTX (4.5 mg/mL in DMSO), genistein (20 mg/mL in DMSO) were prepared, aliquoted and stored in amber glass vials at -20^{0}C and thawed and diluted on the day of experimentation. To prepare the working solutions suitable for animal parenteral injection, the original stock solutions were diluted in saline. Riboflavin and FMN were dissolved from powder into saline and ko143 was dissolved in corn oil the morning of the study experiment.

Separate standard solutions of MTX, 7OH-MTX and MTX-d3 were prepared at a concentration of 1 mg/mL in methanol and stored in an opaque box at -20°C. Intermediate stocks solutions of MTX and 7OH-MTX were prepared at a concentration of 1 μg/mL for each respective compound. The internal standard (MTX-d3) stock solution was prepared at 10 ng/mL in methanol. The final internal standard stock was stored at -20°C in a 15 mL falcon tube covered in aluminum foil. Six calibration standards with concentrations (0.75, 1.5, 3.1, 6.25, 12.5, 25 ng/mL) and three QC levels (1, 8, 16 ng/mL) were prepared in drug-free human breast milk by spiking and serial dilution. All calibration standards and QC samples were stored at -20°C and sample extraction was preformed immediately once thawed.

3.5.5 Liquid Chromatography (LC)

Chromatographic separation was performed on a Shimadzu Prominence UFLC system, which includes binary pumps, autosampler, degasser, and column oven. A sample volume of 5 μL was injected into an biphenyl column (2.0 x 75 mm, 3 μm) at 40^{0}C. The total LC run time was 6 min and liquid flow rate is 0.5 mL/min. LC Gradient Conditions; Solvent A was water with 0.1% formic acid, Solvent B was methanol with 0.1% formic acid, as shown in **figure 3-1** below.

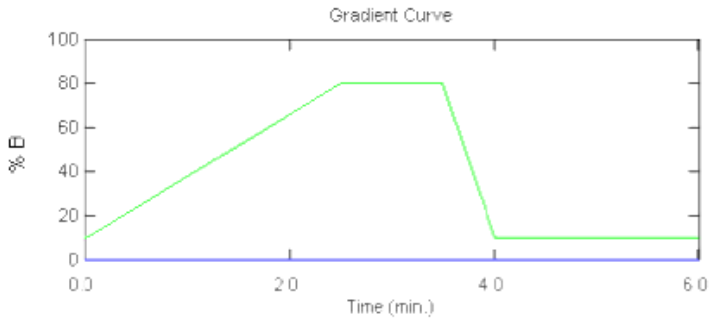


Figure 3-1. The gradient curve (% solvent B over time) for the final liquid chromatography method in the LC-MS/MS assay.

3.5.6 Mass Spectrometry (MS)

Mass spectrometric analysis was run on an Ab Sciex QTrap 5500 equipped with an ESI source. The final optimized conditions for analysis were as follows: ESI voltage: 5000; HSID Temp: 300°C; nebulizer gas setting: 400; drying gas setting: 120; heating gas setting: 350; source temp: 250°C; dwell time (ms): 100; pause time (ms): 5. The following MRM transitions were selected: MTX: 455/308.1, 7OH-MTX: 471/324.1, and the internal standard MTX-d3 458/311.1. Optimized conditions for declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CxP) for each respective transition are shown in **table 3-1**.

Table 3-1. Final quadrupole parameters for each transition for MTX, 7OH-MTX and MTX-d3

Compound	Transitions	DP (volts)	CE (volts)	EP (volts)	CxP (volts)
Methotrexate	455 → 308	45	29	10	39
	455 → 175	45	53	10	22
7-Hydroxymethotrexate	471 → 191	45	29	10	18
	471 → 324	45	15	10	52
Methotrexate -d3	458 → 311	36	27	10	12

3.5.7 Assay Precision

The precision of this method was determined from the analysis of breast milk-spiked samples of equimolar concentrations of MTX and 7OH-MTX at three different quality control levels: low (1 ng/mL), medium (8 ng/mL) and high (16 ng/mL). Intra- and inter-day precision were determined by calculating the percent CV from the mean and standard deviation of each level of QC. Intra-day precision was calculated on a single day using 20 replicates at each concentration level. Inter-day precision was performed over 12 runs across 12 months, the total number of runs performed for study validation and analyzing study samples.

3.5.8 Determination of Linear Range

Seven equally spaced intermediate concentrations levels were prepared by proportionately mixing high and low pools from the dilution scheme included in the Clinical & Laboratory Standards Institute (CLSI) EP6-A Appendix A[199]. Samples were run in triplicate and plots of the mean measured value were compared against the expected value. Polynomial regression analysis was used to evaluate linearity.

3.5.9 Limit of Quantification (LoQ)

The functional LoQ was defined as the lowest measurable concentration of MTX and 7OH-MTX. The LoQ was calculated using the precision profile approach as defined by CLSI EP17-A2[200]. Standard 1 (0.78 ng/mL) was serially diluted three times in a 1:1 ratio with blank milk to yield expected concentrations of (0.39, 0.2, and 0.1 ng/mL). Samples were each run in 5 replicates and the sample with a 20% CV was defined as the functional limit of quantification as per the precision profile approach criteria[200].

3.5.10 Carryover

Two approaches to measure assay carryover were used. To examine column retention of the analyte, a high standard (25 ng/mL) was injected, followed by three blanks. A sequence of 4 high

(a) standards and 4 low (b) standards were run and the percentage carryover was determined by the equation from **figure 3-2** below[201].

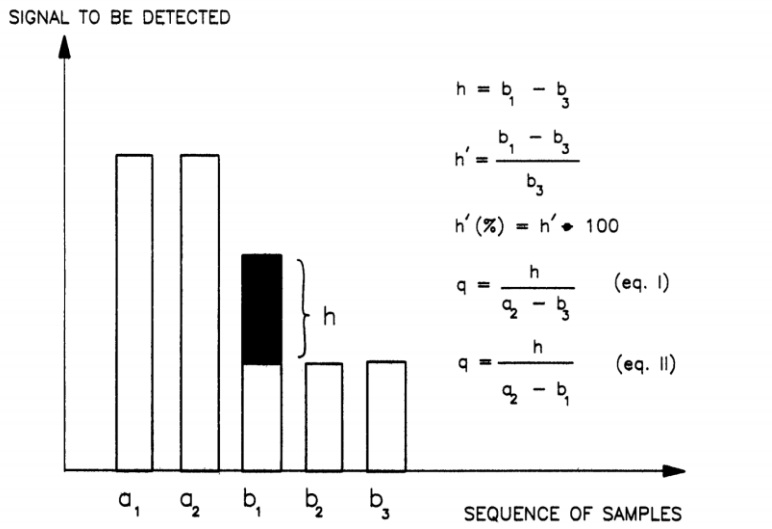


Figure 3-2. Equations used to calculate percent carry-over (open access distributed under the Creative Commons Attribution License)

3.5.11 Method Comparison

Due to the unavailability of a comparative method for 7OH-MTX, the method comparison study was carried out using solely MTX values. Comparisons were carried out with a total of 36 patient plasma samples using MTX values generated from the clinically validated Abbott ARCHITECT chemiluminescent assay and plotted against the calculated patient concentration from our method. Twenty-six serum samples were obtained from patients undergoing high-dose MTX treatment for malignancy at the Hospital for Sick Children. Five external quality assurance samples, provided by the Canadian Association of Pathologists proficiency program, were also used in our method comparison analysis. Serum samples were spiked into breast milk at a 1:10 dilution to achieve calculations within our calibration range. To not alter the final matrix, volumes of <10% of the total volume were spiked into milk.

3.5.12 Trienzyme Digest

50 µL of protease (10 mg/mL) was added to 100 µL human breast milk in 200 µL phosphate buffered saline (1% ascorbate). Samples were incubated for 2 hours at 37^0C, followed by heating at 100^0C for 5 minutes to deactivate enzyme activity. Samples were allowed to cool on ice for another 5 minutes, at which point 100 µL of α-amylase (20 mg/mL), 50 µL human plasma folate conjugase (pooled human plasma), and another 200 µL phosphate buffered saline (1% ascorbate) were added and left to incubate for 3 hours at 37^0C. Samples were centrifuged at 5000 x g for 10 minutes and extracted by following the protocol above using organic solvents. Samples were analyzed via the LC-MS/MS method described here.

3.5.13 Determination of Plasma Concentrations

Measurement of MTX in mouse plasma was performed on the Abbott ARCHITECT i2000 platform using a one-step immunoassay using chemiluminescent microparticle immunoassay technology.

3.5.14 Riboflavin Measurement

Measurement of riboflavin and FMN were carried out by the Analytical Facility for Bioactive Molecules at The Hospital for Sick Children's Research Institute. 250 µL methanol was added to mouse milk samples, vortexed and then placed on ice for an hour. Samples were then centrifuged at 10,500 x g for 15 minutes. The supernatant from each sample was removed and aliquoted into clean tubes. Another 250 µL methanol was added to the first tubes, then followed the same procedure as above. The supernatants from the methanol fractions were evaporated and then reconstituted in methanol/ 50 mM ammonium acetate. 10 µL was injected into a Dionex Ultimate 3000 series HPLC and chromatographic separation was performed on a Nova-Pak C18 column (150 x 3.9 mm, 4 µm). Flow was 0.8 mL/minute with an isocratic mobile phase of 50 mM ammonium acetate and methanol (75/25). Riboflavin and FMN were detected by the Dionex RF 2000 with fluorescence excitation wavelength of 372 nm and emission wavelength of 520 nm.

3.5.15 Animal Lactation Model

Female mice (lactation days 8-12) were obtained from Taconic Inc. or Jackson Laboratories housed and handled according to the Lab Animal Services' Animal Use Protocol at SickKids Research Institute. The animals used in these experiments were wild-type FVB or Bcrp knockout (Bcrp -/-) aged 7 weeks. Animals were kept in a temperature-controlled environment and received water and chow ad libitum. Immediately after delivery, litter size was controlled to maintain 6 pups. If dams did not produce a litter of 6, they were cross-fostered. On experimental days, litters were euthanized between 4-5 hours prior to milk collection, to ensure that the mammary glands had sufficient volumes of milk for collection.

3.5.16 Determination of Bcrp-Mediated Transport of MTX

In order to determine if Bcrp is involved in transporting MTX into milk, we used a mouse lactation model with two groups: wild-type (WT) with Bcrp inherently overexpressed, and Bcrp knockout mice (Bcrp -/-) As shown in figure 3-3, mice were given an IP injection of 0.75 mg/kg methotrexate at the beginning of the experiment (time 0)[202] . Plasma concentrations were used to determine whether an injection successfully reached systemic circulation (e.g. mice were rejected from analysis if plasma concentrations were < 22 ng/mL (0.049 μmol/L)), the clinical assay cutoff.

Forty-five minutes post-MTX dose, an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) was administered in order to anaesthetize the mouse for milk and plasma collection. Immediately following, mice were given a subcutaneous dose of 5 IU oxytocin (250μL) to facilitate milk let-down. Sixty minutes post-MTX dose, milk was collected via suction or manual expression and plasma was collected by cardiac puncture. Once plasma was collected, mice were euthanized by cervical dislocation.

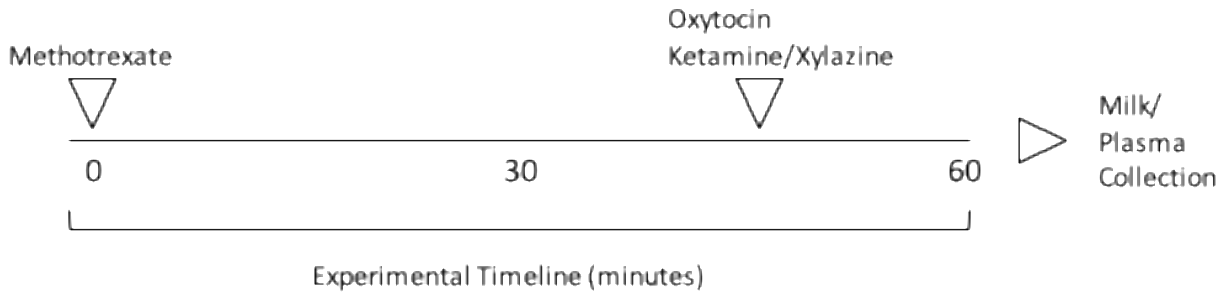


Figure 3-3. Experimental timeline used to determine the role of Bcrp in excreting MTX into milk.

3.5.17 Riboflavin-MTX Interaction

To determine the influence of co-administering a Bcrp substrate or inhibitor, mice were pre-treated with riboflavin (20 mg/kg), FMN (100 mg/kg), ko143 (20 mg/kg), or genistein (20 mg/kg) 30 minutes prior to MTX administration, shown in figure 3-4. The experimental timeline for anesthesia and milk/plasma collection were the same as above.

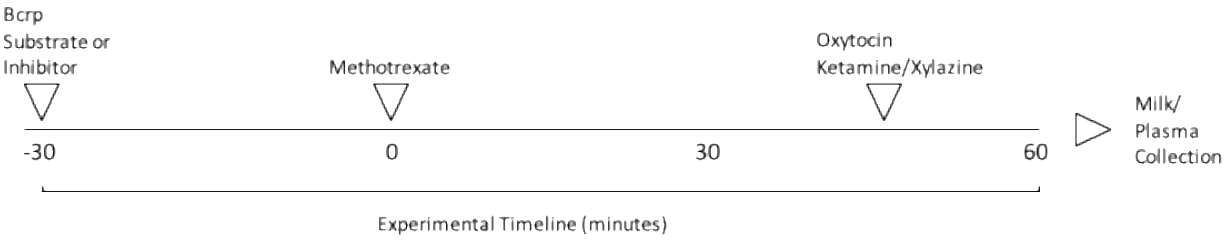


Figure 3-4. Experimental timeline used to determine the effects of co-administering a Bcrp substrate or inhibitor on MTX milk and plasma concentrations.

3.5.18 Statistical Analysis

Comparison between two groups was accomplished by using a student's t-test. Differences of $P < 0.05$ were considered to be statistically significant. N=3-6 per group of mice.

3.6 Escitalopram

3.6.1 Study Population

A total of 18 lactating women taking ESC provided informed consent and were enrolled in the DLAC Study.

3.6.2 Software

All simulations (adult and infant) were completed using PK-Sim® v6.3 (Bayer Technology Services, Leverkusen, Germany), which implements a whole-body PBPK model consisting of 15 organs. Organs were structured into the default small molecule mode of four compartments: interstitial, plasma, red blood cells, and intracellular space.

3.6.3 Reagents

Citalopram (the racemic enantiomer of ESC) was purchased from Cerilliant (1 mg/mL in methanol) and the internal standard, citalopram-d6, was purchased from Toronto Research Chemicals (1 ng/mL in methanol). All solvents and other reagents were analytical or mass spectrometry grade.

3.6.4 Analytical Methods

ESC concentrations in breast milk were measured by LC-MS/MS using the Thermo TSQ ESI in positive mode with a Kinetex 2.6 µm F5 100Å, 100 x 2.1 mm column. The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% formic acid in water) for a total 7-minute sample run time. Solvent A was delivered initially at 30%, held for 0.5 minutes and increased to 95% via 4.5-minute gradient and held at 95% for another minute, then decreased back to 30% in another minute to re-equilibrate the column to starting conditions. The flow rate was 300 µL/min and the injection volume was 20 µL. The transition ions were 325-->109 and

331-->109 for citalopram and citalopram-d6, respectively. Final mass spectrometry settings are shown in table 3-2 below.

Table 3-2 Final mass spectrometry settings for the ESC LC-MS/MS method

Setting	**Compound**	
	325 ESC	311 D-ESC
Spray Voltage	5000	
Vaporizer Temperature (°C)	350	
Sheath Gas	30	
Ion Sweep	0	
Aux Gas	10	
Capillary Temperature (°C)	325	
Tube Lens	102	112
Skimmer	-5	-5
	Transition 325 → 109	Transition 311 → 109
CP	1.5	
CE	27	31

3.6.5 LC-MS/MS Assay Performance

The assay performance characteristics (precision, linearity, ion suppression) were analyzed in accordance with CLSI guidelines[203]. Performance studies were determined using spiked breast milk controls and standards. Within-day precision was assessed by running 20 replicates of each level of quality control milk samples and calculating the coefficient of variation for each level. Samples were run in one batch; therefore, inter-day precision was not calculated. Linearity was evaluated by preparing proportionate dilutions of high and low control pools. Six pools were run in triplicate and the measured mean was plotted against the expected value. Ion suppression and matrix effect was carried out by extracting blank milk, injecting into the HPLC and directly infusing ESC in methanol into the mass spectrometer across the chromatographic run.

3.6.6 Sample Preparation

50 µL each of hexane, acetonitrile and methanol containing citalopram-d6 internal standard were added to 100 uL standard or patient breast milk. Samples were vortexed well and centrifuged at 9000 rpm for 5 minutes. 100 µL supernatant (under the fat disc) was removed and 100 µL methanol was added. Samples were vortexed and centrifuged at 9000 rpm for another 5 minutes. 100 µL of the supernatant was removed, dried down and reconstituted in 300 µL 70/30 0.1% formic acid/acetonitrile and injected into the HPLC.

3.6.7 Simulating Infant Daily Dose

Measured breast milk concentrations at a maternal dose of 20 mg/day ESC (the highest dose indicated for depression in the product monograph) was used along with feeding-related parameters from the literature (**table 3-3**) to stochastically simulate the infant daily doses in a large population of infants. A daily dose of ESC was estimated by multiplying the randomly-generated parameters: volume of milk (mL/feed) and frequency of feeds (feeds/day) based on the previous studies [204,205], and milk ESC concentration (ng/mL) measured in the present study. This result was then weight-adjusted (divided by kg) to generate an infant dose in mg/kg/day (equation 3-1). The simulation was further adapted so that frequency of feeds was dependent on the volume of the feed. If the volume of feed was greater than the mean (76 mL/feed), a frequency was selected that was less than the mean (11 feeds/day) and vice versa to avoid unrealistic outliers.

Table 3-3. Feeding-related parameters used to generate the infant daily dose (data will be show in ESC results section)[42,204]

Parameter	Mean	SD
Infant Weight (kg)	5.43	1.3
Volume of Milk per Feed (mL/feed)	76.0	12.6
Frequency of Feeds (feeds/day)	11	3
Milk [ESC] at 20 mg dose (ng/mL)	49.8	17.5

$$\textit{Infant Dose (mg/kg/day)} = \frac{volume\ of\ milk\ (mL) \times frequency\left(\frac{feeds}{day}\right) \times milk\ concentration\ (\frac{mg}{mL})}{weight\ (kg)}$$

Equation 3-1. The equation used to generate the total infant daily dose based on the above literature values and variation.

3.6.8 PBPK Model Development and Simulation

An infant PBPK model was developed using the workflow published by Maharaj et al. **(Figure 3-5)** [206] to simulate plasma exposure after administering daily doses of ESC via breast milk in our virtual infant model.

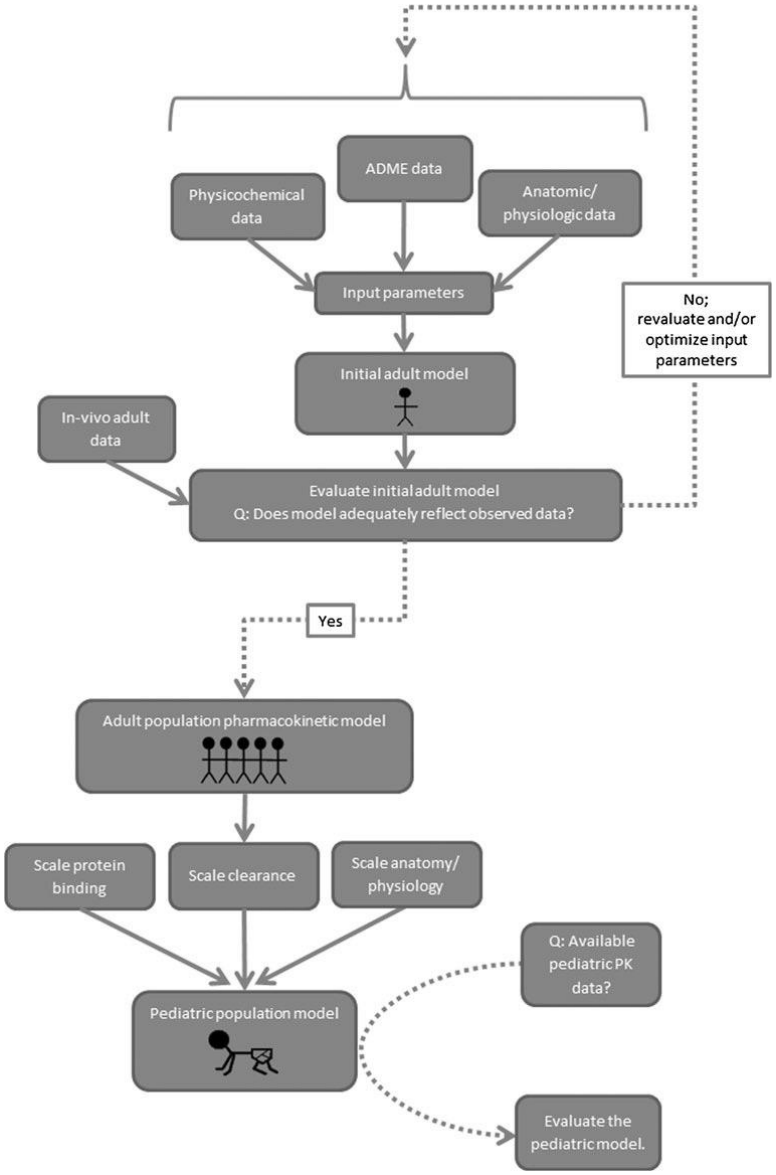


Figure 3-5. Pediatric and infant PBPK model development (with permission from Maharaj et al. [206]).

The first step in infant model development is to construct an adult PBPK model using data on: the physicochemical properties of ESC, adult pharmacokinetic data from the literature, and physiological data on the population of interest. The physicochemical properties of ESC used to build the model were also obtained from the literature and shown in **table 3-4**[167].

Table 3-4. Physicochemical properties of ESC used in building the PBPK model

Parameter	Value
Lipophilicity (logP)	3.5
Molecular weight	324.4 g/mol
Halogens	1 Fluorine
pKa	9.78 (base)
Solubility[a]	35.78 g/L
Oral bioavailability	80%
Fraction unbound in adults	45%

[a] Solubility of ESC oxalate in distilled water

Tissue: plasma partition coefficients were calculated using the Rodgers and Rowland algorithm in PK-Sim® v6.3[207]. Physicochemical parameters of the drug were kept consistent between the adult and infant models, as drug-specific properties are not age-dependent.

To construct the initial model, we utilized data from adult studies examining the pharmacokinetics of ESC following IV administration. Simulations were performed using the data set from Sogaard et al.[168]. In order to fit the model and minimize the difference between the simulated and literature values[168], lipophilicity (logP) of ESC was optimized to 2.85 and used in all further simulations. This allowed for the model-predicted maximum concentration (C_{max}), half-life, volume of distribution (V_d) and area-under-the-curve (AUC) to fit the observed values in the literature (**table 3-5**).

Table 3-5 Model-predicted values vs. observed values after IV optimization

Parameter	**Model-Predicted**	**Sogaard et al.**[168]
C_{max}	57.6 nM	58.6 ± 15.8 nM
Half-life	30.6 h	26.6 ± 7.11 h
V_d	15.5 L/kg	20 L/kg
AUC	1130 nmol·h/L	1102 nmol·h/L

Next, the pharmacokinetics following oral administration of ESC were examined. The drug-specific parameters that were optimized in the IV model simulation remained constant, except those pertaining to oral absorption. ESC solubility was set at the experimental value from Choudary et al.[208], with the optimal shape followed a Weibull function with a 50% dissolution time of 75 min. Intestinal permeability was calculated using PK-Sim® to be 7.22×10^{-5} cm/min. The observed plasma concentrations after oral administration from Malling et al. [209] were compared to oral administration simulations for a population of 1000 healthy adults to visually optimize the dissolution function according to C_{max} and T_{max}. **Figure 3-6**. shows a comparison of the model-fitted oral simulation to the observed dataset from Malling et al.[209].

