
# Maternal medication use and the risk to breastfed infants: A focus on active efflux transporters and changes in their expression during lactation.

by

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A thesis submitted to the Curtin University to fulfil the requirements for the degree of Doctor of Philosophy in the discipline of Pharmacy.

Perth, Western Australia, 2020

# Author's Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from Curtin University Human Research Ethics Committee (EC00262), Approval #HR110/2012.

Signature:

Date: 25th September 2020

# Statement of Contributors

The majority of the work presented in this thesis was completed by the author, Hilai Ahmadzai. However, other individuals require acknowledgement for their contributions to each chapter and the publications arising from this thesis.

**Chapter 2:**

This chapter was written by the author and edited by Dr Andrew Crowe, Associate Professor Lisa Tee, Emeritus Professor Bruce Sunderland and Dr Gaewyn Ellison. The online survey was designed by AHB IT Solutions Pty Ltd. Dr Richard Parsons assisted with the statistical analysis. The Australian Breastfeeding Association (ABA), Ngala and the Child and Adolescent Health Services of Western Australia assisted in participant recruitment by granting permission to advertise a call for participants.

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# Abstract

While the benefits of breastfeeding are numerous, breastmilk can function as a transfer vehicle for xenobiotics and

medicines from the mother to the breastfed infant, inadvertently exposing the nursed infant to potentially toxic substances. Nearly half of the drugs currently available on the Australian market are recommended to be used with caution or contraindicated during lactation, predominantly due to a lack of safety data. Owing to the perceived social and ethical risks associated with drug testing in pregnant and breastfeeding mothers, pharmaceutical companies recommend that their products not be used in this population and prescribing often occurs “off-label”. Determination of drug safety through independent research and clinical trials is hampered by similar ethical challenges. As a consequence, patients and healthcare professionals use lactation safety data from small observational studies or case-reports. Often such studies may lack a robust methodology, making it difficult to provide high quality and conclusive information to lactating women. Like any off-label prescribing, the infant of a mother taking these medicines should be closely monitored for adverse drug reactions (ADRs). However, infant ADRs are inherently difficult to recognise due to their often-non-specific nature. Secondly, establishing a causal relationship between maternal medication use and the appearance of infant ADRs requires trained clinicians. Clinicians often do not receive adequate training in lactation and therefore may not recognise signs of infant ADRs.

A considerable knowledge gap currently exists in lactation drug safety data. Most medicines are classified in a binary manner as either compatible or incompatible with lactation largely based on pharmacokinetic and small *in vivo* studies without long-term follow up of exposed infants. For example, recent research has shown that early life exposure to some antibiotics, usually considered compatible with breastfeeding, can have long term detrimental effects on the growth and development of the infant. Similarly, the safety of many other drugs including the commonly used central nervous system acting drugs such as antidepressants and antipsychotics in lactation is determined based on limited *in vivo* and *in vitro* studies despite a lack of long-term follow-up of exposed infants. One factor contributing to the inadequacy of classifying a drug in this binary manner is that it does not include the inter-individual

variability that exists in the composition of breastmilk which can influence drug kinetics and consequently the amount of the drug excreted in breastmilk. Furthermore, as described below, additional drug transport mechanisms such as active cellular transporters in the lactating mammary gland can also contribute to the variability and the amount of a drug excreted into breastmilk at various stages of lactation.

The aim of this thesis is to expand our current knowledge in lactation-related medicine safety data. The first hypothesis of this work is "infant ADRs are underreported due to the difficulty in recognising and establishing a causal relationship between maternal medicine intake and the infant reaction". To address this hypothesis, an online survey of breastfeeding mothers was conducted where they were asked to provide an account of any ADRs that developed in their breastfed infant that was perceived to have been associated with maternal medicine use and transferred through breastmilk. This national online survey was completed by 339 participants. The results showed that breastfeeding women take medicines frequently with 42% of the respondents using one or more medicines (average 1.42 medicines per respondent) whilst breastfeeding. Lactating women who took medicines expressed concerns for the safety of their breastfed infants and sought professional advice to address their concerns.