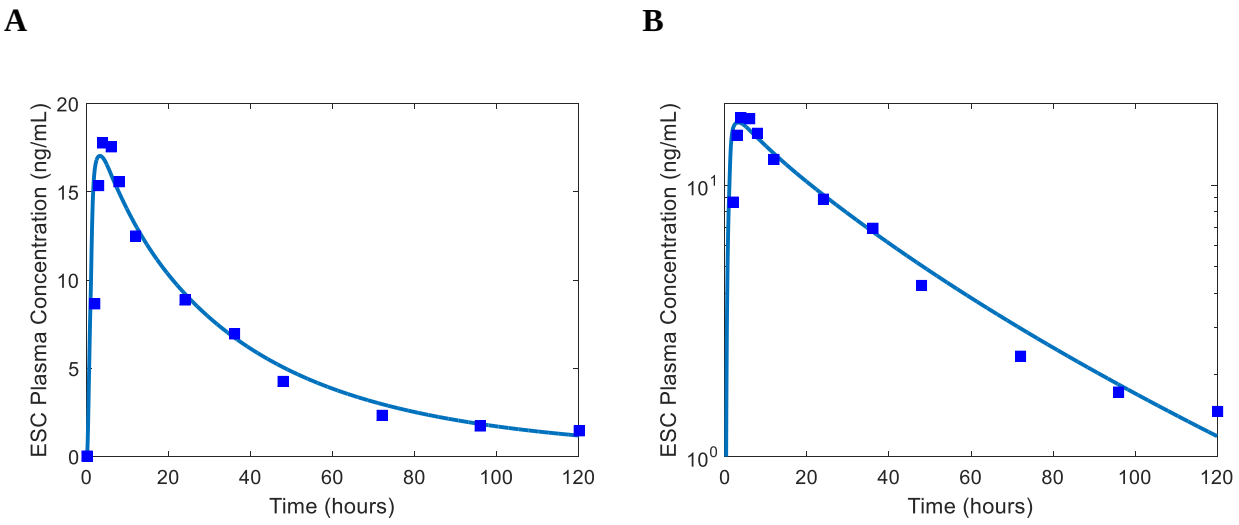


Figure 3-6. Comparison of the model-fitted oral simulation (A: linear; B: semi-log) to the experimental dataset from Malling et al. for healthy adults that was used for model building. (Figure prepared by Dr. Paul Malik using MATLAB)

3.6.9 PBPK Model Evaluation

As shown in **table 3-6**. eight adult human PK datasets from the 5 published studies[168,210–213] were used to evaluate the predictive accuracy of the adult PBPK model for plasma $AUC_{0-\infty}$. The two studies that were used in model development are included in grey (**table 3-6**). Populations in the trials were well-differentiated by age, sex, ethnicity, height, weight and the doses of ESC received. For each of the eight datasets, a virtual population of 1000 individuals was created to match the population in the experimental trial. Plasma concentration-time profiles were simulated for each individual in the population, and a plot of the observed vs. model-predicted values was generated to assess model accuracy. Model accuracy was further assessed by using a bootstrapping technique to determine if the observed mean plasma $AUC_{0-\infty}$ fell within a 95% confidence interval of the simulated means from our virtual populations (**Figure 3-7**).

Table 3-6 Adult pharmacokinetic data used for PBPK model development and evaluation

Trial	Dose	N	Age (years)	Sex (% male)	Ethnicity
Sogaard et al. (2)	10 mg i.v.	8	18-45	100%	European
Malling et al. (3)	20 mg p.o.	16	18-45	50%	European
Periclou et al. (4)	10 mg p.o.	12	18-35	50%	American
Periclou et al. (4)	10 mg p.o.	11	12-17	50%	American
Munoz et al. (5)	20 mg p.o.	20	20-28	50%	Columbian
Sogaard et al. (2)	10 mg p.o. (SS)	17	18-45	50%	European
Sogaard et al. (2)	20 mg p.o.	24	18-45	100%	European
Sogaard et al. (2)	30 mg p.o. (SS)	16	18-45	50%	European
Gutierrez et al. (6)	10 mg p.o. (SS)	18	18-35	50%	American
Areberg et al. (7)	20 mg p.o.	8	51-67	75%	European

SS = steady state

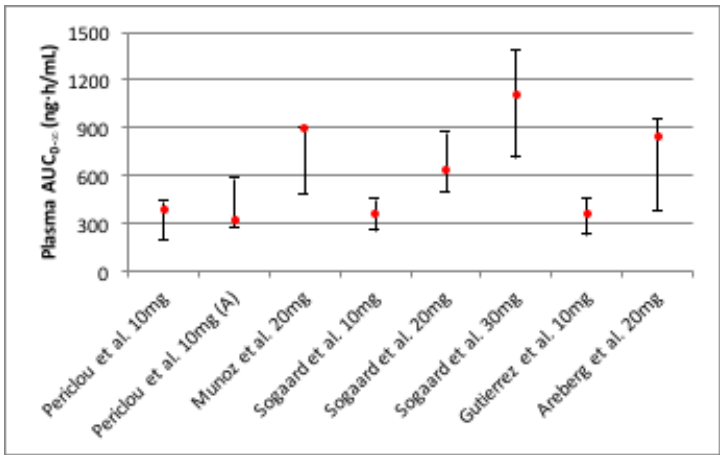


Figure 3-7. 95% confidence intervals generated by the model (error bars) and mean data from the literature (table 3-6) in red circles. (Figure prepared by Dr. Paul Malik).

3.6.10 Final Infant PBPK Model

Once the model was evaluated, physiology and anatomy were adapted to represent infant parameters according to the knowledge base in PK-Sim®. Parameters such as body weight, height, organ weights, blood flows, cardiac output, total body water, and lipid and protein concentrations were scaled by established age-dependent algorithms[214,215]. The age-dependent activities of CYP3A4, CYP2D6 and CYP2C19 enzymes and their inter-individual variability within infant populations were based on the default ontogeny functions in PK-Sim®. Drug-

specific physicochemical properties (molecular weight, lipophilicity, etc.) did not change between the adult model and the infant model adaptation.

It was estimated that 10% of ESC clearance is renal glomerular filtration, based on the percent unchanged in urine and oral bioavailabity, with remaining clearance attributed to metabolism by CYP3A4, CYP2D6 and CYP2C19 enzymes in the liver and intestines according to the relative contributions defined by von Moltke et al. [167,168,216]. The relative expressions of CYP enzymes in each tissue were obtained from the GENEDB/Zanger database which was based on RT-PCR measurements [217]. Total hepatic clearance in PK-SIM was 608 μmol·mL/min, and the default values in PK-Sim, with default intrinsic clearance and expression values as follows, CYP3A4: 49 mL/min and 4.3 μM; CYP2D6: 426 mL/min and 0.40 μM; CYP2C19: 296 mL/min and 0.76μM[216]. The variability was also considered for each enzyme, with the following CV %: CYP3A4: 125%; CYP2D6: 35%; CYP2C19: 100%[218].

3.6.11 Simulating Infant Plasma Concentrations

A virtual population of 1600 term infants was generated that consisted of the following age groups (100 infants per group): each of the first 4 weeks of life, and each of the subsequent 12 months of life. Shorter intervals between age groups were selected for the neonatal period to ensure that the rapid growth and maturation that occur in early life were appropriately investigated. Simulation of plasma ESC levels in the virtual infant population was performed by stochastically assigning each infant a single dose of the infant daily doses calculated above. In this simulation, we assumed no gestational ESC exposure. We then predicted plasma $AUC_{0-\infty}$ of each infant after the single dose. Approximation of the infant daily dose as a single dose, instead of divided doses, is acceptable because the desired output is the steady-state average plasma concentration ($C_{av,ss\ plasma}$), which is unchanged whether the dose is divided or not as long as dose per time is the same. $C_{av,ss\ plasma}$ is calculated by dividing "AUC during a dosing interval at steady state" with the dosing interval. Because $AUC_{0-\infty}$ after a single dose is equivalent to AUC during a dosing interval at steady state, we calculated $C_{av,ss}$ as follows:

$$C_{av,ss} = AUC_{0-\infty} / 24\ h$$

Finally, to evaluate the infant model, the model-predicted $C_{av,ss}$ in infants were compared to those from the study by Rampono et al. who had examined infant ESC plasma concentrations following breastfeeding[171].

3.6.12 Statistical Analysis

Comparison between foremilk and hind milk was accomplished by a paired student's t-test (two-tailed). Differences of $P < 0.05$ were considered to be statistically significant. To compare the influence of pre-, post- or between-feeds on milk concentrations, a one-way ANOVA was conducted. To examine the significance of the correlation between dose and milk concentrations, Pearson's r was calculated; $p<0.05$ was considered to be significant.

3.7 Infliximab

3.7.1 Study Population

Five women taking IFX for Crohn's Disease or Ulcerative Colitis provided consent and enrolled in the DLAC Study.

3.7.2 Materials

Capture (HCA212) and detection (HCA213P) anti-IFX antibodies for free IFX, HISPEC diluent, and ELISA BSA were purchased from Bio-Rad Laboratories Inc. Quantablu and MaxiSorp 96-well plates were purchased from ThermoFisher Scientific and Tween®20 were purchased from Sigma Aldrich. IFX (10 mg/mL) was acquired from the Hospital for Sick Children Pharmacy.

3.7.3 Preparation of Standards

Confirmed drug-free milk was donated by healthy donors to the DLAC study. To prepare assay calibrators and QC, 1 mL of milk was aliquoted into 1.5 mL Eppendorf tubes and centrifuged at 2500 x g at 4°C for 10 min in order to separate the lipid and aqueous phases of milk. The milk lipid layer congealed and was removed using a transfer pipette, then discarded. The aqueous phase of milk was obtained for preparation of assay calibrators and QC. Two intermediate IFX stocks (50 µg/mL and 1000 ng/mL) were prepared in water. A calibration curve was prepared by spiking the intermediate stocks into milk to prepare a high and low level. Proportionate dilutions of high and low control pools were used to prepare the following concentrations of standards: 5, 66.25, 127.5, 187.5, 250 ng/mL. QC stocks were prepared by diluting the high and low stocks to 37.5 (low), 150 (medium) and 225 (high) ng/mL. Standards were stored at 4°C prepared fresh every two months.

3.7.4 Preparation of Patient Samples

Upon arrival to the lab (pre-analytical procedures discussed in detail in section 3.4), patient samples were immediately aliquoted into 1.5 mL Eppendorf tubes and frozen at -80°C until they were analyzed. Samples remained in the 35 mL collection containers and not aliquoted if they arrived un-thawed and were only thawed and aliquoted prior to sample analysis. Patient samples were prepared using a similar method to standard preparation; samples were centrifuged at 2500 x g at 4°C for 10 min and the aqueous phase of milk included in the analytical protocol.

3.7.5 Analytical Methods

The experimental protocol was carried out as per Bio-Rad Laboratories Inc. instructions. The capture antibody (HCA212) was prepared in PBS at 1 µg/mL and 100 µL was aliquoted into the required number of wells and incubated overnight at 4°C. The wells were washed 5 times with PBS with 0.1% Tween®20. Antibody binding was blocked 200 µL 5% BSA and allowed to incubate for 1 hour at room temperature, followed by washing 5 times with PBST. Next, 100 µL each of calibrator, quality control and patient sample were run in duplicate (unless otherwise

stated) and incubated for 1 hour at room temperature. This was followed by another plate washing with PBST before the addition of 100 µL HRP-conjugated detection antibody (2 µg/mL). Following a 1 hour incubation at room temperature, wells were washed 10 times and 100 µL QuantaBlu (substrate + peroxide solution) was added. Fluorescence was measured after 30 minutes at 325 and 420 nm.

3.7.6 Assay Performance

The assay performance characteristics were analyzed in accordance with CLSI guidelines. Performance studies were determined using spiked breast milk controls and standards. Within-day precision was assessed by running 20 replicates of each low, medium and high QC milk samples and calculating the coefficient of variation for each level. Between-day precision was calculated using the same method; however, CVs were calculated from each QC level following 8 runs. (LoQ) was carried out by serially diluting the low calibrator into the aqueous phase of blank milk. Samples were run in 5 replicates and the concentration yielding a 20% coefficient of variation (CV) was determined as the LoQ. Reproducibility of the standard curve and the analytical measuring range (AMR) were assessed by analyzing linear regression of 8 standard curves (5, 66.25, 127.5, 187.5, 250 ng/mL) from 8 different runs on 8 separate days. Standard curve reproducibility and AMR were defined where the regression line deviated from the mean at CVs of less than 10%, with R^2 >0.98. The run was rejected, and patient samples repeated if the QC failed to yield the expected value.

Chapter 4
Methotrexate

4 Methotrexate Chapter Outline

In Chapter 4, the results from the methotrexate (MTX) project are discussed. The results from the LC-MS/MS method validation study are first described, followed by a discussion of the outcomes from the DLAC and animal studies. Finally, the chapter ends with a summary of the results. Insights and implications on MTX safety during lactation are critically discussed in the discussion chapter (chapter 7).

4.1 LC-MS/MS Method Validation

4.1.1 Chromatography

Various chromatographic conditions were explored in order to achieve appropriate separation of MTX (with and without internal standard, d3) and metabolite, 7-hydroxymethotrexate (7OH-MTX). A chromatogram obtained from analysis of a milk calibrator are shown in **figure 4-1**. Retention times were as follows for MTX, 7OH-MTX and MTX-d3, respectively: 3.77 min, 3.91 min and 3.77 min.

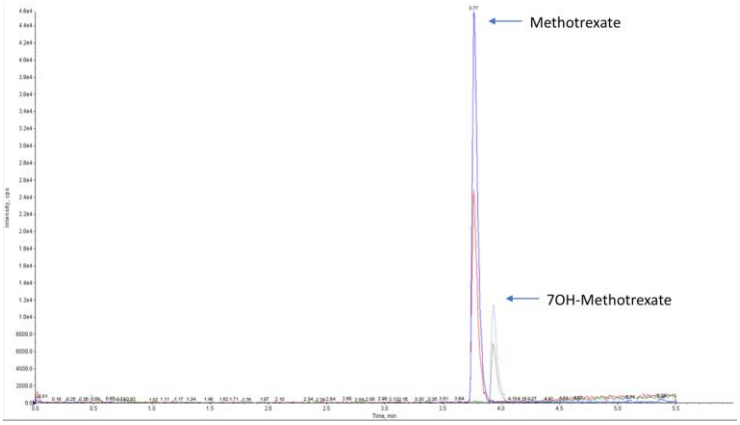


Figure 4-1. Chromatographic separation of MTX (blue peak), 7OH-MTX (grey peak) and MTX-d3 (red peak).

4.1.2 Precision

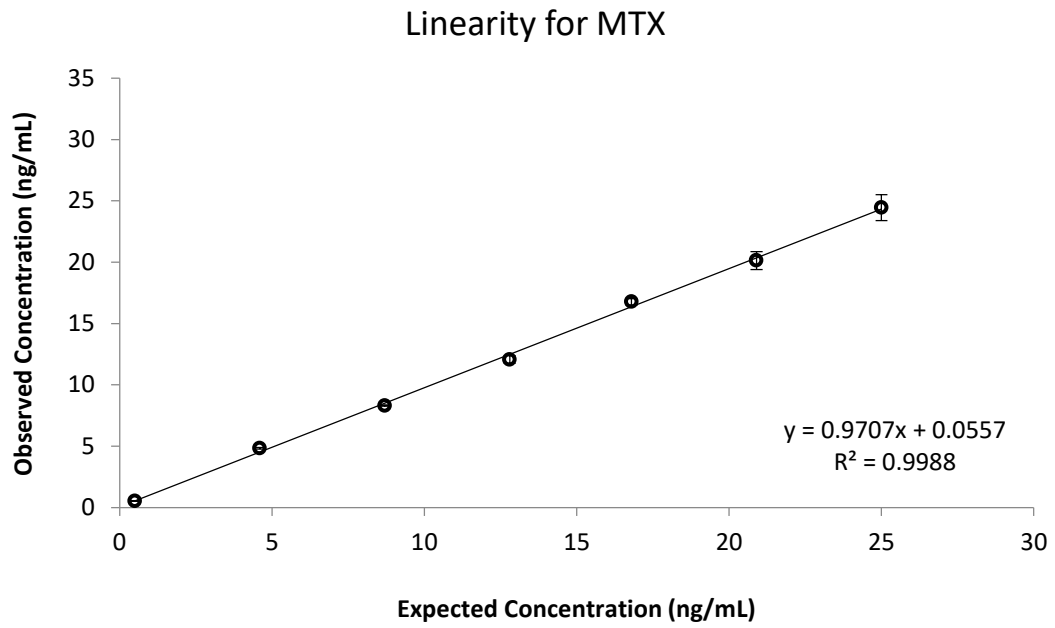
The total precision for both MTX and 7OH-MTX were calculated using QC samples with expected concentrations of 1, 8, and 16 ng/mL and is shown in **table 4-1**. Acceptance criteria were established as CVs ≤15%, as per the CLSI Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline C62-A[203]. Within-day precision was calculated by running 20 replicates of each level and calculating the % CV for each. The CVs for low, medium and high QCs were: 6.9%, 2.9% and 3.4%, respectively for MTX; and 8.7%, 2.6% and 5.9%, respectively for 7OH-MTX. Between-day precision was assessed across 12 runs over 12 months. The CVs for between-day precision were: 12.2%, 3.6% and 4.6%, respectively for MTX; and 12.3%, 8.7% and 9.2%, respectively for 7OH-MTX. Therefore, the CVs for both analytes at each QC level fell well below our stated cut-off of 15% and were considered to be acceptable in our validation criteria.

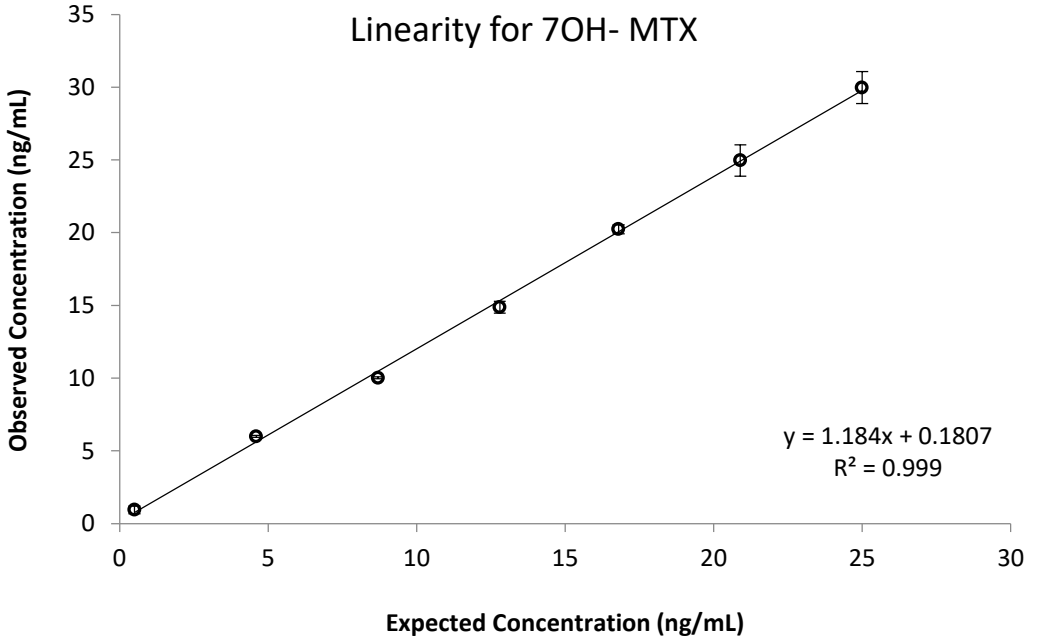
Table 4-1. LC-MS/MS assay precision: low (1 ng/mL), Med (8 ng/mL), High (16 ng/mL)

	Within-day			Between-day		
	MTX			**MTX**		
Level	Low	Med	High	Low	Med	High
(ng/mL)	1.0	8.0	16.0	1.0	8.0	16.0
N	20	20	20	12	12	12
Mean	0.8	8.0	18.4	0.9	7.8	16.9
SD	0.1	0.2	0.7	0.1	0.3	0.8
CV (%)	**12.5**	**2.5**	**3.8**	**11.1**	**3.8**	**4.7**
	7OH-MTX			**7OH-MTX**		
Level	Low	Med	High	Low	Med	High
(ng/mL)	1.0	8.0	16.0	1.0	8.0	16.0
N	20	20	20	12	12	12
Mean	1.0	8.1	18.6	1.1	7.9	17.3
SD	0.1	0.2	1.1	0.2	0.6	1.7
CV (%)	**10.0**	**2.5**	**5.9**	**18.2**	**7.6**	**9.8**

4.1.3 Linearity

Linearity was determined by running 7 equidistant sample pools in triplicate and comparing the mean value against the expected concentration. Based on the plotted expected versus observed concentrations shown in **figure 4-2A** and **4-2B**, the method was linear over the range of 0.5-25 ng/mL (slope: 0.97, intercept: 0.05, R^2 0.99) for MTX. For 7OH-MTX, the method was linear over the same range as MTX (slope: 1.18, intercept: 0.18, R^2 0.99).





(B)

Figure 4-2. Linearity plots of (A) MTX and (B) 7OH-MTX. Open circles represent the mean from 3 replicates, error bars represent the standard deviation.

4.1.4 Limit of Quantification (LoQ)

Based on previous reports from Johns et al.[156] and Thorne et al.[157],we anticipated milk concentrations to be in the low ng/mL range; thus, requiring an LC-MS/MS with additional

sensitivity to be developed and validated. In order to validate the LoQ for this method, the precision approach was used. A low standard (6.25 ng/mL) was diluted in blank milk to achieve concentrations of 0.78, 0.39, 0.20 and 0.1 ng/mL. Samples were run in 5 replicates and CVs were calculated as shown in **table 4-2** for MTX and 4-X for 7OH-MTX. The concentration that yielded a CV < 20% was 0.1 ng/mL for MTX and 7OH-MTX and was defined as the functional LoQ for this method. The chromatogram of MTX at the LoQ is shown in **figure 4-3**.

Table 4-2. The calculated CVs used to determine the LoQ of MTX

	MTX			7OH-MTX		
Expected Concentration (ng/mL)	Mean	SD	% CV	Mean	SD	% CV
0.10	0.11	0.02	18.1	0.83	0.04	4.8
0.20	0.25	0.02	8.0	1.00	0.05	5.0
0.39	0.48	0.02	4.2	1.35	0.23	17.0
0.78	0.97	0.03	3.1	1.70	0.19	11.2
6.25	6.35	0.21	3.3	5.45	0.19	3.5

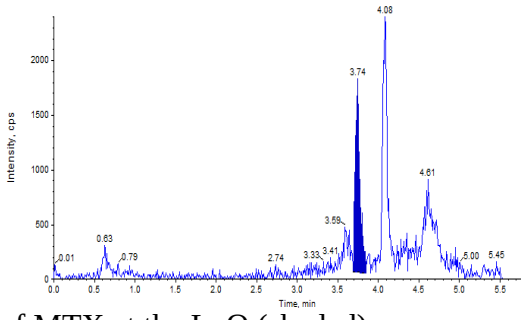


Figure 4-3. Chromatogram of MTX at the LoQ (shaded).

4.1.5 Carryover

Due to the fact that our LC-MS/MS method was extremely sensitive, determining carryover was essential for ensuring accurate results. To examine the extent of carryover, two approaches were used. First, column retention was assessed by injecting a high calibrator, followed by three

subsequent blank samples. There was no peak or any indication of analyte retention on the column noted in the first blank as shown in **Figure 4-4A** (high calibrator) and **4-4B** (blank).

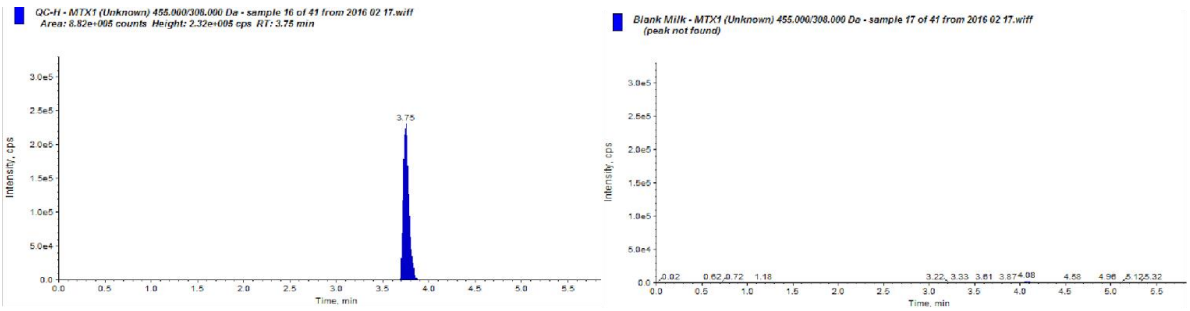


Figure 4-4. (A) Injection of high calibrator and (B) subsequent injection of blank milk indicates no notable carryover in the LC-MS/MS method

The next approach utilized the equation shown in **table 4-3** following 4 injections of the high calibrator and 4 subsequent injections of the low calibrator. As seen in **table 4-3** below, carryover was 0.3%. Overall, there was no remarkable carryover of any metabolites in our LC-MS/MS method.

Table 4-3. Carryover calculation

Level		Expected Concentration	Calculated Concentration
High	a1	25 ng/mL	24.1
High	a2	25 ng/mL	24.0
High	a3	25 ng/mL	25.3
High	a4	25 ng/mL	24.2
Low	b1	0.78 ng/mL	0.86
Low	b2	0.78 ng/mL	0.89
Low	b3	0.78 ng/mL	0.89
Low	b4	0.78 ng/mL	0.87
		% Carry Over	0.26

4.1.6 Ion Suppression

The extent of ion suppression at MTX, MTX-d3 and 7OH-MTX retention times was determined. Blank human milk and blank mouse milk were extracted and injected into the instrument to

follow the final chromatographic conditions, while an equimolar mixture of all analytes was directly infused into the mass spectrometer. Although ion suppression was significant at various retention times, **figures 4-5** and **4-6** show a total ion chromatogram (TIC) indicating no noteworthy interference with ionization of any MTX analytes in human milk and mouse milk, respectively.

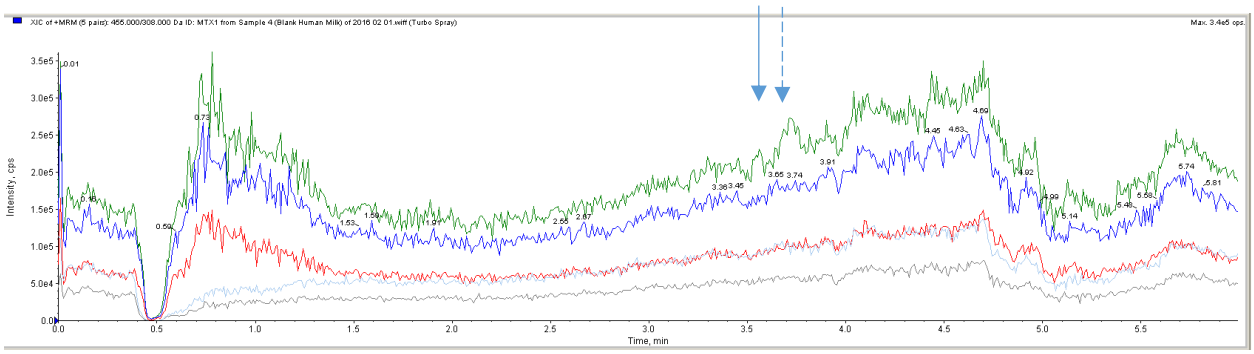


Figure 4-5. TIC ion suppression study in human milk. The arrows denote MTX (solid line) and 7OH-MTX (dashed line) elution times, signifying no ion suppression.

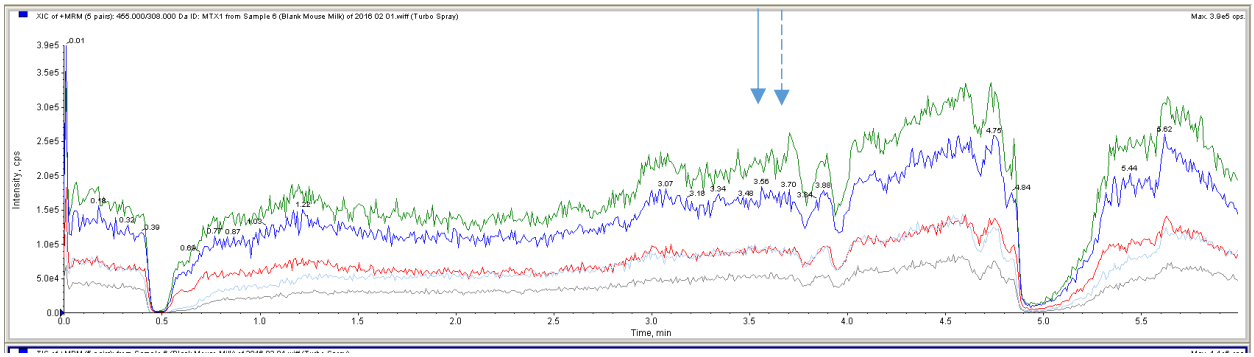


Figure 4-6. TIC ion suppression study in mouse milk. The arrows denote MTX (solid line) and 7OH-MTX (dashed line) elution times, signifying no ion suppression.

4.1.7 Method Comparison

Comparison of the LC-MS/MS assay with the Abbott ARCHITECT chemiluminescent assay was performed. For the comparison, the spiked milk samples were run in singleton on the LC-

MS/MS and plotted against to the previously reported clinical laboratory data from the Abbott assay. The LC-MS/MS method compared very well and yielded an r^2 of 0.99. (slope: 0.97, intercept: 0.08) between the two methods (**figure 4-7**). To further asses the agreement between methods, a plot graphing the absolute difference between the LC-MS/MS method (y-axis) and the Abbott ARCHITECT method (x-axis) is shown in **figure 4-8**. The maximum difference in absolute values was 3.34 ng/mL (mean: 0.19 ± 0.87 ng/mL) (**figure 4-8**). **Figure 4-9** displays the percent difference between the two values obtained by both methods. The majority of samples did not exceed a different of 10% between the two analytical methods, meeting our validation cut-off criteria and further supporting that our LC-MS/MS method was comparable with a clinically validated test.

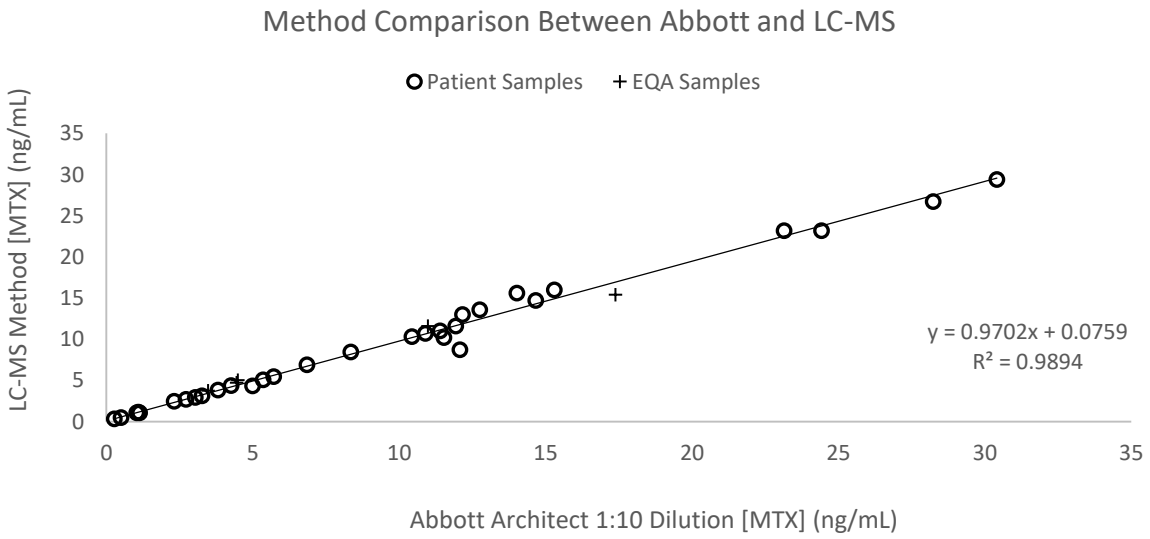


Figure 4-7. Method comparison plot comparing the Abbott Architect immunoassay (x-axis) to the LC-MS/MS method (y-axis) (n=36)

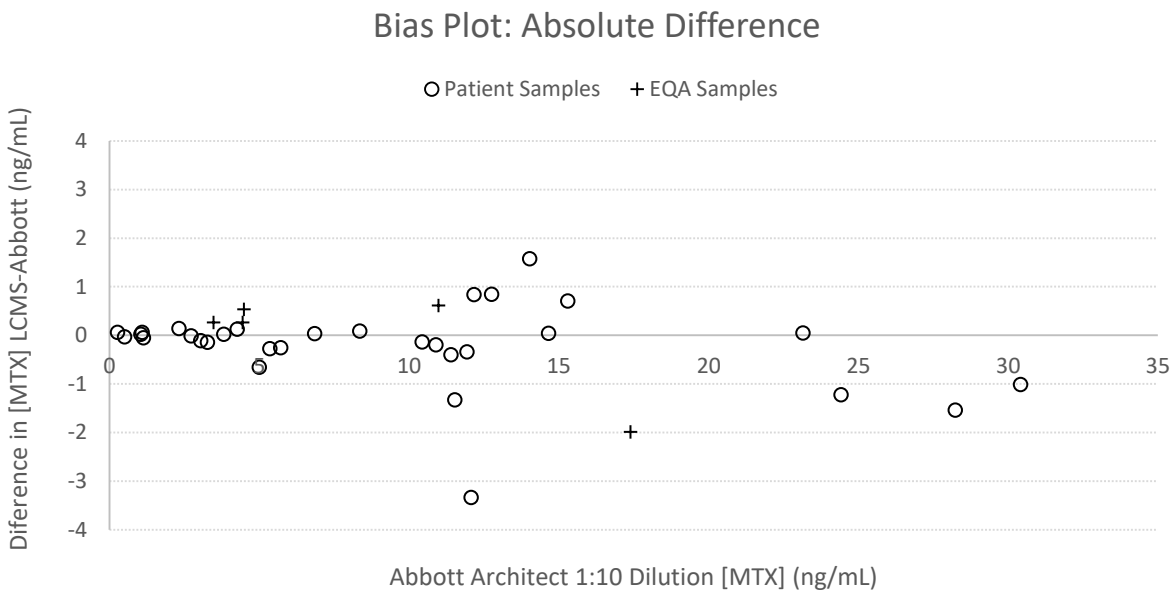


Figure 4-8. Bias plot showing the absolute difference between the Abbott Architect and LC-MS/MS methods (n=36)

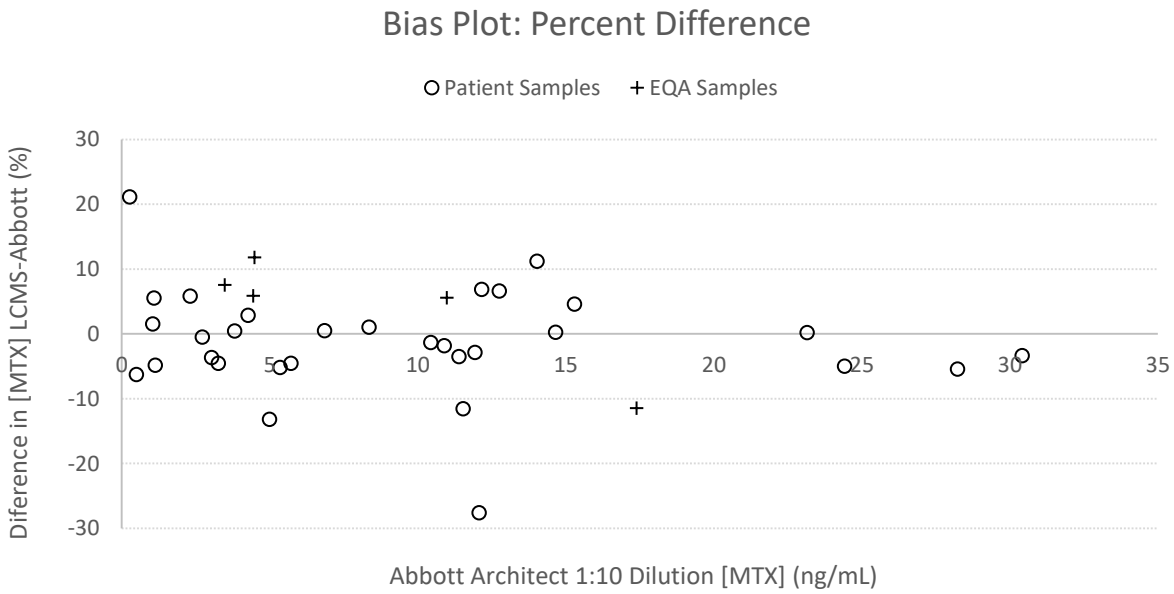


Figure 4-9. Bias Plot showing the percent difference between the Abbott Architect and LC-MS/MS methods (n=36)

4.2 Patient Characteristics

Two participants receiving low-dose MTX therapy for rheumatoid arthritis (RA) were recruited as part of the DLAC study at SickKids Hospital. Both patients received a subcutaneous (SC) dose of 25 mg weekly. Other patient characteristics including age, weight, and concomitant medications are shown in **table 4-5**.

Table 4-5. Characteristics of patients involved in the DLAC study

	Patient 1	**Patient 2**
Dose	25 mg weekly	25 mg weekly
Route	SC	SC
Age (years)	37	32
Weight (kg)	59	-
Indication	RA	RA
Concomitant Medication	Vitamin B_{12}, folic acid (2 days after MTX administration)	Bupropion, fluoxetine, prednisone, folic acid, ferrous sulfate, docusate sodium, hydroxidine, multivitamins

4.3 Measurement of MTX and 7OH-MTX in Human Breast Milk

Figure 4-10 shows the time concentration profile of MTX in milk from the two women included in the study. The data shown for patient 1 represents the average MTX milk concentration between the foremilk and the hindmilk, while the profile from patient 2 represents the concentration of milk from an emptied breast, which corresponds to the entirety of a single feed. Quantifiable concentrations of MTX above the LoQ of 0.1 ng/mL were measured in 13 of 16 milk samples collected (range: 0.2 ng/mL - 2 ng/mL; mean= 0.89 ng/mL).

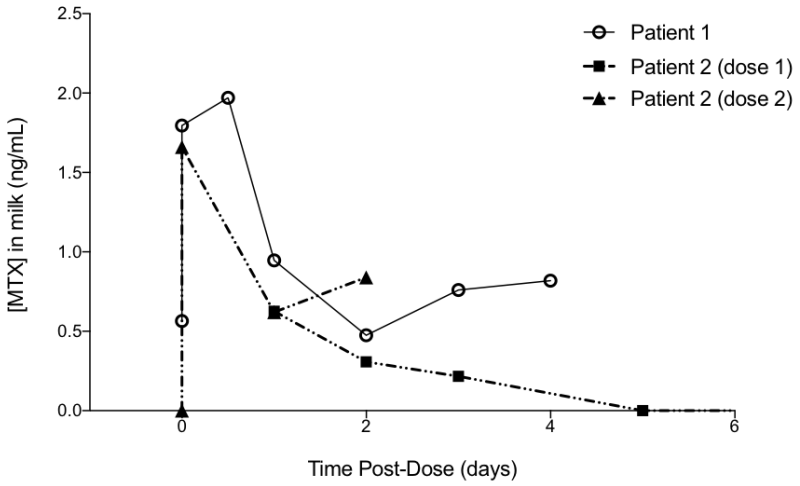


Figure 4-10. Time-concentration curve of MTX in human breast milk. Patient 1 (circles, dashed line) received a dose of 25 mg SC weekly. Patient 2 (squares: dose 1; triangles: dose 2) also received a dose of 25 mg SC weekly.

The metabolite, 7OH-MTX, was quantifiable only in the foremilk of patient 1, but not detectable in any time points in patient 2, which is not surprising given that patient 2 had just initiated her treatment. The peak concentration of 7OH-MTX was 0.88 ng/mL, observed at 24 hours post-MTX dose (**figure 4-11**).

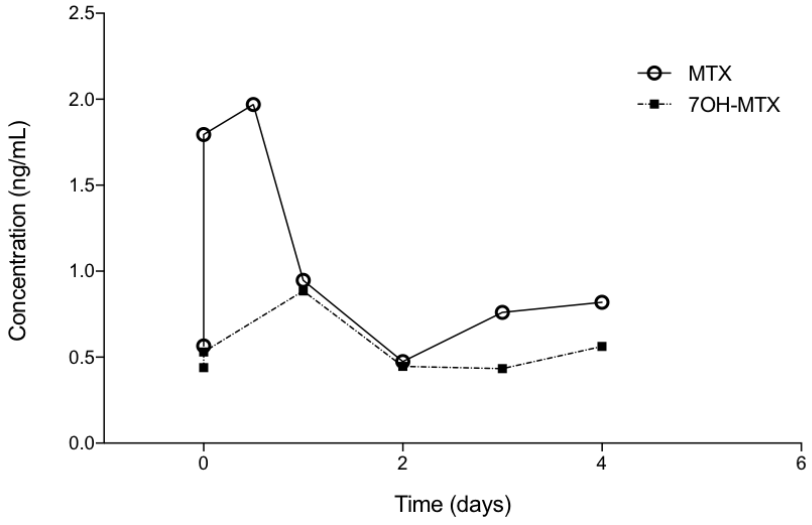


Figure 4-11. Time-concentration curve of MTX and metabolite, mean foremilk and hindmilk 7OH-MTX in human breast milk from patient 1. 7OH-MTX was undetectable in patient 2.

Milk composition varies across a feed; the lipid content is lower in the foremilk (beginning of feed) and higher in the hindmilk (end of feed). Based on the physicochemical properties of the drug, the concentration could theoretically vary throughout an individual feed[37,93]. We measured the concentration of MTX (**figure 4-12A**) and 7OH-MTX (**figure 4-12B**) in both foremilk and hindmilk. **Figure 4-12A** shows that measurable differences were seen in MTX concentrations between the foremilk and hindmilk; however, the difference was not statistically significant. **Figure 4-12B** displays the statistically significant differences (P <0.05) between the foremilk and hindmilk concentrations of 7OH-MTX.

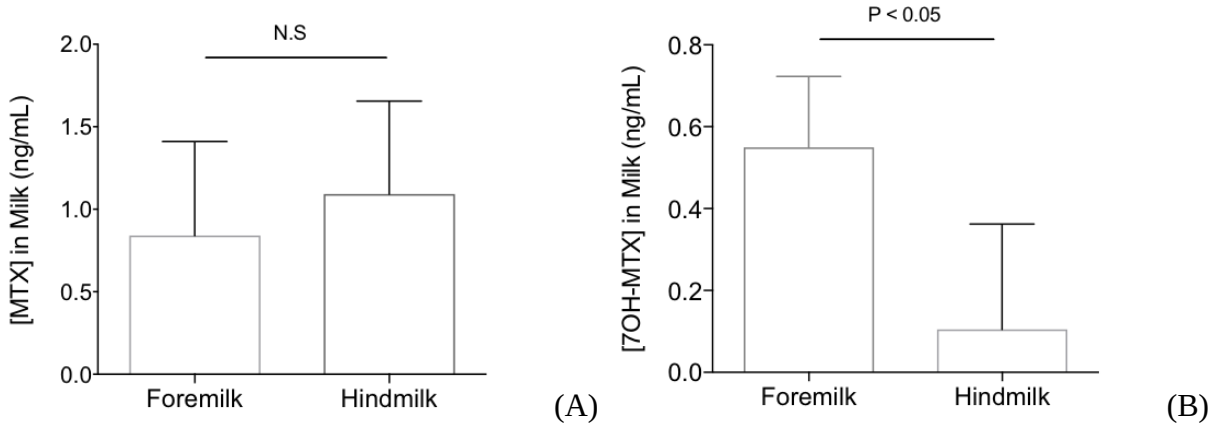


Figure 4-12. Plots of MTX (A) and 7OH-MTX (B) in foremilk and hindmilk from patient 1. 7OH-MTX was not detectable in hindmilk after the 1-hour sampling time-point. Bars represent the mean ± SD. Paired t-test; P <0.05 was considered to be significant.

4.4 Increase in Measureable MTX following Trienzyme Digestion

We wanted to determine whether there is a pool of uncharacterized MTX in milk, existing in the polyglutamated form, that was previously not able to be measured by standard analysis. MTX and folates share a number of enzymatic targets and transporters[219]. There is evidence showing a large proportion of total folates in milk exist in the polyglutamate form. Measurable folate content was significantly increased following treatment with folate conjugase and even further increased following treatment with α-amylase, protease and conjugase (tri-enzyme digest)[220]. Based on the structural similarity of MTX and folates, a tri-enzyme digest was performed on the milk samples provided by patient 1, in order to determine if there was an increase in measurable

MTX by our LC-MS/MS method (measures monoglutamate MTX only). Following treatment with a tri-enzyme digest, an increase in measurable MTX was noted (**Figure 4-13**). This increase became apparent at 24 h post dose and showed a maximum of 1.7 ng/mL (0.82 ng/mL without digest) at 96 hours post-dose.

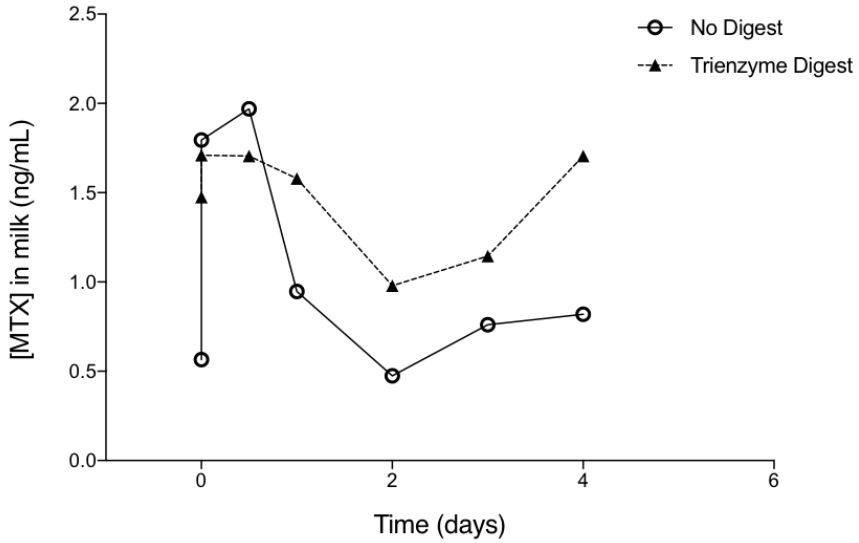


Figure 4-13. Time-concentration curve of MTX milk concentrations following a trienzymatic digest using protease, α-amylase and conjugase (triangles, solid line) and without digest (open circles, dotted line) in patient 1.

4.5 Time-Concentration Curve of MTX in Mouse Milk

To determine peak sampling time for MTX measurement in mouse milk, mice were administered MTX at time zero and milk was collected 30, 60 and 120 minutes post-injection. The peak concentration was noted 60 minutes post-dose (**Figure 4-14**). This 60-minute collection time-point was used in all subsequent mouse experiments.

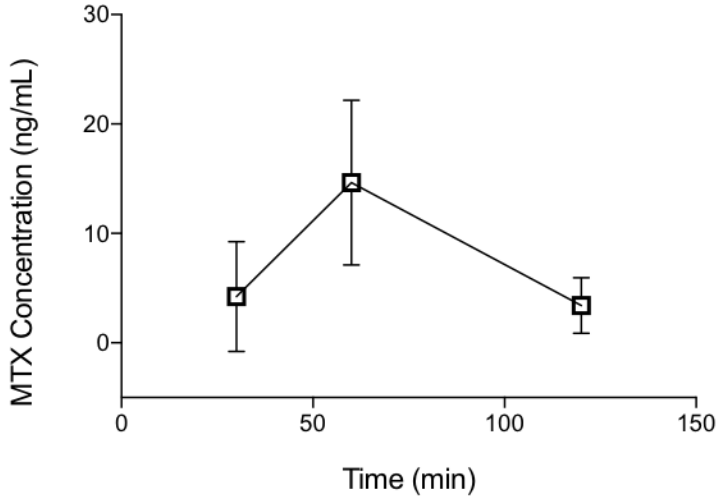


Figure 4-14. Time-concentration profile of MTX in mouse milk. Mean ±SD, n=3

4.6 Pharmacokinetics of MTX in Milk and Plasma of Bcrp-/- and FVB Mice

To confirm the involvement of Bcrp in mediating systemic and mammary clearance of MTX, we determined the plasma and milk concentrations of MTX in lactating wild-type FVB and Bcrp -/- knockout mice (**Figure 4-15**). Consistent with previous findings in Bcrp -/- mice [149,221], the plasma concentration of MTX (mean ± SD) at 60 min post IP injection in Bcrp -/- mice was 275.4 ± 42 ng/mL, which was 1.5-fold higher ($P<0.01$) than those in wild-type mice (181.7 ± 42 ng/mL), as seen in **figure 4-15**.

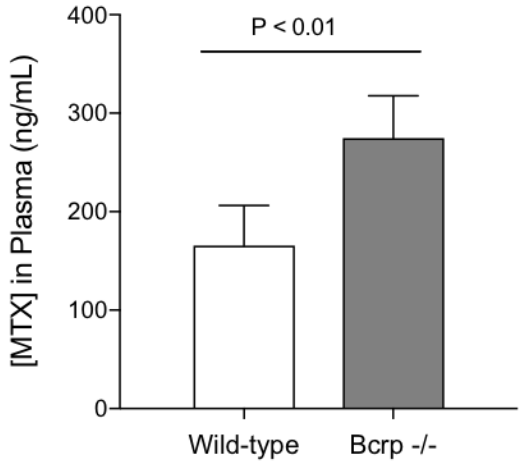


Figure 4-15. Plasma MTX concentrations in wild-type (n=5) or Bcrp -/- (n=6) mice. Data represent the mean ± SD. P < 0.05 was considered to be significant (two-tailed unpaired t-test).