A total of 23 participants (6.7%) reported noticing an ADR in their breastfed infant that they attributed to the use of maternal medicines. The average age of infants at the time of the perceived ADR was 25.6 days (95% CI; 4 – 85 days), which aligned with evidence that infant ADRs mostly occur under 2 months of age. Over half of the reported ADRs (n=16) had a positive Naranjo score linking the maternal drug as a possible or probable cause of the infant ADRs. Antibiotics and opioids were identified as the most common Adverse Drug Reaction (ADR) causing drugs. Only ten participants reported the ADR to their clinician. This survey highlighted the high incidence of medicine use in breastfeeding women and the occurrence of ADRs in breastfed infants. It also highlighted that despite mothers reporting these perceived reactions to their clinician, they were unlikely to be further reported to regulatory authorities such as the Australian Therapeutics Goods Administration (TGA), which can contribute to the perceived notion that breastfeeding related ADRs are uncommon. In line with current literature, this study confirmed that antibiotics and

some centrally acting drugs (such as antidepressants and antipsychotics) which are associated with a negative impact on the neurobehavioral development of children are commonly used by breastfeeding women (6.2% and 8.5% respectively), further strengthening the need for long term follow up of children exposed to maternal medicines via breastmilk.

In order to highlight the problems associated with the current binary classification of drugs as “compatible” or otherwise with breastfeeding, and the systemic deficiencies associated with the identification and reporting of ADRs in breastfed infants, the case of an opioid related breastfeeding infant ADR is reported. This case not only demonstrated a lack of clinician awareness of breastfeeding related ADRs but also how they may be managed in clinical practice, and the impact of such ADRs on parents and healthcare resources.

As mentioned earlier, drug safety in breastfeeding is largely determined by predictive physiologically based pharmacokinetic modelling (PBPK) and *in vitro* studies. This approach takes into account the physical and chemical properties of the drug and simulates its behaviour in a compartmentalised model of plasma and milk, providing a prediction of the amount of the drug in breastmilk compared to plasma; also called milk to plasma ratio. Milk to plasma ratio is a fundamental consideration in the determination of safety of a drug in lactation. However, there have been many instances where the actual measured breastmilk drug levels have been higher than predicted (e.g., nitrofurantoin, amisulpride, cimetidine) using this methodology. Inter-individual variability and active efflux transporters embedded on the epithelial cell membranes of the mammary gland are thought to be the cause of this discrepancy. Evidently, the expression of active transporters varies between the resting and the lactating states of the mammary gland. In lactation, these active transporters concentrate nutrients and vitamins in breastmilk, increasing nutritional value of breastmilk. Unfortunately, they can also facilitate the extrusion of drugs and xenobiotics into breastmilk which can place the suckling infant at risk of toxicity. Although efflux transporters have been implicated in the transport of drugs to breastmilk, the exact nature of their role remains unexplored. Animal studies have shown that active transporters such as the Breast Cancer Resistance Protein (BCRP) that is overexpressed during gestation and lactation both in animals and humans, play

a key role in the concentration of various veterinary drugs in the milk of dairy animals. Additionally, expression of transporters has been shown to be lactation-stage dependent indicating that the amount of a drug substrate that these transporters excrete in milk fluctuates. However, no such studies have been conducted in humans. Therefore, the current labelling of drugs identifying them as 'compatible with lactation' or otherwise is inadequate especially when the drug of concern is a substrate of a mammary gland active transporter.

In order to investigate the role of active transporters as a significant transport mechanism for drugs into breastmilk, a study was conducted where breastmilk was used as a non-invasive tool to gain access to the cellular changes that occur in the functional mammary gland as lactation progresses. This novel longitudinal study investigated the expression of active efflux transporters in the lactating mammary gland and how these changes might affect the passage of actively transported drugs into breastmilk. Milk samples (n=88) from healthy lactating participants (n=22) were collected at different timepoints, starting at 1-month post-partum and then at 3, 5, 9 and 12 months post-partum. Taqman gene technologies, iTRAQ proteomics and immunochemistry, were used to characterise and analyse breastmilk. Changes in the gene and protein expression of 4 active transporters namely BCRP, Multi Drug Resistance 1 (MDR1), Multi Resistance Protein (MRP1) and Multi Resistance Associated Protein (MRP2) were measured. The expression of BCRP during all stages of lactation was found to be significantly higher than the other three transporters and peaked at the mid-way point of 5 months. Proteomic analysis (iTRAQ) of the same samples did not detect the presence of active transporters (potentially due to the relatively high presence of many other proteins in milk overshadowing less abundant transporter proteins such as BCRP) but showed a number of other differentially expressed proteins in the milk samples over time. It is proposed that since BCRP showed highest activity around five months post-partum, this time period could potentially represent the highest risk for ADRs in breastfed infants, particularly for drugs that are substrates of BCRP. However, the degree of inter-individual variability (potentially affected by maternal genetic and epigenetic factors) was significant. This necessitates a personalised approach to medicine safety in breastfeeding. Further studies to establish a functional role of BCRP in drug transport are needed.