4.7 Determining the Role of Bcrp in Transporting MTX into Milk

To explore the involvement of Bcrp in secreting MTX across the mammary epithelium, milk was collected from the same lactating mice immediately prior to plasma collection and 60 minutes following MTX administration. Despite higher plasma MTX concentrations, Bcrp -/- mice showed 1.7-fold lower MTX concentrations in milk (7.8 ± 2 ng/mL) than wild-type (13.8 ± 6 ng/mL), as seen in **figure 4-16** (P<0.05).

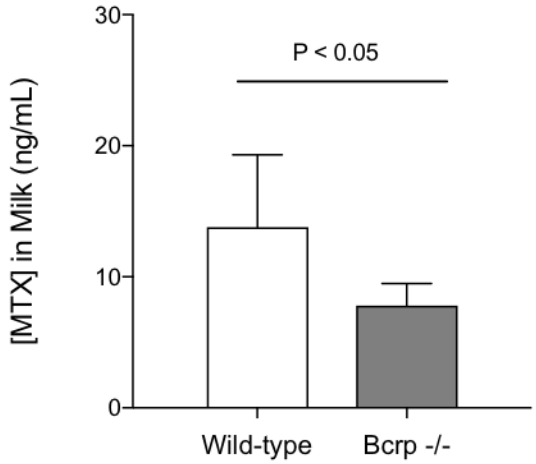


Figure 4-16. Milk MTX concentrations in wild-type (n=5) or Bcrp -/- (n=6) mice. Data represent the mean ± SD. P < 0.05 was considered to be significant (two-tailed unpaired t-test).

The milk-to-plasma (M/P) ratio was significantly lower (2.9-fold; P <0.05) in the Bcrp-/- mice (0.03 ± 0.008) than in the wild-type (0.08 ±0.05), as shown in **figure 4-17**. These findings illustrate a significant role of Bcrp in both MTX systemic clearance and mammary secretion. Although M/P ratio of MTX is already low likely due to its anionic ionization characteristics, it is noteworthy that lack of mammary Bcrp results in further reduction of MTX secretion into milk, further highlighting the role of Bcrp in excreting MTX into milk.

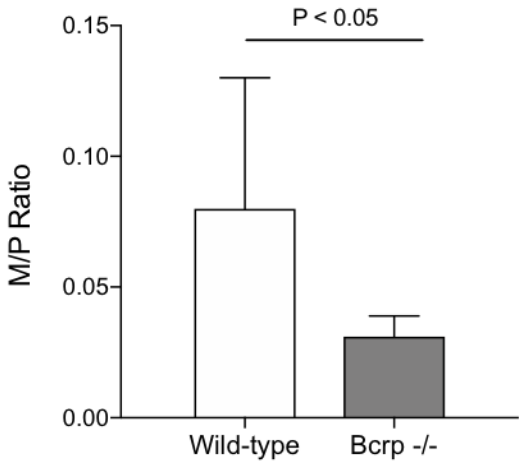


Figure 4-17. Milk-to-plasma ratio between wild-type (n=5)and Bcrp -/- (n=6) mice. P < 0.05 was considered to be significant (two-tailed unpaired t-test)

4.8 MTX-Nutrient Interaction Study

To investigate the effects of the co-administration of a Bcrp substrate or inhibitor on MTX disposition in plasma and milk, wild-type FVB mice were treated with Bcrp inhibitors (ko143 or genistein) or substrates (riboflavin or FMN) 30 minutes prior to MTX administration. Milk and plasma were collected 60 minutes following MTX dosing. Following administration of 20 mg/kg riboflavin or 100 mg/kg FMN, the concentration of MTX in milk significantly decreased by 69% and 55% (4.3 ng/mL ± 2.1 and 6.2 ng/mL ± 3.4), respectively, compared to wild-type mice (**Figure 4-18**)**.** This decrease in milk MTX concentrations was similar to, or more than the reduction of MTX in milk of Bcrp -/- mice (**Figure 4-16**); however, in contrast to the findings in plasma MTX levels in these mice, pre-treatment with of riboflavin or FMN did not significantly differ from MTX plasma concentrations in wild-type mice (riboflavin: 126.9 ± 59 ng/mL and

FMN: 210 ± 37 ng/mL vs. control: 181.7± 42.3 ng/mL) (**figure 4-19**), despite the noted decrease in milk concentrations in the same mice (**figure 4-20**). The milk-to-plasma ratio in the groups treated with riboflavin or FMN were greater than 2.5-fold lower than the mice treated with MTX alone; although, this reduction in the MTX m/p ratio was not considered to be statistically significant (**figure 4-20**).

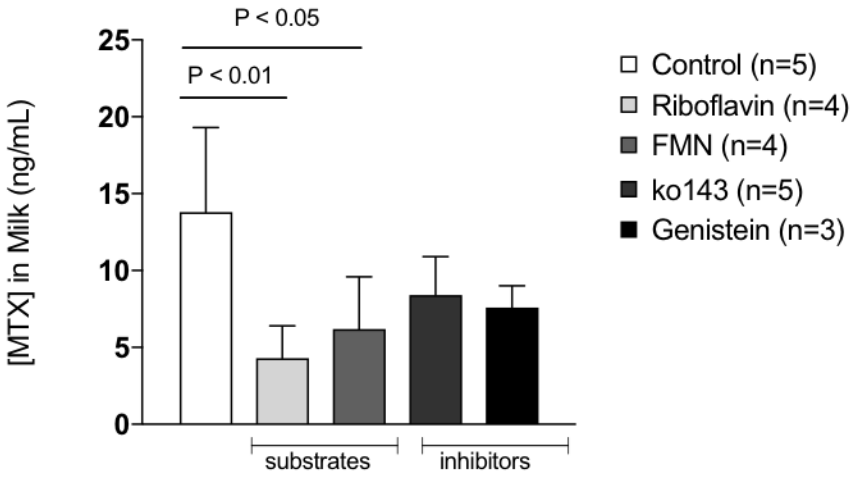


Figure 4-18. Milk MTX concentrations in wild-type mice co-administered a Bcrp substrate or inhibitor. Data represent the mean ± SD, n = 3-5. P < 0.05 was considered to be significant (two-tailed unpaired t-test).

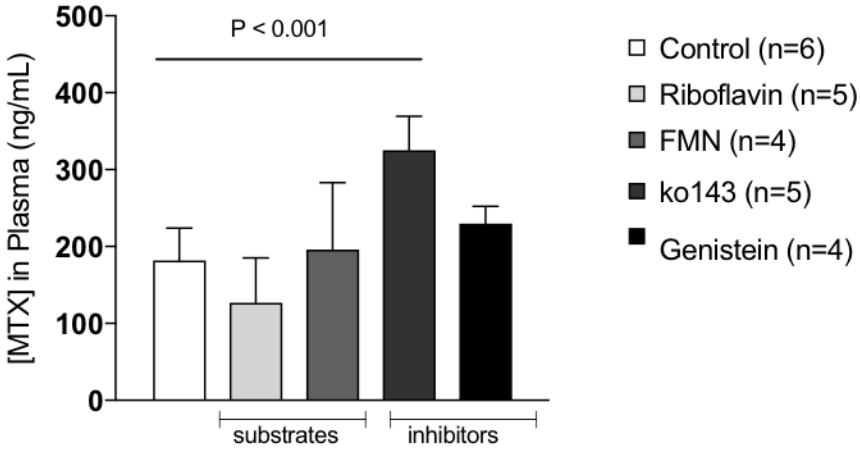


Figure 4-19. Plasma MTX concentrations in wild-type mice co-administered a Bcrp substrate or inhibitor. Data represent the mean ± SD, n = 3-5. P < 0.05 was considered to be significant (two-tailed unpaired t-test).

Two other groups of wild-type mice were treated with the Bcrp inhibitors, ko143 (20 mg/kg) or genistein (20 mg/kg) 30 minutes before MTX injection. Milk concentrations decreased following administration of the Bcrp inhibitors, ko143 or genistein, although not significantly. Consistent with the Bcrp -/- mice, the mice treated with ko143 had significantly higher plasma concentrations (309.4 ±44 ng/mL vs. 181.7± 42.3 ng/mL; $P < 0.001$) compared to control. The plasma concentrations in the mice administered genistein were only slightly increased (226.8 ng/mL± 19.3), suggesting that systemic clearance of MTX may not be affected by co-treating with genistein. The MTX m/p ratio was decreased in the mice treated with genistein and ko143-treated mice, although the study was not powered to show a significant difference, as shown in **figure 4-20** below.

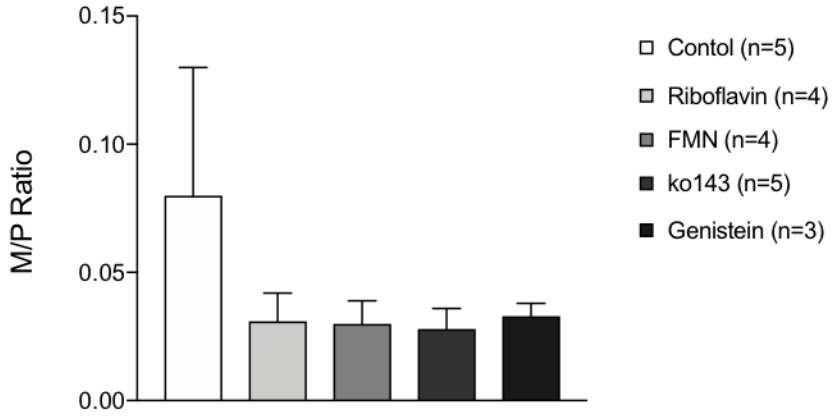


Figure 4-20. Milk-to-plasma ratio between control (MTX alone) and co-treatment with riboflavin (20 mg/kg), FMN (100 mg/kg), ko143 (20 mg/kg) or genistein (20 mg/kg).

4.9 Effects of Riboflavin Concentrations in Milk Following MTX Administration

To determine whether MTX transport across the mammary epithelium could reduce excretion of riboflavin into milk, we measured milk and plasma concentrations of riboflavin following co-administration with MTX. The same experimental protocol was followed as above; mice were treated with 20 mg/kg riboflavin 30 minutes prior to MTX (0.75 mg/kg) administration and milk and plasma collected 60 minutes later. As expected, following IP administration of riboflavin, an

increase in plasma concentrations corresponded with an increase in milk concentrations (r = 0.96); p =0.04), as shown in **figure 4-21**.

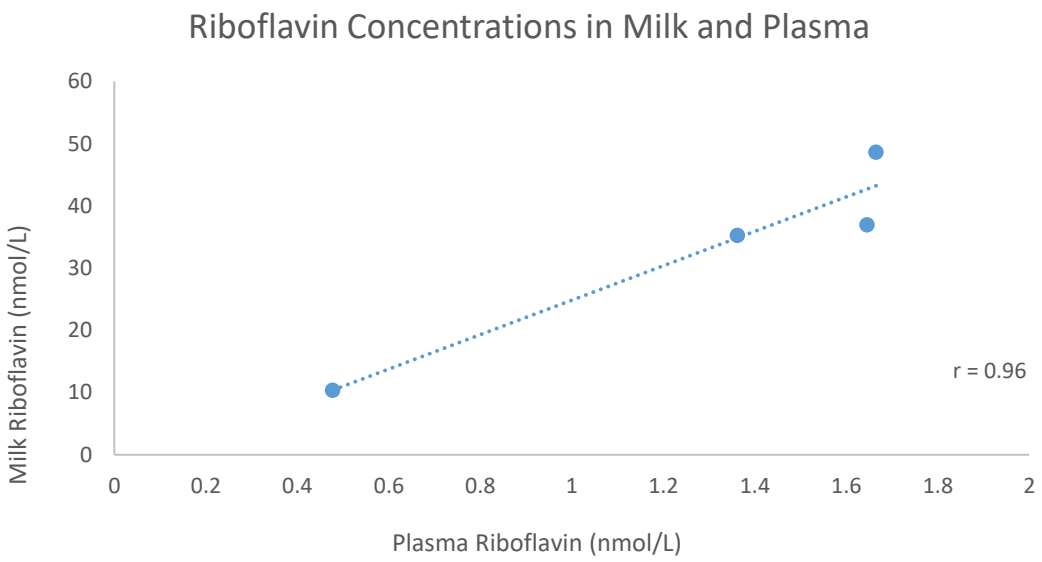


Figure 4-21. Milk vs. plasma concentrations of riboflavin following administration of 20 mg/kg riboflavin and 0.75 mg/kg of MTX (n=4). P<0.05 was considered significant.

As we have demonstrated, milk concentrations of MTX significantly decrease following co-administration of riboflavin (P<0.05; **figure 4-18**), without affecting plasma concentrations of MTX. Interestingly, a significant correlation between riboflavin concentration and MTX concentration in milk was not shown, as shown in **figure 4-22** (r = -0.84; p= 0.15); however, an inverse trend was noted. Findings from these experiments will be further explored in the discussion section, Chapter 7.

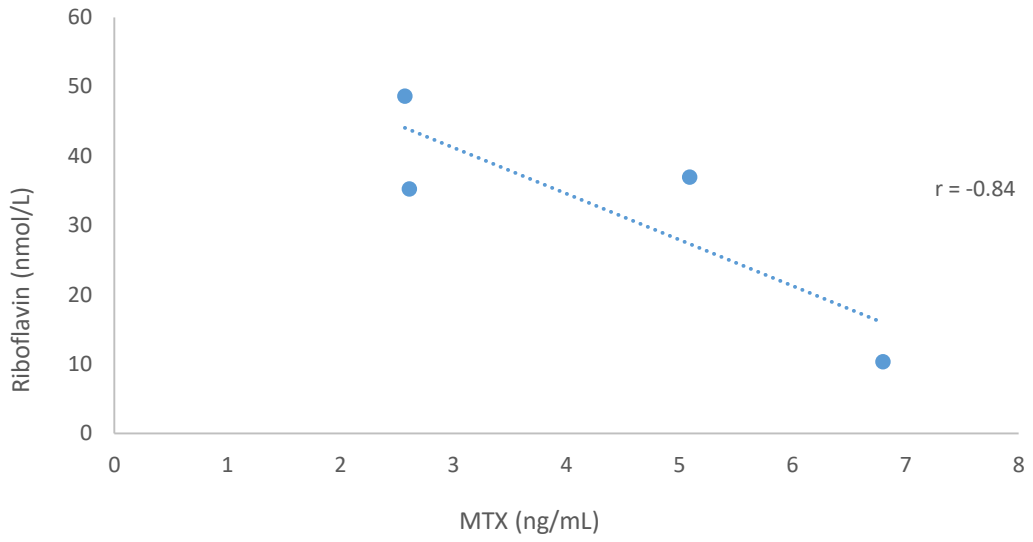


Figure 4-22. The correlation of milk MTX and riboflavin concentrations following administration of 20 mg/kg riboflavin and 0.75 mg/kg of MTX (n=4). P<0.05 was considered significant.

4.10 MTX Results Summary

The aims of this project were three-fold: (1) to better understand the extent of MTX excretion into human milk and describe the potential risk to the nursing infant; (2) characterize the role of Bcrp in actively transporting MTX into mouse milk; and (3) to explore the use of high-dose riboflavin to inhibit transport of MTX into milk in mice. Based on our findings, infant exposure to MTX species through milk is relatively low, following maternal weekly immune-modulating doses (10-25 mg/week). Our mouse model revealed that Bcrp mediates MTX efflux into milk. Further, co-administration with high-dose riboflavin during lactation may reduce the transport of MTX into milk and subsequently decrease infant exposure of the drug. These interesting findings will be further explored in the discussion section.

Chapter 5
Escitalopram

The content in this section has been accepted to The Journal of Clinical Pharmacokinetics, April 2018.

Sarah R. Delaney, Paul R. V. Malik, Cristiana Stefan, Andrea N. Edginton, David A. Colantonio, Shinya Ito. "*Predicting escitalopram exposure to breastfeeding infants: integrating analytical and in silico techniques*" [ahead of print]

5 Escitalopram Chapter Outline

Chapter 4 discusses the results from the escitalopram (ESC) project. First, the validation parameters from the LC-MS/MS method optimized to measure ESC and metabolite in milk are discussed. Next, the milk pharmacokinetics of ESC and metabolite are described, followed by the results from the simulation examining total infant daily dose (taking into account variability in feeding-related parameters in an infant population). The last section describes the outcomes from the physiologically-based pharmacokinetic (PBPK) modelling and explores the *in-silico* results of infant systemic exposure to ESC through breast milk.

5.1 LC-MS/MS Method Validation

An LC-MS/MS method was developed and optimized to measure ESC and metabolite, desmethylescitalopram (D-ESC), in human breast milk. Within-day precision, linearity and ion suppression were considered as part of our method validation study. Validation parameters were carried out in accordance with CLSI guidelines. The calibration range was 5-1000 ng/mL for both ESC and metabolite. **Figure 5-1** shows the calibration curves for both ESC (slope: 0.005, intercept: -0.006, R^2: 0.99) and D-ESC (slope: 0.003, intercept: -0.005, R^2: 0.99) from the run measuring the batch of patient samples. Due to the unavailability of a comparative method, no further quality assurance measures were assessed.

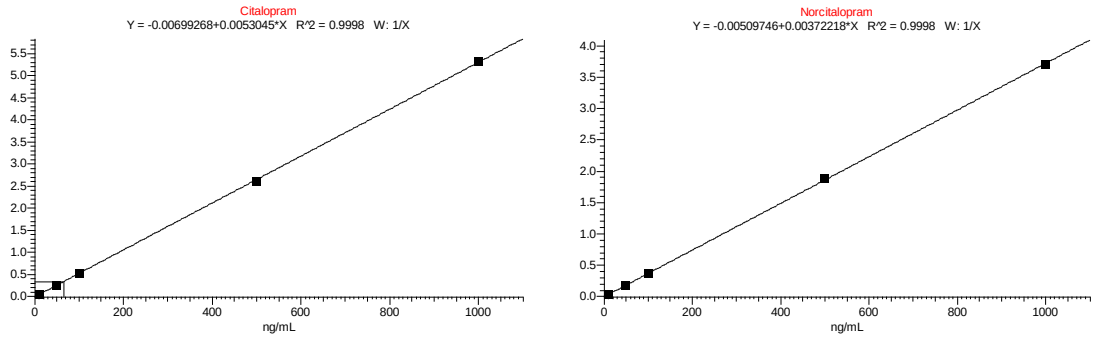


Figure 5-1. Standard curves from citalopram (escitalopram) and metabolite norcitalopram (desmethylescitalopram)

5.1.1 Chromatography

Chromatographic separation and detection of citalopram (racemic enantiomer of ESC), norcitalopram (racemic enantiomer of D-ESC) and their respective deuterated (d6) internal standards was achieved. **Figure 5-2A and 5-2B** show the chromatograms of citalopram and norcitalopram, respectively, from the final chromatographic method. Retention times were 3.08 minutes for ESC (and ESC-d6) and 2.87 minutes for D-ESC (and D-ESC-d6).

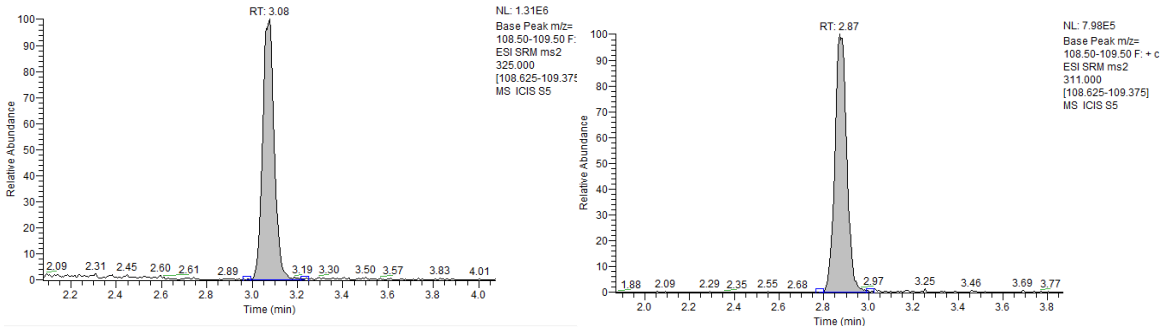


Figure 5-2. Chromatogram of (A) citalopram (racemic ESC) and (B) norcitalopram (racemic D-ESC).

5.1.2 Precision

To determine the assay precision, three quality control levels (5, 300 and 800 ng/mL) were run in 20 replicates. As shown in **table 5-1**, the total precision for each of the three levels were: 3.7%, 4.7% and 4.2%, respectively for low, medium and high controls for ESC. Precision for D-ESC

was 3.1%, 3.3% and 4.4%. The patient samples were run in one batch to minimize variability between runs and eliminating the need to explore between-day assay precision.

Table 5-1. Total within-day precision of ESC and D-ESC (n=20).

Within-Day Precision						
	ESC			D-ESC		
Level (ng/mL)	Low (5)	Med (300)	High (800)	Low (5)	Med (300)	High (800)
Mean	5.1	289.9	718.8	4.8	292.5	712.2
SD	0.2	13.6	30.2	0.1	9.5	31.6
CV (%)	3.9	4.7	4.2	2.1	3.2	4.4

5.1.3 Linearity

Linearity was assessed by running six equally spaced pools in triplicate and plotting the expected concentration versus mean observed result from the three runs. Based on linear regression analysis, the method was considered linear over the range of 5-1000 ng/mL for both ESC (slope: 1.06, intercept: 7.1, R^2: 0.99) and D-ESC (slope: 1.01, intercept: 5.9, R^2: 0.99), as shown in **figure 5-3A** (ESC) and **5-3B** (D-ESC) below.

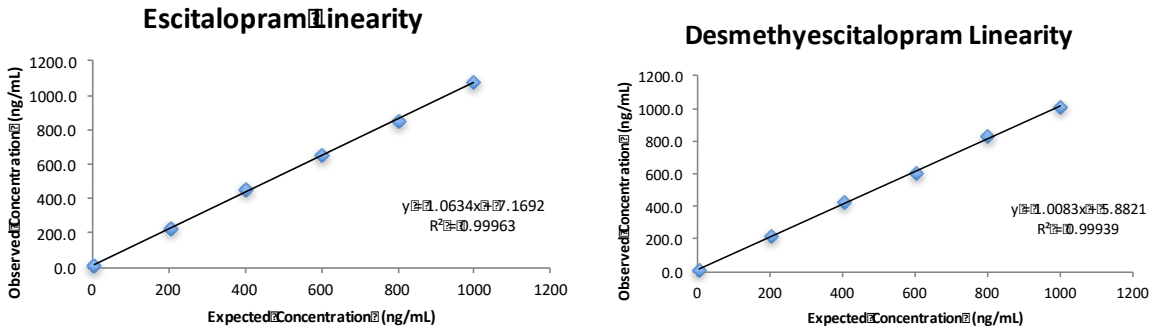


Figure 5-3. Linearity for (A) ESC and (B) D-ESC. Points represent the mean value for each level (n=3).

5.1.4 Ion Suppression

Ion suppression was assessed by injecting an extract of blank milk into the instrument and directly infusing ESC/D-ESC in methanol across the chromatographic run. No ion suppression was noted at the ESC, D-ESC or respective internal standard peak elution times.

5.2 Patient Characteristics

Eighteen lactating women taking ESC for various indications (e.g. depression, generalized anxiety disorder, etc.) were recruited through the DLAC study. A total of 104 milk samples were collected. All demographic information on the participants from the study are listed in **table 5-2**. All women were considered to have steady-state plasma concentration of ESC, based on inclusion criteria. Two participants were receiving co-medication with another antidepressant, bupropion. The mean participant age was 32.6 years (range: 25.7 – 40.3 years), weight was 70.8 (range 49.9 – 102 kg), and height was 1.66 meters (1.50 – 1.76 meters), with a calculated mean BMI of 25.6 (range: 19.3 – 37.5). The median daily dose of ESC was 10 mg (range 2.5 – 30 mg), representing 61% of the studied population. The mean daily weight-adjusted dose was 0.2 mg/kg (range 0.04 – 0.4 mg/kg).

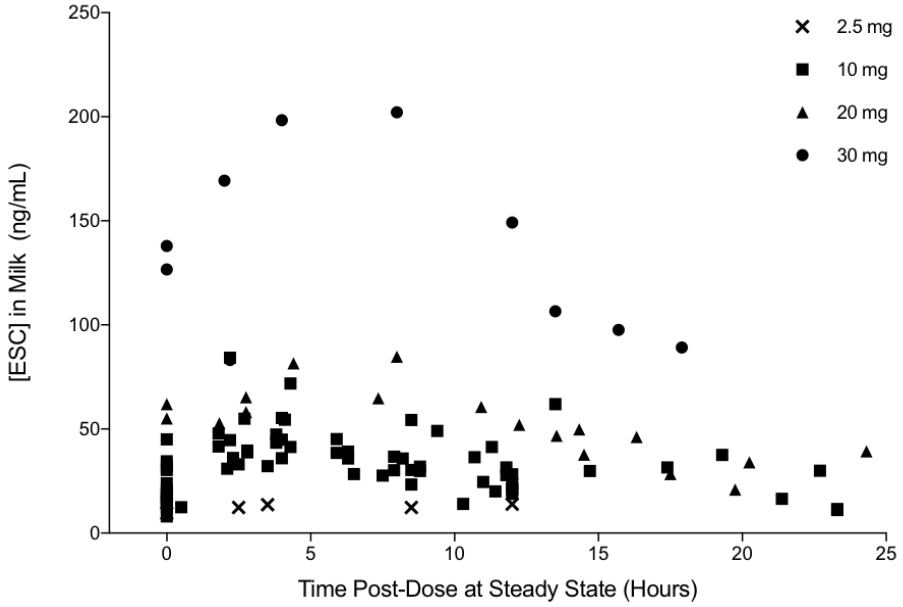
Table 5-2 Characteristics of participants in the ESC DLAC Study. (n=18 women; 104 breast milk samples).

	Mean ± SD	**Range**
Age (years)	32.6 ± 4	25.7 – 40.3
Body Weight (kg)	70.8 ± 12.6	49.9 – 102
Height (m)	1.66 ± 0.08	1.5 – 1.76
BMI (%)	25.6 ± 4.2	19.3 – 37.5
Dose (mg)	10*	2.5 – 30
Weight-Adjusted Dose (mg/kg)	0.2 ± 0.1	0.04 – 0.4

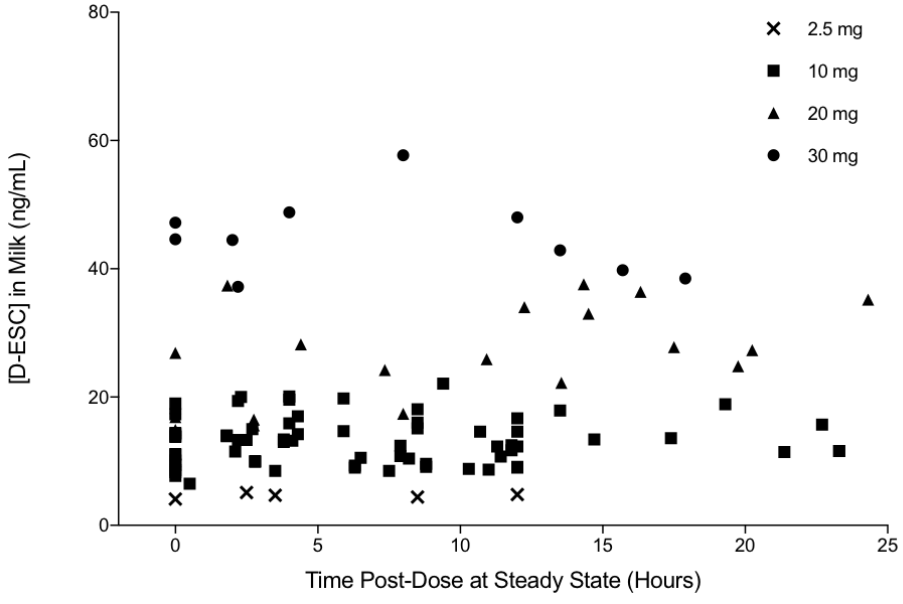
* Median

5.3 Measured ESC Milk Concentrations

The pooled time-concentration profile of ESC and D-ESC for each sampling time point provided by all 18 women is shown in **Figure 5-4A** and **5-4B**, respectively. As shown in **table 5-3**, the highest milk concentration of ESC in each of the dose groups ($_{high}C$) was approximately equal for the 10 and 20 mg/day dosing regimens (84.3 and 84.7 ng/mL, respectively). The time to reach $_{high}C$ was defined as $_{high}T$ (hours). As anticipated, the highest milk metabolite (D-ESC) concentrations from the 20 mg/day group (37.6 ng/mL) were nearly double that of the 10 mg/day group (22.1 ng/mL). Note that $_{high}C$ and $_{high}T$ are defined on a basis of a dose group, but not each subject. The average steady-state ESC milk concentration (Cav) across the dosing interval for each patient was higher in the women receiving 20 mg daily (mean ± SD: 49.8 ± 18 ng/mL), compared to those receiving 10 mg (32.9 ± 15 ng/mL). D-ESC metabolite Cav in milk was exactly 2-fold higher in the 20 mg group (26.0 ± 7 ng/mL) versus the 10 mg group (13.1 ± 4 ng/mL). Interestingly, the women receiving 30 mg daily had 2-fold higher Cav (136.0 ± 43 ng/mL) and $_{high}C$ milk ESC concentrations (202.2 ng/mL) compared to the women receiving 20 mg daily. Similarly, the Cav milk concentrations of D-ESC from the women receiving 30 mg/day (44.9 ± 6) was nearly 2-fold higher than those receiving 20 mg daily (26.0 ± 8). $_{high}C$ milk profiles of D-ESC were 1.5-times higher between the 20 mg group (37.6 ng/mL) and 30 mg group (57.7 ng/mL). Investigations into the kinetics of ESC and D-ESC in milk will be discussed in Chapter 7.



(A)



(B)

Figure 5-4. Measured concentrations of (A) ESC and (B) D-ESC in breast milk from 18 lactating women taking 2.5 – 30 mg ESC daily. The scatter plot is shown per dose group, but not per subject: 2.5 mg (X), 10 mg (squares), 20 mg (triangles), 30 mg (circles). (sample n= 104).

Table 5-3. Observed pharmacokinetic measures of ESC and D-ESC in breast milk

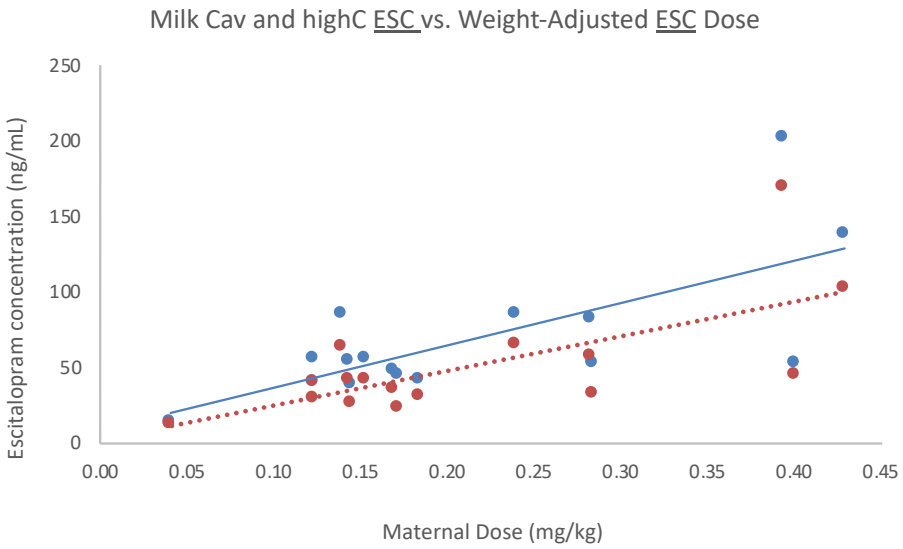
		ESC		
ESC Dose (mg)	**N**	**Milk $_{high}$C (ng/mL)**	**Milk $_{high}$T (hours)**	**Milk C_{av} (ng/mL)**
2.5	1	13.9	12.0	12.4 ± 2
10	11	84.0	2.2	32.9 ± 15
20	4	84.7	8.0	49.8 ± 18
30	2	202.2	8.0	136.0 ± 43

		D-ESC		
ESC Dose (mg)	**N**	**Milk $_{high}$C (ng/mL)**	**Milk $_{high}$T (hours)**	**Milk C_{av} (ng/mL)**
2.5	1	5.1	3.5	4.6 ± 0.4
10	11	22.1	9.4	13.1 ± 4
20	4	36.6	14.3	26.0 ± 8
30	2	57.7	8.0	44.0 ± 6

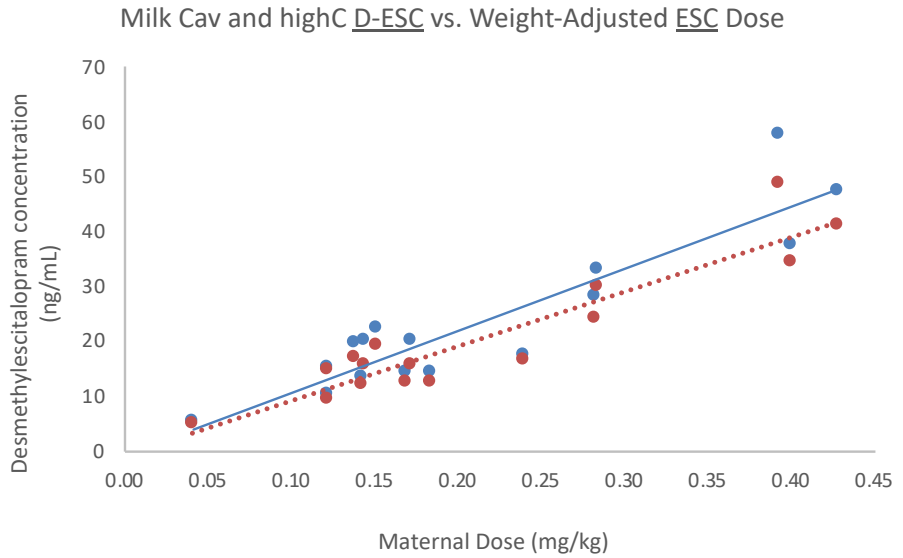
5.4 Relationship Between ESC/D-ESC Milk Levels and Maternal Dose

To examine the linear relationship between dose and milk concentrations, the weight-adjusted dose was calculated for each participant and plotted against Cav and $_{high}$C milk concentrations of ESC and D-ESC. **Figures 5-5A** and **5-5B** display the mean milk concentrations of ESC (A) and D-ESC (B) plotted against the weight-adjusted dose of ESC for each participant included in the study. As anticipated, there was a statistically significant correlation between weight-adjusted ESC dose and Cav milk concentrations of ESC (r: 0.69; p=0.003) or D-ESC (r: 0.93; P<0.0001) showing a linear trend between weight-adjusted dose with both parent drug and metabolite concentrations in milk.

The weight-adjusted dose and $_{high}$C milk concentrations of ESC and D-ESC were plotted to determine whether there is a relationship between the two parameters. Consistent with the Cav milk concentrations, there was a statistically significant relationship between milk ESC and D-ESC $_{high}$C and weight-adjusted dose, as shown in **figure 5-5A** and **5-5B** below. Pearson r was 0.71 (p=0.002) for ESC (**figure 5-5A**) and r: 0.92 (p<0.0001) for D-ESC (**figure 5-5B**).



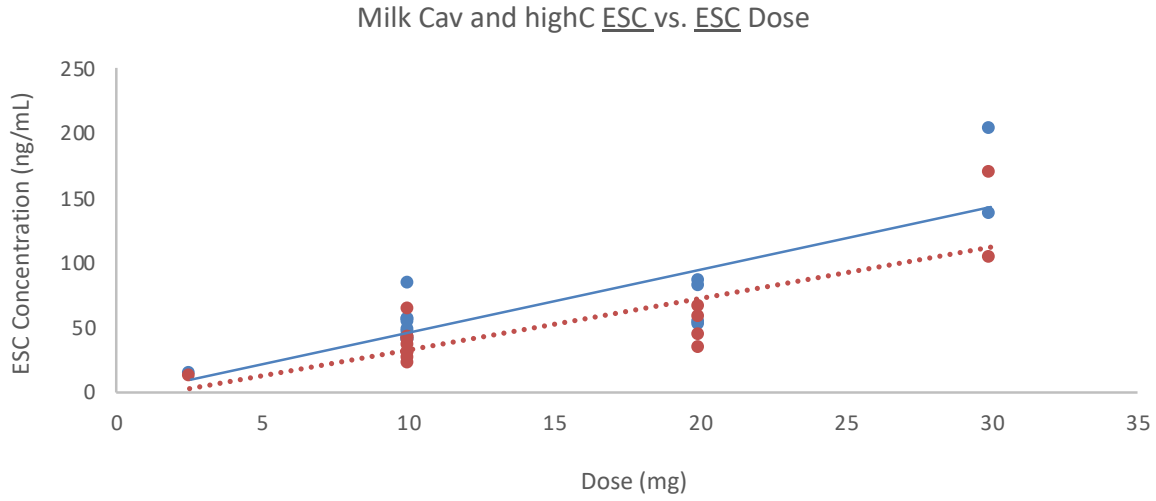
(A)



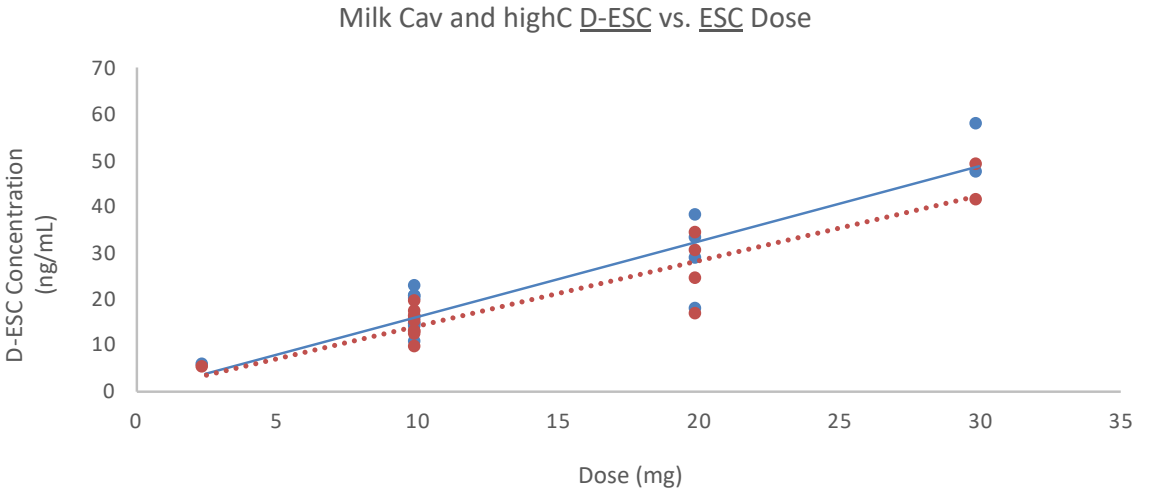
(B)

Figure 5-5. Relationship between (A) ESC and (B) D-ESC milk concentrations vs. weight-adjusted maternal dose (mg/kg) (n=18). Blue dots, solid line ($_{high}$C); red dots, dotted line (Cav)

Next, the relationship between the absolute dose (in mg), and Cav and $_{high}$C milk concentrations was explored. **Figure 5-6A** and **5-6B** shows the plotted dose vs. concentration of ESC and D-ESC, respectively, for both Cav and $_{high}$C. as seen with the maternal weight-adjusted dose, the Cav and $_{high}$C of ESC in milk showed a statistically significant correlation with an increase in dose for both Cav (r: 0.81; p=0.0001) and $_{high}$C (r: 0.84; p<0.0001) (**Figure 5-6A**). Further, the milk metabolite concentration follows a similar trend showing that an increase in dose corresponds with an increase in D-ESC Cav (r: 0.93; p<0.0001) and $_{high}$C (r: 0.92; p<0.0001) (**Figure 5-6B**).



(A)



(B)

Figure 5-6. Relationship between (A) ESC and (B) D-ESC peak milk concentrations vs. maternal dose (mg) (n=18). Blue dots, solid line ($_{high}$C); red dots, dotted line (Cav)

5.5 Differences in Concentration Between Foremilk and Hindmilk

In order to determine whether milk composition influences levels of ESC and D-ESC in milk, we collected a total of 17 paired foremilk (beginning of feed; more aqueous) and hindmilk (end of feed; more lipid-rich) samples from 4 women and measured concentrations of parent compound and metabolite. One woman was receiving a daily dose of 20 mg daily, while the remaining 3 women were receiving 10 mg daily. **Figure 5-7A** and **5-7B** show that there was a statistically significant difference between foremilk and hindmilk concentrations for ESC and D-ESC, respectively (paired t-test, $P < 0.05$). Higher concentrations were measured in the hindmilk for both ESC and D-ESC.

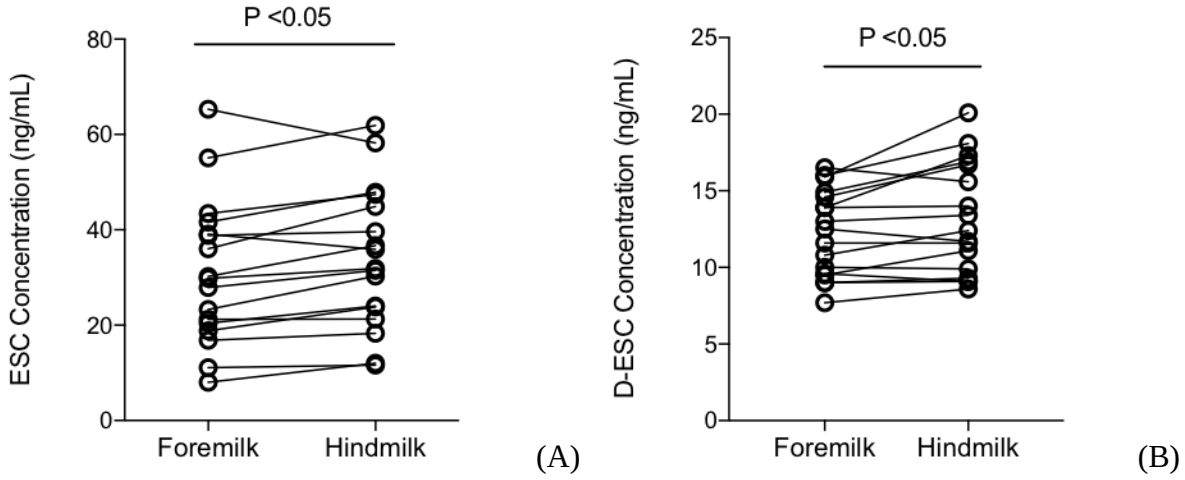
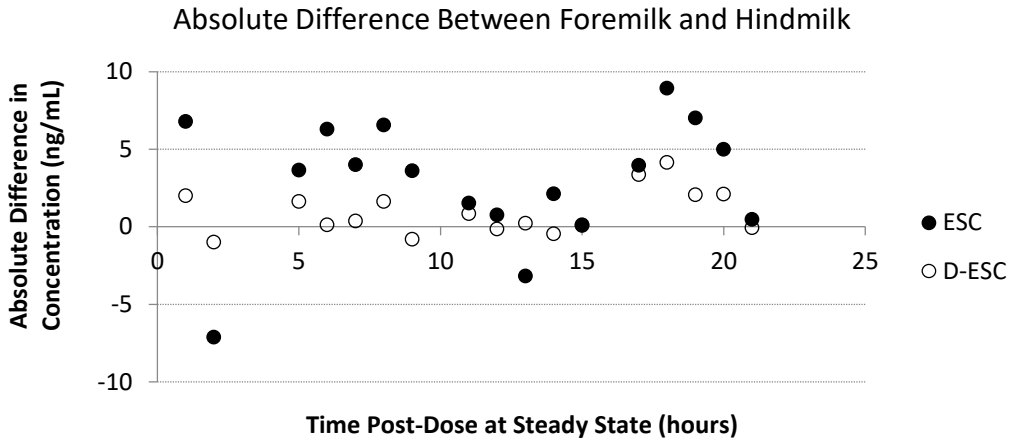
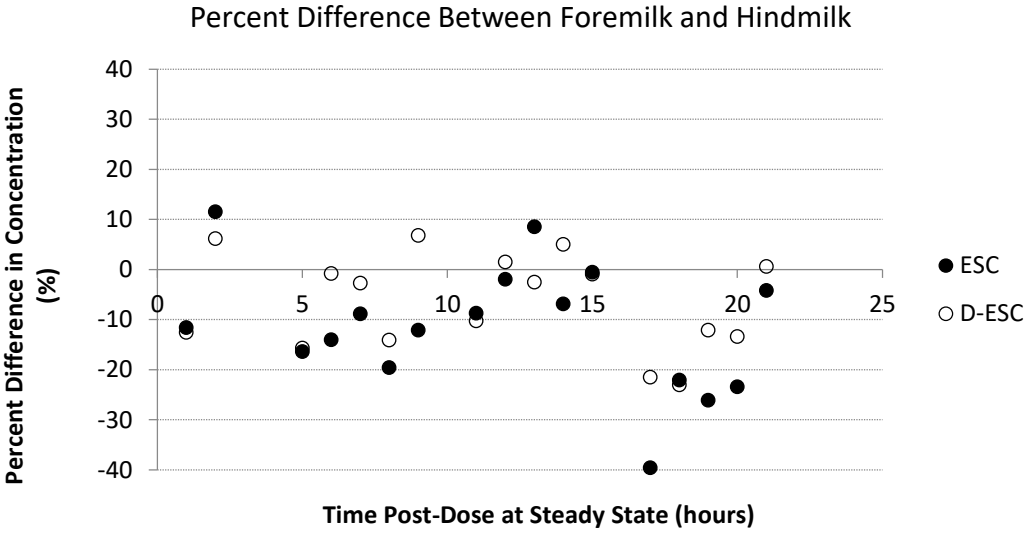


Figure 5-7. The difference between foremilk and hindmilk in (A) ESC (left plot) and (B) D-ESC (right plot). Each point represents either foremilk or hindmilk. $P < 0.05$ was considered to be significant (n= 17 paired milk samples).

Further, the absolute difference and percent difference between the foremilk and hindmilk for each sample were plotted and shown in **figure 5-8A** and **5-8B**, respectively. The difference in absolute concentrations shown in **Figure 5-8A** did not exceed 10 ng/mL for ESC and 5 ng/mL for D-ESC (**figure 5-8A**) which converts to a percent difference generally not exceeding 30% for ESC and 20% for D-ESC (**figure 5-8B**).



(A)



(B)

Figure 5-8. (A) Absolute difference and (B) percent difference in concentration between hindmilk and foremilk at collection time points post-dose (N=17 from 4 women; average of 4 samples from each woman).

Despite these statistically significant differences between foremilk and hindmilk, the relative difference in concentration was 8.8% for ESC and 4.6% for D-ESC, which suggests that sampling phases have no substantial impact on infant exposure estimates.

5.6 Determining Sampling Time Bias

Each participant included in the study indicated whether they collected their milk before feeding their infant (pre-feed; full breast with intent to feed their infant), after feeding (post-feed; mostly emptied breast) or between feeds (not feeding their infant). **Figure 5-9** shows the distribution of concentrations ESC (**figure 5-9A**) and D-ESC (**figure 5-9B**) depending on the time of sampling specified by the participant. A one-way ANOVA was conducted to compare the influence of sampling phase on milk concentrations. There was no statistically significant difference in concentrations depending on the time or phase of sampling, further supporting that there is no sampling bias and that infant exposure estimates should not be affected.

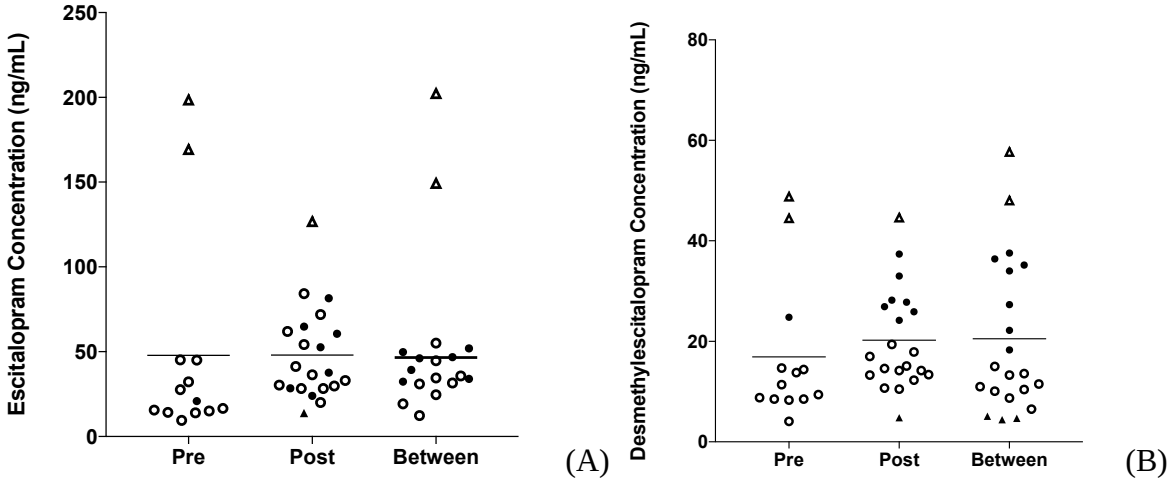


Figure 5-9. (A) ESC and (B) D-ESC shows the distribution of milk sampling – pre-, post-, and between-feeds and effects of sampling phase on milk concentrations. 2.5 mg (dark triangles), 10 mg (open circles), 20 mg (dark circles), 30 mg (open triangles). One-way ANOVA $P<0.05$ was considered to be significant.