Considering the inter-individual variability of BCRP expression, the use of breastmilk derived epithelial cells obtained directly from breastfeeding mothers as a potential personalised model for drug transport studies was evaluated. Culturing primary human mammary epithelial cells (HMEC) has been limited due to their unpredictable *in vitro* culture behaviour. Primary HMEC have characteristically low growth rate and poor viability in culture medium. In this study, a commercially available HMEC culture medium, HuMEC Ready Medium® (Life Technologies) designed for mammary epithelial cell lines was modified and used for breastmilk derived primary cells. With this medium the time for the cells to reach confluence was reduced from 35 days to approximately 22 days (range 14-26 days). The use of this medium promoted the growth of cells from all stages of breastfeeding ranging from 14 weeks post-partum to 78 weeks post-partum, reducing the variability that was previously thought to have been caused by inter-individual differences. Using RT-PCR Taqman gene technologies, RNA expression of active transporters from cultured cells were compared with their primary counterparts taken from freshly expressed breastmilk. Cultured cells exhibited a significantly lower expression of BCRP compared to primary uncultured epithelial cells, confirming that the characteristics of breastmilk derived epithelial cells change when propagated in culture medium. Therefore, breastmilk derived cultured cells are not suitable as a drug transport model and perhaps the use of cultured primary cells to study drug transport mechanisms may not be appropriate.

The studies described herein add to the available body of data, particularly highlighting the role of active transporters such as BCRP by way of a supplementary route for the passage of drugs and xenobiotics from mother to baby through breastmilk. The need for better lactation related safety data and pharmacovigilance is also highlighted since this study demonstrated that the reports of infant ADRs attributed to breastfeeding is an underrepresentation and the actual numbers are likely to be much higher. With government policy aiming to promote breastfeeding, the issue of medication safety in lactation is likely to intensify, increasing the importance of having quality lactation safety data and appropriate pharmacovigilance.

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# Publications

**Publications arising from this work**

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Ahmadzai, H. and Tee, L. and Crowe, A. 2014. Pharmacological role of efflux transporters: Clinical implications for medicine use during breastfeeding. World Journal of Pharmacology. 3 (4): pp. 153-161.

The role of active efflux transporters in the lactating mammary gland. (Manuscript in preparation). Hilai Ahmadzai, Lisa Tee and Andrew Crowe.

Perceived infant adverse drug reactions due to maternal medication use: A population-based study (Manuscript in preparation). Hilai Ahmadzai, Lisa Tee and Andrew Crowe.

Optimising the growth of breastmilk derived mammary epithelial cells using modified culture medium. (Manuscript in preparation). Hilai Ahmadzai, Lisa Tee and Andrew Crowe.

**Presentations arising from this work**

Ahmadzai H, Crowe AP, Tee L. The perceived impact of medicines, foods and substances taken by the mother on their breastfed baby. In World Congress of Basic and Clinical Pharmacology conference. Kyoto, Japan, 1-6 July 2018.

Ahmadzai H, Crowe AP, Tee L. A longitudinal study investigating changes in the expression of efflux transporters in lactating mammary epithelial cells and their impact on transfer of drugs from mother to baby via milk. In World Congress of Basic and Clinical Pharmacology conference. Kyoto, Japan, 1-6 July 2018.

Ahmadzai H, Crowe AP, Tee L. The perceived impact of medicines, foods and substances taken by the mother on their breastfed baby. Oral presentation. In 43rd SHPA Medicines Management conference 2017. Sydney, Australia, 16-19 December 2017.

Ahmadzai H, Crowe AP, Tee L. The role of efflux transporters in the transfer of drugs from mother to breastfed infant via breastmilk. In Joint Australasian Society of Clinical and Experimental Pharmacologist and Toxicologist and Australasian Pharmaceutical Sciences Association 2017 conference. Brisbane, Australia, 5-8 December 2017.

Ahmadzai H, Crowe AP, Tee L. The perceived impact of medicines, foods and substances taken by the mother on their breastfed baby. In Joint Australasian Society of Clinical and Experimental Pharmacologist and Toxicologist and Australasian Pharmaceutical Sciences Association 2017 conference. Brisbane, Australia, 5-8 December 2017.