5.7 Simulation of Total Infant Daily Dose

Due to the fact that desmethylescitalopram does not have significant biological activity at the serotonin receptor, we opted to not take it into account moving forward in generating of the total infant daily dose and in our infant PBPK plasma simulation. The measured ESC concentrations

in breast milk from women taking 20 mg daily were used with infant feeding parameters from the literature to simulate the total infant daily ESC dose with applicable variability within each parameter. This dose was used because it was the maximum dose described in the product monograph for postpartum depression[222].

To estimate the daily dose of ESC that an infant would consume via breast milk (whose mothers were taking 20 mg ESC daily), volume of milk (mL/feed), frequency of feeds (number of feeds per day), and milk ESC concentrations (ng/mL) were multiplied and then weight-adjusted to generate a dose in mg/kg/day. The mean and standard deviation of the weight-based doses generated were 0.0078 ± 0.0032 mg/kg/day (**Figure 5-10**). The daily doses generated from this simulation was subsequently used as the "dose" to simulate the plasma $AUC_{0-\infty}$ in the infant PBPK model (section 4.8).

The RID was calculated by dividing the generated infant dose by the mean maternal weight-normalized dose and multiplying by 100. Base on these calculations, breastfeeding infants would be exposed to a dose of ESC, equaling 2.6% of the maternal weight-adjusted dose, which falls well below the accepted limit of 10% being compatible with breastfeeding[223].

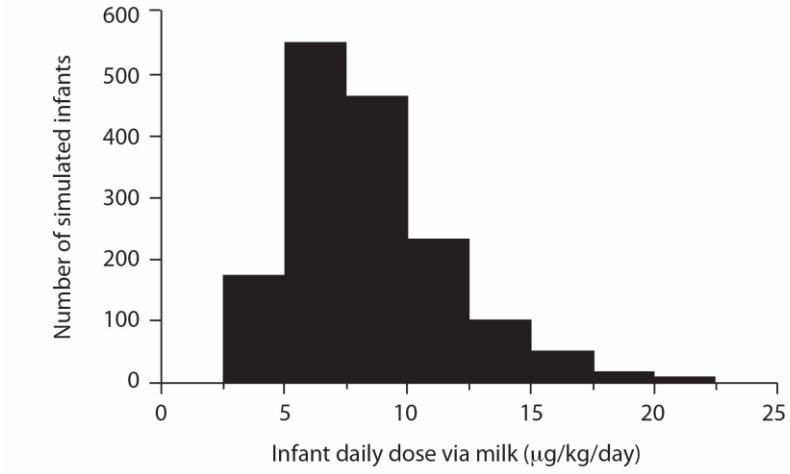


Figure 5-10. Histogram of the simulated daily ESC doses for an infant population whose mothers were taking 20 mg ESC daily. (with permission from Delaney et al.[224]).

5.8 Infant PBPK Model Simulations

In order to understand the extent of infant plasma exposure to ESC via breast milk (using data from figure 5-10), the final infant PBPK model (model development and validation discussed in materials and methods, chapter 3) was used to simulate plasma $AUC_{0-\infty}$ in 1600 virtual infants ranging from birth (term) to 12 months old. Virtual populations of infants were generated and the pharmacokinetic profile was predicted using the infant PBPK model in PK-Sim® after administration of the simulated total infant daily doses.

As shown in **figure 5-11**, the resulting mean and range of infant $AUC_{0-\infty}$ from the simulation was 12.0 (range: 2.7 – 71.2) ng·h/mL. The mean predicted infant $AUC_{0-\infty}$ represents 1.7% of the mean adult $AUC_{0-\infty}$ after a dose administration of 20 mg. Interestingly, infant exposure did not vary widely between age groups across the first 12 months of life.

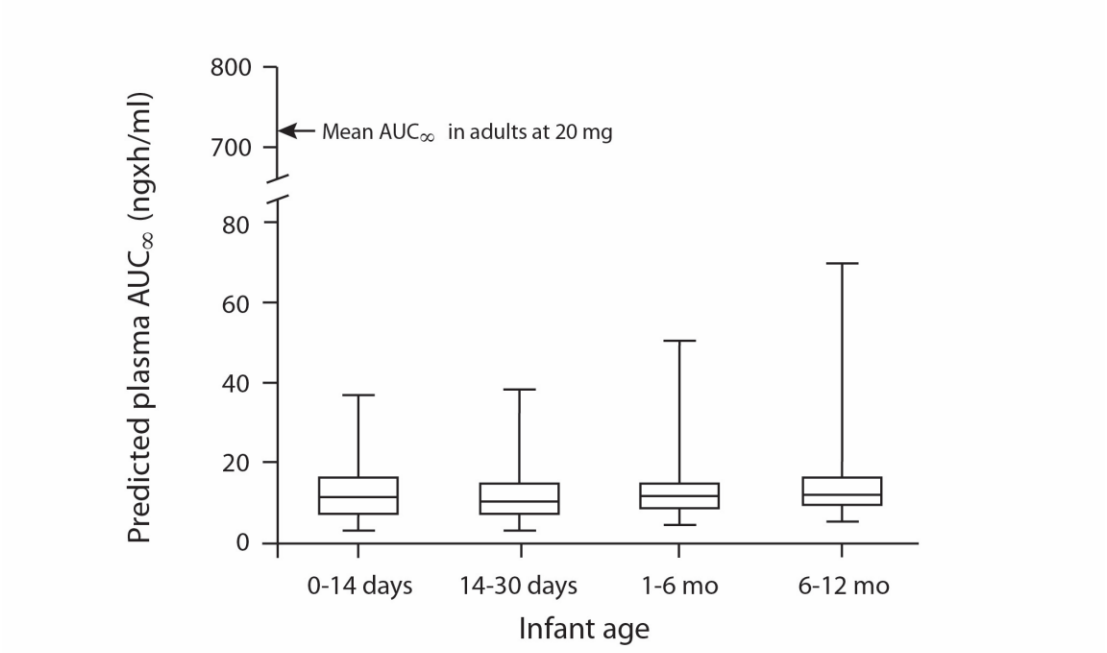


Figure 5-11. Simulated plasma $AUC_{0-\infty}$ for infants birth – 12 months (n=1600). Box edges mark the first and third quartile and contain the median; bars illustrate the minimum and maximum. Red dashed line represents the mean maternal $AUC_{0-\infty}$. (with permission from Delaney et al.[224])

The average plasma concentrations at steady state were calculated for the infant populations and compared to the plasma concentrations measured in 5 infants by Rampono et al.[171]. ESC plasma

concentrations were undetectable in four breastfeeding infants and 3 ng/mL in one breastfeeding infant whose mothers were taking 10 – 20mg ESC daily[171]. The mean and range of the model-predicted steady state average plasma concentrations ($C_{av,ss}$) in infants whose mothers were taking 20 mg ESC daily had a mean and range of 0.5 (range: 0.1 – 3.0) ng/mL (**figure 5-12**). The predicted values from our simulation are consistent to those described by Rampono et al[171]; therefore, it is reasonable to assume that our *in-silico* model has good predictive accuracy.

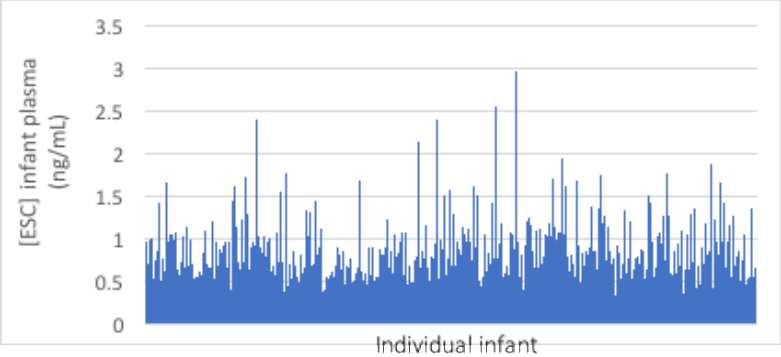


Figure 5-12. Steady-state plasma concentrations for all simulated infants aged birth- 12 months. N=1600. mean and range of 0.5 [0.1 – 3.0] ng/mL.

5.9 ESC Results Summary

The data generated from this study helps fill in the gaps on safety and compatibility of ESC therapy during lactation and serves as a proof-of-concept approach to examine risk assessment of infants exposed to other drugs through breast milk. Using an analytical method optimized for quantification of ESC in breast milk, we were able to confidently measure ESC concentrations in the milk of 18 women taking 2.5 – 30 mg daily. With this data, we used PBPK modeling to simulate infant plasma concentrations following exposure to ESC via breast milk, which accounts for the unique physiology of infants. Based on the infant exposures generated, our study demonstrates that ESC use during breastfeeding should be considered safe and that the benefits of pharmacological PPD therapy can outweigh the risk of adverse events in infants. Inferences from these data will be further explored in the discussion (Chapter 7).

Chapter 6
Infliximab

6 Infliximab Chapter Outline

This chapter will discuss the results from the IFX project. To date, there has been no analytical method optimized to quantitate IFX in breast milk. First, the results from the assay developed and validated to measure IFX in milk are described. IFX quantitation in human milk samples from 5 patients will be discussed and summarized to end the chapter.

6.1 Assay Validation Results

Common analytical methods used to measure IFX are ELISA assays optimized for quantification in serum. In a clinical setting, IFX concentrations < 1 μg/mL in serum are considered to be undetectable[197]. Based on previous reports in the literature, IFX levels in milk were found to be highly variable, ranging from not detectable to 300 ng/mL[192,195]. These data highlighted the need to develop an analytical approach sensitive enough to quantitate IFX in milk; a method more sensitive than what is currently used for plasma IFX determination. The analytical measuring range used in our assay was generated from the literature, with a calibration range from 5-250 ng/mL. Here, the method precision, LoQ, and linearity/reproducibility of the standard curve were assessed for IFX specifically in human milk. Effort was made to follow CLSI guidelines for each aspect of validation; however, inadequate resources contributed to the lack agreement with the criteria.

6.1.1 Precision

As shown in **table 6-1**, total precision for IFX was determined using low (37.5 ng/mL), medium (150 ng/mL), and high (225 ng/mL) QC samples. Acceptance criteria were established as CVs ≤20%[225]. Within-day precision was calculated by running 12 replicates of each low, medium, and high QC level and determining the CV and were: 2.9%, 8.3% and 5.4%, respectively.

The stability of reconstituted, refrigerated IFX has been assessed for up to 6 weeks[226]; therefore, a fresh QC lot was prepared within that timeframe. In order to account for inter-lot imprecision, two different QC preparations were also included in the calculation for between day imprecision. CVs were 18.9%, 10.1% and 14.0% for low, medium and high QC levels, respectively.

Table 6-1 Within- and between-run precision of IFX quantitation. Quality control levels: low (37.5 ng/mL), medium (150 ng/mL), and high (225 ng/mL).

	Within-Day (n=12)			Between-Day (n=8, 2 lots)		
Level	Low	Med	High	Low	Med	High
Mean	41.3	134.0	181.6	33.8	144.9	212.2
SD	1.2	11.2	9.7	6.4	14.6	29.7
CV (%)	**2.9**	**8.4**	**5.3**	**18.9**	**10.1**	**14.0**

6.1.2 Limit of Quantification (LoQ)

To date, there have been several attempts to measure IFX in breast milk. Many groups reported undetectable levels of infliximab in breast milk, while others reported concentrations in the range of 10 – 300 ng/mL[190–195]. Until this study, analytical methods optimized for quantification of IFX in serum have been used to measure milk concentrations in the literature. These assays utilized an analytical measuring range approximately 100-fold higher than the expected concentrations in breast milk. In order to get a comprehensive understanding of infant exposure across the dosing interval, it was important to establish a reliable assay LoQ. A low standard 25 ng/mL was serially diluted in two different batches blank milk to achieve the concentrations 12.5, 6.3, 5, 3.1, 2.5, 1.5, 1.25 and 0.63 ng/mL. Samples were run in 5 replicates and the CVs were calculated as shown in **table 6-2**. The functional LoQ was defined as the concentration that yielded a CV of < 20%. The lowest dilution (0.63 ng/mL) that we examined yielded a CV of 7.7% and was defined as the LoQ. We did not feel the need to further dilute and explore the reproducibility at lower concentrations, as each of the 106 patient samples yielded concentrations greater than our lowest dilution.

Table 6-2. Limit of quantification of IFX measurement in breast milk

Expected (ng/mL)	Measured	SD	%CV
25	18.7	0.6	3.2
12.5	6.6	0.2	3.0
6.25	4.7	0.3	6.4
5	3.4	0.3	8.2
3.125	3.8	0.1	2.6
2.5	2.1	0.2	9.5
1.5	3.4	0.04	1.1
1.25	1.7	0.2	11.8
0.625	1.3	0.1	7.7

6.1.3 Reproducibility of the Standard Curve and Analytical Measuring Range

A 5-point standard curve was produced (5, 66.25, 127.5, 187.5, 250 ng/mL) by spiking IFX into pooled human milk samples. The analytical measuring range was defined by linear regression analysis of 8 standard curves from 8 different runs on 8 separate days. **Figure 6-1** displays the expected vs. observed concentrations and their respective linear regression across each run. Quantification of IFX was linear from 5-250 ng/mL (mean R^2= 0.999; slope= 0.99 ± 2.5; y-intercept = 0.69 ± 1.4).

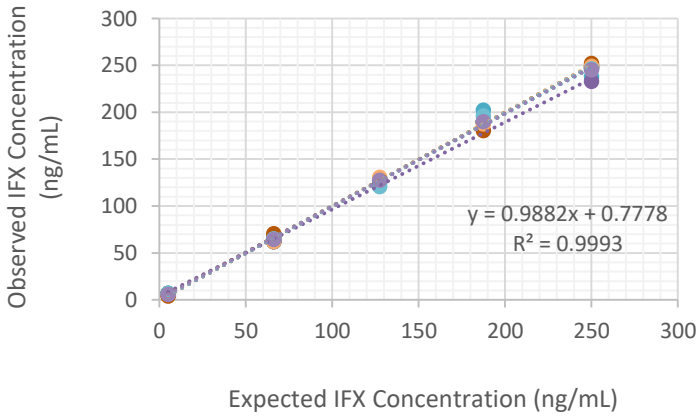


Figure 6-1. Reproducibility of standard curves used to quantitate IFX in breast milk. mean R^2= 0.999; slope= 0.99 ± 2.5; y-intercept = 0.69 ± 1.4).

6.2 Patient Characteristics

A total of 5 participants receiving IFX therapy were recruited as part of the DLAC Study. IFX concentrations were determined in a total of 106 breast milk samples (19-22 samples per patient) using an in-house validated ELISA assay. Milk was collected prior to infusion and up until 26-36 days post-dose in order to assess infant exposure as comprehensively as possible across the dosing interval.

All demographic information is included in **table 6-3**. Women received a dose of 300-400 mg every 8 weeks for maintenance therapy. Each participant disclosed taking concomitant medication or supplements while they collected milk. Three of the women reported other diagnoses including: asthma, depression, anxiety and Reynaud's. In all 5 cases, breast milk samples were collected prior to receiving IFX infusions (trough concentrations). Interestingly, there was high variability of trough concentrations in breast milk, ranging from 6 – 42.6 ng/mL. The peak milk concentrations were noted between 1-4 days post-infusion and ranged from 103.3-312.2 ng/mL. The mean milk concentrations across the sample collection interval range between 54.7 – 106.1 ng/mL. Inferences on the variability in milk concentrations will be discussed in Chapter 7: Discussion.

Table 6-3. Characteristics of 5 lactating women receiving IFX for IBD

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Patient Diagnosis	Colitis	Crohn's and Colitis	Crohn's	Crohn's	Crohn's
Dose (mg)	400	300	400	400	300
Frequency	q8 weeks	q8 weeks	q8 weeks	q8 weeks	q8 weeks
Other Diagnoses			Asthma, depression	Anxiety	Reynaud's Syndrome
Co-medication	Imuran (75 mg/day), Materna, vitamin D, fish oil	Prenatal vitamins	Citalopram (20mg/day), multivitamins, probiotics, vitamin D	Zoloft, Pregvit, calcium, vitamin D	Prenatal vitamins, Nyaderm
Age (years)	31	32	34	34	37
Race	Caucasian	Caucasian	Caucasian	Ashkenazi Jewish	Caucasian
Weight (kg)	59.0	65.8	69.7	73.0	56.0
BMI	23.0	22.7	21.8	27.5	20.8
Weight-adjusted dose (mg/kg)	6.8	4.6	5.7	5.5	5.4
Trough Milk Concentration (ng/mL)	7.0	6.0	3.1	42.6	11.6
Mean Milk Concentrations (ng/mL)	102.3	69.9	54.7	106.1	100.7
Max Milk Concentration (ng/mL)	283.5	129.5	103.3	223.0	312.2
Time to peak (days post-infusion)	3	4	1	3	1

6.3 IFX Concentrations in Breast Milk up to 36 days Post-Infusion

Each of the 5 patients recruited for this study collected a total of 19-22 samples (including trough samples) post-IFX infusion. **Figure 6-2** displays the time-concentration profile from all 106 milk samples up to 36 days post-infusion. IFX concentrations peaked between days 1 – 4 post-dose up to the final collection time points (days 26-36 post-infusion). Interestingly, the peak milk concentration (312.2 ng/mL post-infusion day 1) was seen in a patient receiving only 300 mg every 8 weeks, whereas the other patient receiving 300 mg displayed a peak milk concentration

of 129.5 ng/mL on the 4th day post-infusion. Each patient had quantifiable IFX levels at trough and on their final sample collection day.

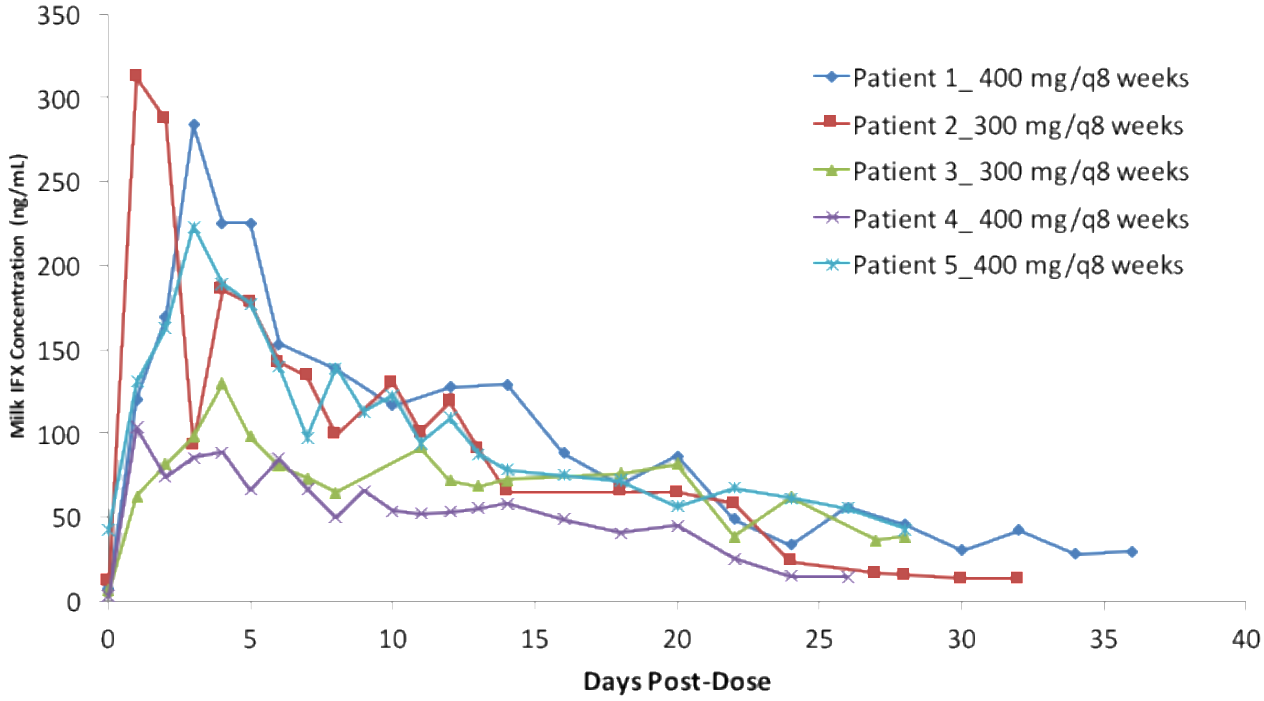


Figure 6-2. Time-concentration profile of IFX in milk from 5 lactating women with IBD. N=106 (19-22-time points per patient).

6.4 Influence of Dose on IFX Milk Concentrations

In order to understand whether there is a dose-dependent influence in milk concentrations, the weight-adjusted dose (mg/kg) was plotted against the peak and mean milk IFX concentrations from each study participant. Interestingly, as shown in **Figure 6-3**, there was no correlation between dose and both peak and mean milk concentrations, with r = 0.47 (p = 0.53) and 0.38 (p = 0.43), respectively. This will be further explored in the discussion chapter.

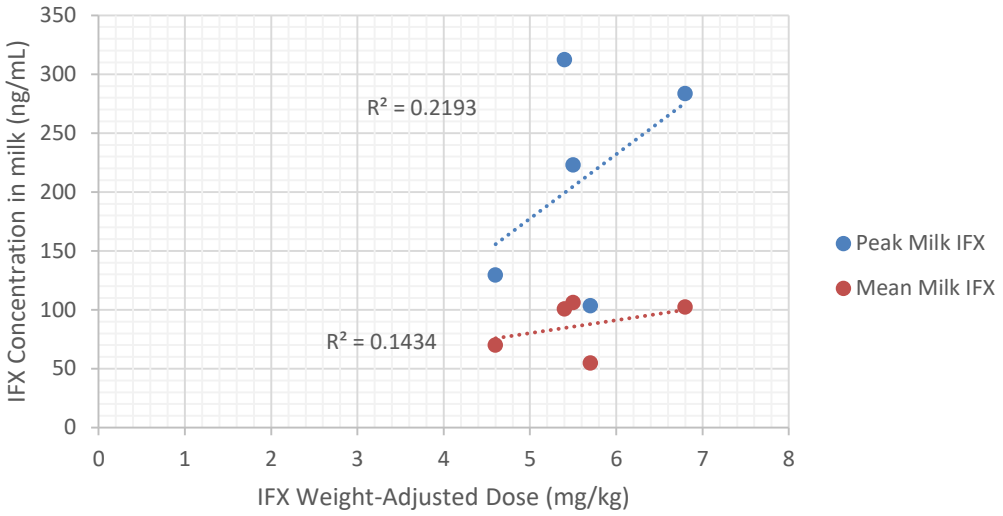


Figure 6-3. Relationship between weight-adjusted dose and peak or average milk concentrations

6.5 Infant Exposure Estimates

Our data shows that there are quantifiable IFX concentrations in milk up to 8 weeks post-infusion (trough). The highest IFX milk concentration measured in our cohort was 312.2 ng/mL from a woman receiving 300 mg (5.4 mg/kg). If an infant ingests a total of 150 mL milk per kg body weight per day at a concentration of 312.2 ng/mL IFX, the estimated maximum dose the infant would be exposed to is 0.047 mg/kg/day. Based on the mean milk concentrations from all 5 patients (86.7 ng/mL), the mean infant dose across the sampling interval is 0.0013 mg/kg/day. IFX clearance in neonates and infants remain unknown.

6.6 IFX Summary

The primary aims of this study were to (1) validate an analytical method to quantitate IFX concentrations specifically in human breast milk and (2) gain a better understanding of infant exposure across the dosing interval. This study was the first to optimize a method to measure IFX levels in milk for an extended period post-infusion. Based on our findings, breastfed infants are exposed to a maximum daily dose of 0.047 mg IFX through milk. Despite the low concentrations found in breast milk, the implications of these data are not well understood, as it

is remains unknown whether IFX is absorbed through the infant gastrointestinal tract. These findings will be critically discussed in Chapter 7: Discussion.

Chapter 7
Discussion

7 Discussion Chapter Outline

This chapter summarizes all the findings from all three sub-studies of this thesis and discuss their implications in a broader context. Conclusions will be drawn from each respective project. The chapter will end by discussing limitations, future directions and an overall conclusion from this thesis.

7.1 Methotrexate

Low-dose MTX is currently the most widely used disease-modifying agent to treat rheumatoid arthritis [121]. MTX at a dose greater than 15 mg/week is a preferred treatment due to the clinical efficacy and safety[127,227]. Adverse effects of MTX are generally considered to be dose-dependent and infrequently observed in patients undergoing low-dose treatment for RA[228].

Although a large number of women with RA undergo remission during pregnancy, nearly all patients experience a disease flare after giving birth and need to resume treatment [126,229]. While there are no data on breastfeeding rates and duration in patients with RA, over 50% of women with inflammatory bowel disease did not breastfeed their children, likely due to the presumed risk of exposing their infants to their medication[5]. Further, despite the known clinical benefits of MTX and case reports suggesting marginal infant exposure, the American Academy of Pediatrics (AAP) classifies MTX as a drug that "may interfere with cellular metabolism" of the nursing infant and is listed as contraindicated during breastfeeding[153,154]. Therefore, women experiencing a disease flare while breastfeeding are discouraged from treatment with MTX.

The aims of this project were three-fold: (1) to better understand the extent of MTX excretion into human milk and describe the potential risk to the nursing infant; (2) characterize the role of Bcrp in actively transporting MTX into mouse milk; and (3) to explore the use of high-dose riboflavin to inhibit transport of MTX into milk in mice.

7.1.1 MTX in Breast Milk: Understanding Infant Exposure Estimates

To meet the first aim of the study, we developed and validated a sensitive LC-MS/MS that allowed us to accurately quantitate MTX concentrations in milk. Our method limit of quantification was 0.1 ng/mL, which is over 200 times more sensitive than the MTX assays used in a clinical setting (LoQ = 22.7 ng/mL).

Milk was collected and analyzed from two women receiving a weekly of 25 mg MTX. Our findings indicate that anti-rheumatic doses of MTX result in a peak concentration of about 2 ng/mL 24 hours post-dose. Following this 24-hour sampling time, MTX concentrations progressively decrease over 1-2 days post-dose. Due to the fact that polyglutamate-conjugated MTX emerges after 24 hours post-dose, and because total MTX (conjugated and unconjugated MTX) concentrations are likely to be about 1- 2 ng/mL, it is prudent to assume that MTX concentrations in milk, which could be available to the infant, are about 2 ng/mL throughout a dosing interval of anti-rheumatic weekly dose.

For an infant consuming 150 mL/kg of milk per day, whose mother is taking immune-modulating weekly doses of MTX, an estimated daily dose of MTX that the infant would be exposed to through milk is 0.3 μg/kg/day (150 mL/kg/day x 2 ng/mL = 300 ng/kg/day) or a weekly cumulative dose of 2 μg/kg/week. The MTX dose typically administered to children suffering from juvenile idiopathic arthritis is between 10- 20 mg/m^2/week[230]; therefore, in clinical settings of maternal RA treatment, assuming a typical dose of 500 μg/kg/week, infant exposure to MTX in milk is 250-fold lower than what is used therapeutically. Further, the estimated human reference dose (based on 1/100th of the animal the no observed adverse effect level (NOAEL)) is 3 μg /kg/day[231]. The maximum dose of 2 ng/mL would yield a dose 10-times lower than the estimated safe human dose per day.

We were the first to explore the presence of various MTX species (e.g MTX-Glu) and metabolites (e.g. 7OH-MTX) excreted into milk. The hydroxylated MTX metabolite, 7OH-MTX, was only detectable in one patient, with a peak concentration of 0.88 ng/mL, which should not contribute to increased infant exposure. Interestingly, the MTX metabolite was only quantifiable in the more aqueous foremilk samples, which conflicts with the reports that 7OH-MTX is 3-5 times less water soluble than the parent compound[232].

Nearly 30% of folate species in milk exist in a polyglutamated form[233]. MTX is an analogue of folate; therefore, we speculated that MTX may also become enzymatically modified to form MTX-glutamates in the lactating mammary epithelium, which may then underestimate available MTX sources for the infant. It is known that measurable folate concentrations increase following a trienzyme digest[220]; therefore, we carried out this method to release glutamate residues and re-measured MTX (monoglutamate) concentrations in milk. While our findings indicate a measurable increase in MTX concentrations following digestion, interestingly, this increase was not sustained across the dosing interval. The difference in extent of polyglutamation across the sampling interval could be due to changes in plasma concentrations. McGuire et al. found that the synthesis of MTX polyglutamates was both extracellular MTX concentration- and time-dependent[234]. Another factor that could contribute to these differences could be the variability seen in our analytical method. Although we developed a precise and sensitive method, there may be minor differences in measured concentration accounting for the discrepancy seen here. Taken together, it is reasonable to assume that total MTX equivalent (i.e., a sum of MTX, 7-OH MTX and MTX conjugates) in milk is relatively constant within a weekly dosing interval, ranging about 2-3 ng/mL.

7.1.2 Bcrp-Mediated Riboflavin-MTX Interaction

Aside from Bcrp, multiple transporters, such as organic anion transporters (OATs) and multidrug resistant proteins (MRPs) contribute to the clinical pharmacokinetics of MTX[138,235]. However, many of these transporters are functionally down-regulated or have undetectable expression in the mammary epithelial cells during lactation, while Bcrp is highly induced[66,95].

The results from this animal study suggest that Bcrp mediates MTX efflux in to milk. Bcrp-/- mice had significantly lower milk MTX concentrations ($p<0.05$) despite having significantly higher ($P< 0.01$) plasma levels. Further supporting the finding of Bcrp-mediated transepithelial transport in the mammary gland was the fact that co-administering Bcrp substrates or inhibitors reduced MTX concentrations in milk. It is reasonable to believe that the other ABC drug transporters are either not contributing or play a minor role in MTX efflux, as their expression is down-regulated during lactation.

Although our findings suggest that Bcrp mediates MTX transport into milk, efflux was not completely eliminated in the Bcrp-/- mice, suggesting an alternate pathway and/or mechanism such as diffusion or endocytosis may be involved in transporting MTX across the mammary epithelium. MTX is a low-affinity substrate for the folate binding protein (FBP) and can interfere with folate binding to FBP in cow's milk [236]. An abundance of FBP is found bound to folates in milk[237]. Overall, the extent of MTX efflux into milk is minimal, but perhaps MTX binding to FBP accounts for another mechanism of entry into milk.

Our results suggest that treating wild-type mice with riboflavin or FMN competitively inhibited milk MTX efflux without increasing MTX plasma levels. We postulated that this may be a mammary-specific phenomenon, where Bcrp transport capacity may be saturated by riboflavin, due to preferential uptake of riboflavin by the mammary gland epithelia. Van Herwaarden et al. reported that riboflavin is highly concentrated in milk at least partially due to Bcrp on the apical side of the mammary epithelial cells[67].

We have previously reported that increased Bcrp expression in the mammary gland during lactation is accompanied by similar induction of the riboflavin transporter, Slc52a2, in the mammary gland[68]. We hypothesize that the lactating mammary gland utilizes a joined Slc52a2 and Bcrp system to preferentially carry riboflavin across the mammary gland epithelia, thereby creating an optimal platform for riboflavin – MTX interaction.

Our findings have important clinical significance, as it suggests that riboflavin co-administration may improve the safety profile of MTX during breastfeeding, without hindering renal clearance. Although the doses of riboflavin and FMN used in the current study were supraphysiological (20 mg/kg and 100 mg/kg, respectively), there is still considerable clinical utility to these findings, as high-dose riboflavin (400 mg; 6 mg/kg) is used clinically for migraine prophylaxis[238].

7.1.3 Conclusion from MTX Study

Currently, MTX treatment while breastfeeding is controversial, largely due to limited safety data. The data generated from this study helps to fill in the gaps of knowledge on MTX excretion into milk and provides insight into using riboflavin to improve the MTX safety profile during lactation. Infant exposure to MTX via milk after maternal weekly doses of 10-25 mg is low and

should not lead to adverse outcomes; however, high inter-individual variability of MTX pharmacokinetics has been described[239], warranting further investigation in a larger cohort of milk and infant plasma measurements.

Our animal models suggest that high-dose riboflavin during breastfeeding may further reduce the transport of MTX into milk and reduce infant exposure levels to MTX. These findings might also be translated to other drugs that are Bcrp substrates and help improve their safety profiles during lactation.

7.1.4 MTX Study Strengths and Limitations

There are many strengths noted in this study. This was the first report to examine the total concentrations of all MTX species in milk, which enabled for a comprehensive understanding of infant exposure. Further, this was the first study investigating that Bcrp is the major transport pathway responsible for excreting MTX into milk. This finding facilitated the MTX-riboflavin interaction study, which found that Bcrp-mediated transport of MTX can be inhibited by riboflavin treatment, without influencing plasma concentrations. Despite already low efflux of MTX into milk, defining a competitive pathway between MTX and riboflavin might improve its safety profile and allow for use during lactation.

Sample size was a major limitation in both the DLAC and animal studies. Due to the contentious nature of MTX use during lactation, it was challenging to recruit more than two patients as part of the DLAC study. Furthermore, while most of the animal study was powered effectively, there were groups of mice that required a larger sample size to see a statistically significant effect. This was also noted in the study examining the correlation between MTX and riboflavin concentrations in milk. In order to truly explore the competitive mechanism, we would have benefited from a larger sample size.

In addition, variability in the LC-MS/MS method may affect infant exposure estimates. The variability around the LoQ (0.1 ng/mL) was approximately 20%, which could lead to an under- or over-prediction of risk. Nevertheless, the impact on infant dose may be considered trivial because of already marginal excretion of MTX into milk.

7.2 Escitalopram

SSRIs such as ESC are the most commonly prescribed antidepressant medication during pregnancy and lactation[240]. The dose administered for the treatment PPD is commonly 10-20 mg/day[222,241]. Existing literature on ESC concentrations in breast milk and infant exposure assessments are limited; however, treatment of PPD is imperative due to the fact that infants of depressed mothers are at an increased risk for developing cognitive and behavioural problems[242,243]. Nevertheless, treatment may be delayed by the non-compliance of patients due to uncertainty about the safety of SSRIs during breastfeeding[244]. Large-scale studies involving intensive infant blood sampling are considered the gold standard to examine infant exposure of drugs in breast milk. Clearly, there are various ethical and practical challenges preventing a study of this nature to be performed. In recent years, modeling and simulation techniques have been emerging as tools to better understand drug exposure in understudies and underrepresented groups such as nursing infants. Using an approach applying drug monitoring data of ESC in milk to PBPK modeling techniques we were able to successfully predict ESC $AUC_{0-\infty}$ in simulated breastfeeding infants, eliminating the need for collecting infant blood samples.

The aims of this study were three-fold: (1) develop an optimized LC-MS/MS method to measure ESC concentrations in milk, (2) generate a total infant daily dose taking variability within feeding-related parameters into consideration, and (3) apply this dose to an infant PBPK model to better understand infant systemic exposure of ESC through breast milk.

7.2.1 ESC Measurement in Breast Milk

Current data on ESC infant exposure through breast milk was generated from a small cohort study based on milk concentrations from 8 women receiving ESC daily[171]. Rampono et al. reported an RID of 5.3% the maternal weight-adjusted dose and were also able to quantitate an infant plasma concentration of 3 ng/mL in one infant[171].

Milk is a complex biological fluid requiring meticulous sample preparation and extraction prior to analysis. Here, we optimized and validated a robust analytical method for milk measurement of ESC and measured concentrations in the breast milk of 18 lactating women (n= 104 samples). Our patient population was receiving a range of doses from 2.5 - 30 mg daily. ESC has linear

plasma pharmacokinetics in the range of 10-30 mg/ day[245]. In our study, a linear increase in milk concentrations was observed for women receiving up to 20 mg daily. However, two patients receiving a dose of 30 mg had 2.7-fold higher milk ESC concentrations compared to the 20 mg group. Given this interesting finding, it is reasonable to assume that genetic polymorphisms are a major contributing factor to the variability in milk concentrations. The prevalence of poor and extensive metabolizing CYP2C19 alleles are 15.3% and 21.5%, respectively; therefore, there is a probable presence of variant alleles in the 18 women recruited for this study[246].

7.2.2 Determination of Sampling Bias

Breast milk is a dynamic fluid that changes in composition not only across the feed (foremilk and hindmilk), but also throughout the day[37,93]. Measurement of the two sampling phases is often overlooked in studies examining the safety of drugs during lactation but is essential in determining sampling bias and infant exposure estimates. Based on our random sampling schedule, we were able to comprehensively assess risk of infant ESC exposure across the entire dosing interval (24 hours). We measured a total of 17 paired foremilk and hindmilk samples from four women either receiving 10 or 20 mg daily. Although the differences between foremilk and hindmilk were considered to be statistically significant, the mean difference was approximately 10%, suggesting a negligible effect of milk phase on ESC level monitoring in milk. Furthermore, this difference in concentration between the phases translates to a difference of less than 0.4% of the weight-adjusted RID. These data suggest that milk sampling and measurement would not be affected by sampling phase and that infant exposure is relatively consistent across the feed.

7.2.3 Generating the Total Infant Daily Dose

Conventionally, RID estimates area good parameter for predicting drug safety during lactation and is a measure of the percentage of the maternal weight-adjusted dose that the infant would receive via breast milk. A psychotropic drug with an RID less than 5% of the maternal weight-adjusted dose is considered compatible with lactation[105]. Following the measurement of ESC in 104 milk samples, we were able to generate a mean RID estimate of 2.6%, which, together with

the previous report by Rampono et al.[171], suggest that ESC should be compatible with breastfeeding.

7.2.4 Infant Plasma Exposure

One limitation of solely basic infant exposure estimates on the RID is that this measure does not take into account the cumulative dose that the infant would be exposed to through each feeding, nor does it consider physiological and anatomical differences in an infant that would influence pharmacokinetic parameters.

For our simulation, we considered the variability in feeding-related parameters and our observed milk data, in order to generate an accurate assessment of infant ESC exposure through milk. The data obtained from our infant simulation demonstrate that the systemic exposure level ranged from 0.5% to 5.9% (median 1.7%) of the corresponding adult $AUC_{0-\infty}$ from the literature[168,209,211,212].

The PBPK model-predicted infant plasma $AUC_{0-\infty}$ remained relatively constant across the age-stratified groups throughout the first 12 months of life. Drug elimination pathways, such as enzyme expression and kidney function, typically develop in an age-dependent rate. It is expected that weight-based drug clearance is not fully mature in the neonatal stage, this would suggest that younger infants would have higher plasma $AUC_{0-\infty}$. However, our virtual neonates had similar $AUC_{0-\infty}$ to older infants with fully developed clearance mechanisms. These results may be explained by the fact that ESC elimination primarily occurs in the liver and is mediated by CYP2C19, CYP2D6, CYP3A4, with renal clearance playing a minor role. It is reported that liver expression and activity of CYP2D6 is almost fully mature one week after birth[247]. Another study demonstrated that plasma clearance of the CYP3A4 substrate, sildenafil, is similar to adult clearance by the first 7 days of life[248]. In addition, the liver-body weight ratio is higher in neonates compared to older infants, which, combined with mature CYP activity, could explain the trend in the simulated weight-based ESC $AUC_{0-\infty}$ across the first year of life.

7.2.5 Factors Affecting Infant Exposure

Despite the low $AUC_{0-\infty}$ achieved in our virtual population across the first year of life, it is important to consider the potential factors that could contribute to an increased risk of adverse effects in infants exposed to ESC through breast milk. Systemic exposure of ESC is up to 6-fold higher in individuals who have poor CYP2C19 activity [249,250]. Variability in CYP2C19 alleles activity could potentially affect both mother and infant, as an increase in maternal plasma concentrations could subsequently cause higher infant exposure through breast milk. Infants that are poor metabolizers could be at a higher risk of systemic exposure, regardless of the maternal genotype; however, this relationship is not well defined. Similarly, CYP2D6 variants in the mother or infant could also affect exposure estimates.

There are many other factors to consider when assessing the risk of ESC exposure to the nursing infant. The blood-brain-barrier does not fully develop until after the first 6 postnatal months, which could contribute to higher brain exposure and may have effects on the development of infant serotonergic circuitry[251]. Several groups have looked at the developmental and behavioural consequences following *in utero* and early postnatal exposure to SSRIs. Reports of neonatal serotonergic syndrome suggest that fetuses are exposed to biologically relevant concentrations of SSRIs during the third trimester of pregnancy[252,253]. In one study, postnatal day 2 plasma SSRI concentrations were nearly half of the maternal concentration. The concentrations observed in the study by Laine et al. were significantly higher than the plasma concentrations achieved in our virtual infant population[253]. Based on our data, the extent of infant exposure is unlikely to cause adverse outcomes, as the $AUC_{0-\infty}$ achieved in our virtual infants is a small fraction of the therapeutic concentrations.

Uncertainty and differences in exposure due to genetic variability and immaturity of the blood-brain-barrier warrant performing long-term follow-up following studies examining exposure of ESC though breast milk.

7.2.6 ESC Conclusions

Nursing mothers and healthcare providers are faced with a complex question when considering therapy with ESC for PPD. Mothers could take ESC and continue breastfeeding or abstain from

breastfeeding or elect to avoid ESC altogether in favour of non-pharmacological treatment. We have completed an assessment of ESC exposure in breastfeeding infants and demonstrated that infant $AUC_{0-\infty}$ is expected to be more than 50-fold lower than adult $AUC_{0-\infty}$ following a 20 mg dose. This proof-of-concept framework could serve as a standard for improving risk assessments in the future. This interdisciplinary approach expands on analytical techniques with *in silico* modeling to predict infant drug exposure through breast milk. Using an analytical method optimized for quantification of ESC in breast milk, we were able to confidently measure ESC concentrations in the milk of 18 women taking 2.5-30 mg daily. With this data, we used PBPK modeling to simulate infant plasma concentrations following exposure to ESC via breast milk. Based on the infant $AUC_{0-\infty}$ values generated, our study demonstrates that ESC use during breastfeeding should be considered safe and that the benefits of pharmacological PPD therapy can outweighs the risk of adverse events in infants.

7.2.7 ESC Study Strengths and Limitations

The findings from this project helped bolster the existing literature on milk exposure and RID of ESC, but also serves as a proof-of-concept platform for assessing exposure of infant exposure of other drugs through breast milk. We were the first to translate drug monitoring data of ESC in milk into infant plasma level predictions while overcoming the need for invasive infant blood sampling.

The infant PBPK model is limited by uncertainty. While the method for extrapolating from the adult model was systematic and carefully performed, no infant plasma data was available in the literature to validate the model; therefore, the reliability of the validated knowledge base built into PK-SIM was considered sufficient for this approach. Furthermore, the model gives estimates for plasma concentrations, which are only surrogate markers of pharmacokinetics and pharmacodynamics in the brain. Despite the fact that this is the largest cohort to measure ESC in milk to date, the small sample size of women available for breast milk measurements may be a limitation. Nevertheless, the breast milk measurements at the 20 mg dose were utilized in the simulation because they are linearly consistent with the measurements at the 10 mg dose. In addition, by utilizing the milk concentration from the patients receiving 20 mg/day in our simulation, we were able to assess the infant plasma $AUC_{0-\infty}$ from the highest recommended

dose, which captures milk levels from women receiving lower doses. This assumes that most infants would be exposed to no more than 1.7% of the adult $AUC_{0-\infty}$.

Another limitation our simulation did not consider is intrauterine exposure to ESC. Using antidepressant during pregnancy is also a complex issue requiring an individualized approach to treatment, with many women discontinuing their medication to minimize the risk of neonatal withdrawal symptoms following fetal exposure. While infants who were exposed to SSRIs *in utero* have plasma concentrations nearly 50% of maternal concentrations, our model predictions suggest infant plasma concentrations would likely gradually decrease after birth, even if breastfeeding is initiated, due to lower exposure levels through milk and increased clearance capacity after one week of life.

7.3 Infliximab

Infliximab is a chimeric IgG1 monoclonal antibody that specifically binds and neutralizes the activity of soluble and membrane-bound TNFα[188]. IFX was the first monoclonal antibody to be approved for the treatment of IBD, which is a disease commonly affecting women of childbearing age [175,188]. Overall, there are major inconsistencies and conflicting reports on infliximab excretion into breast milk and thus the safety of this drug during breastfeeding remains unclear. A study by Kane and Lemieux reported that only 44% of women with IBD breastfed their infants (only 29% of women with Crohn's Disease breast fed), which is well below the population mean for breastfeeding initiation. Seventy four percent of women who did breastfeed their infants discontinued their medication prior to breastfeeding, mostly because of fear of medication interactions or because of physician recommendation. This lack of clarity with the detection methods, coupled with the inconsistencies in the reports may contribute to the overall low rate of breastfeeding in women with IBD[5].

7.3.1 Optimizing IFX Measurement in Milk

Common analytical methods used to measure infliximab are immunoassays optimized for quantification serum. There is a large amount of variability in the literature surrounding

excretion of IFX into milk. Three reports attempted to measure IFX in milk and reported that the drug levels were undetectable in milk or infant serum [190–192]. More recently, other groups have been able to quantitate peak IFX concentrations from 100- 475 ng/mL, with serum levels as high as 1700 ng/mL in one infant [194–196].

The analytical measuring range of IFX assays used clinically is typically 100-fold higher than the expected concentration in breast milk, which could account for the variability in the literature. In a clinical setting, IFX concentrations < 1 μg/mL in serum are considered to be undetectable[197]. The groups who were able to quantitate IFX in breast milk noted using dilution as a strategy to mitigate matrix effects; however, they still neglected to optimize their method specifically for milk measurement. Here, it was a priority to optimize and validate the analytical method to generate reliable, reproducible results for IFX quantification in breast milk. The method presented in this thesis had good precision, a reproducible standard curve, and was sensitive enough to quantitate IFX in each patient sample obtained from the study.

Clinically available methods typically use plates coated with TNFα as the primary (capture) antigen to measure free IFX. Milk TNFα concentrations can be up to several thousand-fold higher in milk compared to serum, which may account for the variability in results found in the literature. Due to the enrichment of endogenous TNFα in milk, a pool of unmeasured, undetectable IFX might exist, as the antigen binding fragment may already be bound in an immune complex and unable to bind to the exogenous TNFα on the ELISA plate. This analytical challenge could lead to an underestimated IFX concentration in milk, and subsequent miscalculation infant exposure estimates.

Our immunoassay method did not use TNFα as the primary antibody, but rather a proprietary antibody (HCA212 from BioRad) with an unknown binding site on IFX. The antibody was marketed to measure free drug; thus, suggesting the antibody would bind to the Fab fragment, the same binding site as TNFα, potentially leading to similar analytical shortcomings as discussed above. However, despite fundamental differences between immunoassays and LC-MS/MS assays, a recent study found an excellent quantitative correlation (slope = 0.967; R^2 = 0.970) between an immunoassay and an LC-MS/MS method[197]. While immunoassays like the one developed for this study are believed to only measure free (unbound) IFX, an LC-MS/MS

approach allows for the measurement of total drug, regardless of it is free or bound to TNFα. This correlation suggests that the immunoassay may be analyzing total IFX, not free drug[197]. Based on these findings, it is expected that the analytical approach used here might have actually been measuring total IFX in milk. This would make the abundant concentration of TNFα irrelevant to our analysis and allowed for the generation of accurate infant exposure estimates.

7.3.2 Factors Contributing to Variability in Milk Concentrations

There is large inter- and intra-individual variability in the plasma pharmacokinetics of IFX[188]. Higher IFX clearance has been associated with higher body weight, lower serum albumin levels, and magnitude inflammation, while co-mediation with immunomodulators contributes to lower IFX clearance, leading to higher plasma concentrations[188]. Similarly, we saw large variability in the concentrations of IFX in milk within our 5 study participants and did not find a relationship between weight-adjusted dose and milk concentrations. The factors contributing to the wide inter- and intra-individual variability in plasma may also have an impact on milk levels.

The women who participated in the study were receiving doses ranging from 300-400 mg every 8 weeks. Peak and average milk concentrations were 210.3 ng/mL (range: 103.3 – 312.2 ng/mL) and 86.7 ng/mL (range: 54.7 – 106.1 ng/mL). Interestingly, the peak milk concentration was 312.2 ng/mL from a woman receiving 300 mg daily. While we did not obtain data on her serum albumin levels or magnitude of inflammation, it is reasonable to assume that because she was on a maintenance dose of IFX, it was assumed her IBD was in remission and her inflammation was under control. Her body weight was lower (56 kg); however, her weight-adjusted dose (5.4 mg/kg) was comparable compared to the mean weight-adjusted dose amongst the other patients (5.6 mg/kg). This patient was receiving the antifungal medication, nystatin, during sample collection. It has been reported that nystatin is a pro-inflammatory compound that induces IL-1β, IL-8 and TNFα production by activating toll-like receptors 1 and 2[254]. While no direct drug interactions are known between IFX and nystatin[255], perhaps co-medication with Nystatin is modulating the immune response in this patient causing altered IFX clearance and higher excretion rates into breast milk. This remains unclear due to the limited sample size of patients on this combination treatment.

One study found that concomitant use of the immunosuppressant, azathioprine (Imuran), contributed to higher serum trough concentrations compared to IFX therapy alone[256]. One of the patients in our study was receiving co-medication with azathioprine during sample collection. Her peak, trough and average were 283.5, 7.0 and 102.3 ng/mL, respectively. Her trough concentrations were lower than the mean (14 ng/mL); however, both peak and average milk concentrations were higher than the mean (210.3 and 86.7 ng/mL for peak and average, respectively). These data suggest that co-medication with either an antifungal agent or immunomodulators could contribute to higher milk concentrations, likely due to a decrease in drug clearance and an increase in serum levels.

7.3.3 Understanding Infant Exposure: What is the Risk?

The peak milk concentration seen in our study was 312.2 ng/mL, translating to an infant dose of is 0.047 mg/kg daily. Unfortunately, were not able to obtain any corresponding maternal or infant serum data. In a report by Fritzsche et al., one partially breastfed infant had an infliximab serum concentration of 1700 ng/mL at 3 weeks postpartum and 5 days following the second postpartum infusion[196]. This contradicts the previous report that demonstrated undetectable infant serum infliximab levels[192]. The concentration seen in the serum of this infant following exposure to IFX in breast milk was much lower than what was observed following placental exposure, which was 39.5 μg/mL at 6 weeks postpartum[190]. This finding is especially noteworthy because the infant was partially breastfed, suggesting that there is the potential for infliximab to be absorbed from breast milk through the infant gut and into systemic circulation[190,196]. The transport of anti-TNFα drugs across the placenta has been well-established[190,257,258]; therefore, it is recommended to discontinue treatment of these drugs during the second or third trimester of pregnancy in order to limit infant exposure[258]. It is unknown whether this mother discontinued IFX therapy; however, due to the presumed fetal risk, we believe that it is likely that she stopped treatment. Thus, the infant systemic exposure to IFX was likely via breast milk.

The potential mechanism of IFX drug transfer into milk is currently unknown, but may be similar to the FcRn-mediated pathway of IgG transport into milk[73]. Endogenous IgGs are thought to bind to FcRn basolaterally on the mammary epithelial cell and internalized and then

transported to the luminal side for release[259]. Because IFX is an IgG monoclonal antibody, it may utilize this same transport mechanism into milk. The FcRn is also expressed in a number of neonatal tissues and may be the mechanism responsible for absorption of IFX from the infant gut into systemic circulation[260]. Furthermore, paracellular transport of IgGs may be enhanced during periods of inflammation and during colostrum production, potentially leading to an increase in infant IFX exposure [73].

It has been postulated that IFX treatment during lactation should be considered safe because (1) minute concentrations are excreted into milk, and (2) due to the monoclonal antibody structure of IFX, it would likely undergo proteolytic degradation in the infant gastrointestinal tract. However, there is evidence proposing immunoglobulins are resistant to enzymatic degradation in humans, as immunologically active IgG has been recovered from ileal aspirates and feces[261]. Furthermore, another study found that human milk has the ability to inhibit proteolytic enzymes, which could contribute to differences in protein absorption in an infant[262]. Although speculative, these findings could suggest that IFX (an IgG1 monoclonal antibody) could withstand degradation and have neutralizing activity in the infant digestive tract. While it is unlikely that the concentrations infants are exposed to via milk would suppress infant immune response and development, the local effects on the infant gastrointestinal tract remain unknown.

Another consideration is the effects of IFX on endogenous TNFα levels in milk. IFX directly targets and neutralizes TNFα. It has been well-established that the cytokines present in milk play a role in shaping the immune development of the infant[83]. Recently, a study found that milk-derived TNFα-dependent chemokines play a role in hippocampal development and spatial memory[263]. Taken together, this warrants further investigation of IFX safety during lactation.

7.3.4 IFX Conclusions

We were the first to optimize an immunoassay specifically developed to measure IFX in breast milk. Our data also indicate quantifiable concentrations in milk across the first 36 days post-infusion and at trough measurement. Based on our findings, infants are exposed to a peak dose of 0.047 mg daily, which is consistent with the previous reports in the literature. Based on this low exposure, it is implied that breastfeeding should be compatible with IFX treatment. However,

other reports indicate that infants are able to achieve quantifiable serum concentrations following exposure of IFX through breast milk. Due to these findings and the uncertainty about the effects on the infant mucosa following IFX exposure, the safety of data on IFX use during breastfeeding remains unreliable.

7.3.5 IFX Study Strengths and Limitations

This study was the first to examine IFX concentrations utilizing an analytical method optimized for milk measurement. We were the first to capture IFX milk concentrations beyond 8 days post-infusion and were able to quantitate IFX levels up to 36 days post-dose. This allowed for a more comprehensive understanding of infant exposure across the patient's dosing interval. Due to the large inter- and intra-individual variability in both milk and plasma IFX levels, this study would have benefited from a larger sample size.

As with the other drugs in this thesis, we initially attempted to gain a complete view of all the potential IFX species in milk (free, bound, partially bound). Nevertheless, due to analytical limitations, we were only able to measure free IFX in milk. Despite the claim that our antibodies solely measure "free" drug, we believe that we were actually able to measure "total" drug in milk, based on the published quantitative correlation between immunoassays and LC-MS/MS measurement methods[197]. It is worth noting that we attempted to utilize an LC-MS/MS approach to compare our antibody-based method; however, the available comparative methods did not have the sensitivity required to analyze our samples.

7.4 Future Directions

For the MTX study, increasing the sample size for both human and mouse milk analysis would be beneficial. In addition, exploring the co-administration with oral riboflavin therapy in both humans and mice might help build confidence in the safety MTX during lactation. Once this is established, women suffering from a disease flare in the postpartum period will be able to take advantage of MTX as an effective remission-inducing therapy while being able to breastfeed their infant.

It has been reported that ESC plasma levels are 6-fold higher in individuals with a deficient CYP2C19 allele[249,250]. Such allelic variants in the mother could cause higher milk concentrations and subsequently lead to an increase in infant exposure. Obtaining the maternal genetic data to explore the correlation between of genotype and milk concentrations would be of benefit. Despite the fact that our simulation took population variability in CYP metabolism into account, it is important to explore whether infants that are poor CYP2C19 metabolizers could be at a higher risk of elevated systemic exposure. In addition, infant response to a given plasma concentration of a drug may differ from adults. For example, the blood-brain-barrier is not fully developed in neonates, which could contribute to higher brain exposure of ESC. These factors warrant performing studies examining longer-term cognitive and behavioural follow-up after exposure to ESC in utero and via breast milk.

For the IFX study, due to the analytical uncertainty in our IFX method, it remains unknown whether the assay was indeed measuring "free" or "total" drug. In order to explore the potential presence of various IFX species in milk, a western blot could be carried out to visualize free, bound and partially bound drug. Furthermore, exploring the influence of endogenous TNFα concentrations on IFX levels in milk could be examined. Additionally, determining the role of disease state (e.g. active IBD vs. IBD in remission vs. healthy) on TNFα and IFX concentrations would be interesting in assessing infant exposure. Lastly, a large cohort exploring infant systemic exposure to IFX is essential to determine whether infants are able to achieve noteworthy plasma concentrations of IFX through breast milk. Long-term studies on infant sensitization to IFX and influence on immune development are also warranted.

7.5 Thesis Conclusions

The data surrounding drug safety during lactation are limited. The first step in establishing infant drug exposure estimates is to develop and validate a robust analytical approach to measure drug concentrations in breast milk. This thesis emphasized the importance of understanding milk as a matrix and how it can impact drug measurement and subsequently infant exposure. By utilizing validated analytical techniques, assessment of drug safety was established by exploring human milk data, an animal lactation model, and *in silico* techniques. Hopefully, the techniques

established in this thesis could serve as tools to help with the analysis of other drugs in milk and help provide accurate predictions on infant exposure estimates.

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Appendices

Appendix A – DLAC Study Questionnaire (Version 6, January 23, 2018)

SickKids
THE HOSPITAL FOR SICK CHILDREN

Office Use Only

Participant ID # _____

Drugs in Lactation Analysis Consortium
Study Questionnaire

Please complete all questions to the best of your ability. Thank you.

1. a) **Today's Date**:_____/_____/_____
(mm) (dd) (yyyy)

	Month of Birth (**mm**)	Year of Birth (**yyyy**)
Mother		
Infant		

2. Your baby's age in weeks (Please answer this question if your baby is younger than 6 months old)

_____weeks

3. Your baby is: ☐Female ☐ Male

4. How long was your recent pregnancy (in weeks)?

5. Type of your delivery: Vaginal delivery □ Cesarean section □

6. Did you/your baby experienced any pregnancy-related problems?
Yes □
No □
If "Yes" please explain

7. What was your baby's birth weight?

8. Were you regularly taking any medication during pregnancy?
Yes □
No □
If "Yes" please list the medication(s)

9. Medication in question that you are currently taking (please circle and include dosage):

Bupropion (Wellbutrin)_____mg_____times per day

Citalopram (Celexa) _____mg_____times per day

Methotrexate (Trexall)_____mg_____times per day

Venlafaxine (Effexor) _____mg_____times per day

Ezetimibe (Ezetrol) _____mg_____times per day

Golimumab (Simponi)_____mg every_____days/weeks/months

Infliximab (Remicade)_____mg every_____days/weeks/months

Adalimumab (Humira)_____mg every_____days/weeks/months

Etanercept (Enbrel)_____mg every_____days/weeks/months

Certolizumab (Cimzia)_____mg every_____days/weeks/months

10. When did you start taking this medication? (if you are taking more than one study medication, please record the start date for each separately)

11. What is the illness or health condition for which this medication has been prescribed? (If you are taking more than one medication from the list above, please record the health condition related to each medication separately)

12. Please list all other medications you are currently taking (including supplements, homeopathic remedies, vitamins etc.) (You have the option to refuse to answer):

13. Different ethnic groups may metabolize drugs differently. For this reason, it is important for us to know your/your baby's ethnic origin. Please select the ethnic origin(s) of your biological mother and father. You can select more than one answer.

Biological Mother

□Aboriginal
□Arab/west Asian
□Black
□Chinese
□Filipino
□Japanese
□West Indian
□Refuse to answer
□Korean
□Latin American
□South Asian
□South east Asian
□Caucasian (white)
□Ashkenazi Jewish
□Other: _____

Biological Father

□Aboriginal
□Arab/west Asian
□Black
□Chinese
□Filipino
□Japanese
□West Indian
□Refuse to answer
□Korean
□Latin American
□South Asian
□South east Asian
□Caucasian (white)
□Ashkenazi Jewish
□Other: _____

Your baby's biological father

□ Aboriginal
□Arab/west Asian
□ Black
□Chinese
□Filipino
□Japanese
□West Indian
□Refuse to answer
□Korean
□Latin American
□ South Asian
□ South east Asian
□ Caucasian (white)
□Ashkenazi Jewish
□ Other: _____

14. Is your baby:

☐ Exclusively breastfed

☐ Predominantly breastfed (more than 80% of his/her nutrition)

☐ Breastfed less than 80% of his/her nutrition

15. Does your baby have a long-term illness or health condition that has been confirmed by a doctor?

☐ Yes - please explain:
☐ No
☐ Refuse to answer

16. Does your baby regularly take any prescribed medications?

☐Yes - please explain:
☐ No
☐ Refuse to answer

17. Has your baby had any health issue requiring medical attention?

☐ Yes - please explain:
☐No
☐ Refuse to answer

18. Have you been diagnosed with any <u>other</u> long-term illness or health condition (besides those mentioned in response to question 11) by a doctor?

☐Yes - please explain: _____
☐No
☐Refuse to answer

These measurements will be taken at the appointment, if you choose to visit SickKids for the study procedure. **If completing this portion at home, please fill out to the best of your abilities.**

<u>Your Measurements</u>

weight: _____☐lbs. ☐kg

height: _____cm

These measurements will be taken at the appointment, if you choose to visit SickKids for the study procedure. **If completing this portion at home, please fill out to the best of your abilities.**

<u>Your baby's Measurements</u>

weight: _____☐lbs. ☐kg

length: _____cm

18. Do **YOU** wish to provide a small blood sample as an OPTIONAL part of the DLAC Study? If YES, the maximum amount to be drawn is 5 mL (one tablespoon or 5cc)

☐ Yes
☐ No

19. Do wish to have **YOUR CHILD** provide a small blood sample as an OPTIONAL part of the DLAC Study? If YES, the maximum amount to be drawn is 5 mL (one tablespoon or 5cc)

☐ Yes
☐ No

*Please note that if you choose to have you or your child provide a small blood sample, collection will take place in the SickKids Hospital blood work department.

The study coordinator will complete this table, if you choose to donate a blood sample of you/your baby.

Blood sample	Mother	Infant
DATE	/ /	/ /
TIME	: am/pm	: am/pm

Please indicate on the table below the date and time that the breast milk sample(s) were acquired after last dose of oral medications (Bupropion, Citalopram, Venlafaxine, Ezetimibe or Methotrexate). Please use a separate table for each drug.

Drug Name_____Exact TIME of taking the drug_____am/ pm

Samples	Breast Milk Sampling Specifics		
	Date of Collection (dd/mm/yy)	Time at the **START** of milk Collection (Please circle am or pm)*	Sample Type (Please specify)
Milk Sample 1 (Right before taking medication)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk Sample 2	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk Sample 3	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk Sample 4	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk Sample 5	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**

Please collect samples number 2 to 5 at different time-points between 2 doses of your medication at your convenience. Please make sure to record the exact time of starting milk collection in the above table.
****Please collect the samples at the start of pumping or expressing milk, if you are going to pump a larger volume of milk to store. THANK YOU VERY MUCH FOR YOUR COOPERATION***

Please indicate on the table below the date and time that the breast milk sample(s) were collected after last dose of injectable medications (Golimumab, Infliximab, Adalimumab, Certolizumab, or Etanercept). Please use a separate table for each drug.

Drug Name_____Exact TIME of taking the drug_____am/ pm

Samples	Breast Milk Sampling Specifics		
	Date of Collection dd/mm/yy	Time at the **START** of Milk Collection (please circle am/pm)*	Sample Type (please circle)
Milk sample 1 (Right before injection)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 2 (the day after injection- day 1)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 3 (day 2)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 4 (day 3)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 5 (day 4)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**

Milk sample 6 (day 5)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 7 (day 6)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 8 (day 7)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 9 (day 8)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 10 (day 9)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 11 (day 10)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 12 (day 11)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 13 (day 12)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**

Milk sample 14 (day 13)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 15 (day 14)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 16 (day 16)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 17 (day 18)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 18 (day 20)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 19 (day 22)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 20 (day 24)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**
Milk sample 21 (day 26)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**

Milk sample 22 (day 28)	/ /	am/pm	◯ Pre-feed ◯ Post-feed ◯ Between feeding, less than 2 hours after the last feed** ◯ Between feeding, more than 2 hours after the last feed**

Please collect the samples at different time-points during the day at your convenience. Please make sure to record the exact time of starting milk collection in the above table.
****Please collect the samples at the start of pumping or expressing milk, if you are going to pump a larger amount of milk to store.***

THANK YOU VERY MUCH FOR YOUR COOPERATION.

Appendix B – DLAC Study Consent Form (Version: September 20, 2017)

SickKids
THE HOSPITAL FOR SICK CHILDREN
Research Ethics Board

Research Consent Form – Participant

Title of Research Project: "Drugs in Lactation" Analysis Consortium (DLAC)

Investigator(s):

Principal investigator
Shinya Ito, MD, FRCPC (Tel: 416-813-5781)
Division of Clinical Pharmacology & Toxicology, Hospital for Sick Children

Co-investigators
David Colantonio, PhD, FCACB — Division of Clinical Biochemistry, DPLM, Hospital for Sick Children
Dr. Andrea Edginton, PhD — School of Pharmacy, University of Waterloo

Research Team members
Sarah Delaney, MSc. Graduate Student
Division of Clinical Pharmacology & Toxicology, Hospital for Sick Children

Sholeh Ghayoori Pirsoltan, CCRP (Tel: 416-813-7654 ext. 205675)
Clinical Research Project Coordinator
Division of Clinical Pharmacology & Toxicology, Hospital for Sick Children

Purpose of the Research:

The health benefit of breastfeeding to both mother and infant is widely accepted. For infants, breastfeeding can reduce the risk of infections as well as other diseases, such as diabetes. About 66 to 80 % of breastfeeding women take various medications. So many infants are breastfed by mothers on medications. However, there is not enough information on drug safety during breastfeeding to find out how safe or toxic the drugs are to the babies who are exposed through breast milk. It is important to provide safety information about drug use during breastfeeding.

The purposes of this study is to: 1) determine the safety of the drugs in infants who are exposed through breast milk, based on collected breast milk and (Optionally) blood samples; and 2) to provide safety/toxicity information of various drugs exposed to infants through breastmilk.

Description of the Research:

"Drugs in Lactation" Analysis Consortium (DLAC) is a network of investigators involved in exploring drug safety during breastfeeding. You are being asked to participate in this study because you are taking one or more of the

following medications: Bupropion, Citalopram, Methotrexate, Venlafaxine, Ezetimibe, Golimumab, Adalimumab, Infliximab, Certolizumab or Etanercept.

You have shown interest in participating in DLAC upon your contact with the study coordinator (by phone or email). If you provide consent to take part in this study, you will receive the study package (including the questionnaires, instructions for sample collection, and milk containers) in mail or by a courier service, and will be able to collect samples and complete the questionnaires from home. Alternatively, you may choose to drop by SickKids to pick up the study package.

Participation in this study entails:

- Collecting milk samples: We will ask you to collect breast milk samples (about 5-10 ml or 1-2 teaspoonful each) directly before taking your medication(s), as well as several time-points after taking your medication(s). The frequency of breastmilk you are to collect is outlined in the study questionnaire.
- Filling out the study questionnaires which will take about 20 minutes of your time: The main study questionnaire includes queries about your and your baby's general information (e.g. date of birth, ethnicity, weight and height, etc.), medical history and drug history. We will also ask you to answer some questions about your baby's development on an age-specific standardized questionnaire that will be scored based on the provided answers. If the total score on one or more developmental areas is below the cutoff, you will receive a notification letter from the study investigator, along with a copy of the completed questionnaire and the information summary page, so you may choose to consult with your child's pediatrician or your family doctor, and confirm if any further assessment is required.
  You have the alternate option of filling out the questionnaires with a DLAC research team member at SickKids, if you live within travelling distance from SickKids.
- Returning the specimens: We will arrange the samples to be picked up from your address, when you complete the study procedures. The samples will be delivered to SickKids by pre-paid mail or courier service. Alternatively, you may drop-off the study package at SickKids upon completion of study procedures.
- Donating a small blood sample (one teaspoon = 5 mL) from you and/or your child: Blood samples will provide us with additional important information for our study. Blood sampling is an optional component of the study, and you have the choice to opt in or refuse to donate blood, and only take part in milk collection component. Blood sampling will be completed at the SickKids blood work department. If you are not within traveling distance from SickKids, you do not have to consider giving the optional blood sample for research purposes.

If you are within traveling distance to and from SickKids, please initial next to your preferred options.

_____ I agree to give a small blood sample (one teaspoon, 5mL) as part of this research study

_____ I **do not** agree to give a small blood sample (one teaspoon, 5mL) as part of this research study

_____ I agree to have **my child** give a small blood sample (one teaspoon, 5mL) as part of this research study

_____ I **do not** agree to have **my child** give a small blood sample (one teaspoon, 5mL) as part of this research study

All drug levels will be measured at SickKids and the data will be sent to our analysis center for further analyses. Your privacy will be protected throughout the study.

There is no potential commercial use for these samples. The milk samples will only be studied as part of this research project and will not be used for any other purposes.

Consent to be contacted for Future Research:

We would like your permission to contact you in the future regarding participation in our future research studies.

Please Note: By agreeing to be contacted in future, you are not consenting to participate in any new study, but simply giving us permission to contact you in future and gauge your interest in participation. You can withdraw this consent for future research at any time.

Please initial next to your preference.

_____ Yes, the research team can contact me in the future about future studies.

_____ No, I do not want the research team to contact me in future about future studies.

Potential Harms:

The only anticipated risks associated with participating in this study involve minor discomfort associated with milk and blood sampling. There may be slight discomfort, bruising or redness after blood draw that will usually disappear within a few days. For many participants, applying pressure with a cotton ball immediately after blood donation can help alleviate any bruising or redness.

Potential Discomforts or Inconvenience:

The initial telephone interview, conducted by our study investigators to determine your eligibility and explaining the study is expected to take about 10 minutes. Obtaining breast milk and blood samples will take about 5 minutes per each collection. You are asked to fill in the study questionnaires, which will take about 20 minutes to complete.

Potential Benefits to individual subjects:

You and your child will not benefit directly from participating in this study. However, you will be informed if any drug level is measured at a concentration that may pose a risk to your infant.

A description of this study is available on http://www.ClinicalTrials.gov and the study website. These websites will not include information that can identify you or your child. At most, the website will include a summary of the results. You can search this website at any time.

Potential Benefits to society:

We hope that results of this study will lead to safer use of drugs during lactation.

Alternatives to participation:

Participation in the research is entirely voluntary. There is no social or economic medical disadvantage if you decline to participate in this study.

Confidentiality:

We will respect your and your child's privacy. No information about you or your child will be given to anyone or be published without your permission, unless required by law. For example, the law could make us give information

about your child if he/she has been abused, if he/she has an illness that could spread to others, if he/she or someone else talks about self-harm, or if the courts order us to provide them with information.

The samples (breast milk, blood) and questionnaires will be identified with a unique study ID. Your personal information (name, address, phone number and email address) will be linked to your study ID through a password-protected masterfile, stored on a password-protected computer and kept in a locked office, with limited access only by the principal investigator and study coordinator. Members of the Research Ethics Board (REB) and Research Quality and Risk Management office (RQRM), employees of the funder or sponsor of the study or the regulator of the study may see your data to check on the study. By signing this consent form, you agree to let these people look at your (or your child's) records. We will provide you with a copy of this consent form.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. Following completion of the research study the data will be kept as long as required then destroyed as required by SickKids policy. Published study results will not reveal your or your child's identity.

Participation:

It is your choice to take part in this study (and allow your child to take part in this study); you and your child can stop participating in this study at any time. The care that you and your child receive at SickKids will not be affected in any way whether you and your child decide to take part in this study or not.

New information that we get while you are enrolled in this study may affect your decision to take part or continue in this study. If this happens, we will inform you about this new information, and we will inquire if you are still interested in being part of this study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you and/or your child took part in this study.
If you or your child become ill or are harmed because of study participation, we will treat you and your child for free. Your signing this consent form does not interfere with your (or your child's) legal rights in any way. The study staff, people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

Reimbursement:

We appreciate your support for this research study. A $20 gift card will be offered in recognition of your contribution upon completing the participation. If you choose to visit SickKids for completing the study procedures or donating your and /or your child's blood sample, the transportation or parking expenses for travelling to the hospital will be reimbursed.

Sponsorship:

This research study is funded by Canadian Institute for Health Research. The sponsors of this research study are Dr. Ito and the Hospital for Sick Children.

Conflict of Interest:
Dr. Ito and the other research team members, have no conflict of interest to declare.

Consent to Participate in a Research Study

Study Title: "Drugs in Lactation" Analysis Consortium (DLAC)

By signing this research consent form, I understand and confirm that:

1. The study has been explained to me and all of my questions have been answered.
2. I have the right not to take part in the study.
3. I can stop participating or withdraw from the study at any time without affecting the quality of care my child or my family receives at SickKids.
4. The possible harms and benefits (if any) of this study have been explained to me.
5. I have been told that my medical records will be kept private except as described to me.
6. I know that no identifying information about me will be given to anyone or be published without first asking permission, unless required by law.
7. I have been told that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional responsibilities.
8. I have been given sufficient time to read and think about the information in this consent form.
9. I know that I may ask now, or in the future, any questions I have about the study.
10. I have been told I will be given a signed and dated copy of this consent form.

I consent to participate in this study.

Printed Name of Participant	Participant signature & date

Printed Name of person who obtained consent	Role of person obtaining consent	Signature & date

If you have any questions about this study, please call Dr. Shinya Ito at 416-813-5781.

If you have any questions regarding your rights as a research participant, you may contact the Office of the Research Ethics Board at 416-813-8279 during business hours.

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Instructor name	n/a
Institution name	n/a
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