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# Glossary of Abbreviations

| | |
|---|---|
| ***ABA*** | Australian Breastfeeding Association |
| ***ABC*** | ATPase Binding Cassette |
| ***ABCG2*** | ATPase Binding Cassette G2 |
| ***ADE*** | Adverse Drug Events |
| ***ADME*** | Absorption, Distribution, Metabolism, Excretion |
| ***ADP*** | Adenosine Diphosphate |
| ***ADR*** | Adverse Drug Reaction |
| ***ADRs*** | Adverse Drug Reactions |
| ***ALB*** | Serum Albumin |
| ***ANOVA*** | Analysis Of Variance |
| ***ANXA2*** | Annexin A2 |
| ***ANXA5*** | Annexin A5 |
| ***ATP*** | Adenosine Triphosphate |
| ***BBB*** | Blood Brain Barrier |
| ***BCRP*** | Breast Cancer Resistance Protein |
| ***cDNA*** | Complementary DNA |
| ***CK-14*** | Cytokeratin 14 |
| ***CK-18*** | Cytokeratin 18 |
| ***CK-19*** | Cytokeratin 19 |
| ***cMOAT*** | Canalicular Multi-Specific Organic Anion Transporter |
| ***CNDP2*** | Cytosolic Non-Specific Dipeptidase |
| ***$CO_2$*** | Carbon Dioxide |
| ***CYP2D6*** | Cytochrome P450 2D6 |
| ***CYP450*** | Cytochrome P450 Superfamily |
| ***DDI*** | Drug-Drug Interaction |
| ***DNA*** | Deoxy Ribonucleic Acid |
| ***EGF*** | Epidermal Growth Factor |
| ***ELISA*** | Enzyme-Linked Immunosorbent Assay |
| ***EMA*** | European Medicines Agency |
| ***ENO1*** | Alpha-Enolase |
| ***eTG*** | Electronic Therapeutic Guidelines |
| ***FASN*** | Fatty Acid Synthase |

| | |
|---|---|
| ***FBS*** | Foetal Bovine Serum |
| ***FPVD*** | French Pharmacovigilance Database |
| ***GAPDH*** | Glyceraldehyde 3-Phosphate Dehydrogenase |
| ***GP2*** | UTP-Glucose-1-Phosphate Uridylyltransferase |
| ***GSN*** | Gelsolin |
| ***HBB*** | Haemoglobin Subunit Beta |
| ***HMEC*** | Human Mammary Epithelial Cells |
| ***HuMEC® Ready Medium*** | Human Mammary Epithelial Cell Ready Medium |
| ***iTRAQ*** | Isobaric Tags for Relative and Absolute Quantitation |
| ***JAK2 / STAT5*** | Janus Kinase 2/Signal Transducer and Activator of Transcription Protein 5 |
| ***LDHB*** | L-Lactate Dehydrogenase B Chain |
| ***LYZ*** | Lysozyme |
| ***MATE*** | Multidrug/Toxin Extrusions |
| ***MCF10A*** | Michigan Cancer Foundation Cell Line (Normal Mammary Epithelial Cell Line) |
| ***MCF7*** | Michigan Cancer Foundation Cell Line (Breast Cancer Epithelial Cell Line) |
| ***MDH1*** | Malate Dehydrogenase |
| ***MDR1*** | Multi-Drug Resistance Gene |
| ***MEC*** | Mammary Epithelial Cells |
| ***MedDRA*** | Medical Dictionary for Regulatory Activities |
| ***mg*** | Milligram |
| ***mL*** | Millilitre |
| ***mRNA*** | Messenger RNA |
| ***MRP1*** | Multidrug Resistance Protein 1 |
| ***MRP2*** | Multidrug Resistance Associated Protein 2 |
| ***M:P*** | Milk to Plasma ratio |
| ***NHMRC*** | National Health and Medical Research Council |
| ***OAT*** | Organic Anionic Transporters |
| ***OATP*** | Organic Anion-Transporting Polypeptides |
| ***OCT*** | Organic Cation Transporters |

| | |
|---|---|
| ***OCTN*** | Organic Cation/Carnitine Transporters |
| ***PBS*** | Phosphate Buffered Saline |
| ***PBST*** | Phosphate Buffered Saline with Triton X100 |
| ***PEP*** | Peptide Transporters |
| ***PGD*** | 6-Phosphogluconate Dehydrogenase |
| ***P-gp*** | P-Glycoprotein |
| ***PLIN3*** | Perilipin |
| ***RANZCOG*** | Royal Australian And New Zealand College of Obstetrics and Gynaecologists |
| ***RNA*** | Ribonucleic Acid |
| ***RQ*** | Relative Quotient |
| ***RT-PCR*** | Real-Time Polymerase Chain Reaction |
| ***qRT-PCR*** | Quantitative Real-Time Polymerase Chain Reaction |
| ***SCP2*** | Non-Specific Lipid-Transfer Protein |
| ***SELENBP1*** | Selenium-Binding Protein 1 |
| ***SLC*** | Solute Carrier Protein |
| ***T1*** | Timepoint 1 |
| ***T2*** | Timepoint 2 |
| ***T3*** | Timepoint 3 |
| ***T4*** | Timepoint 4 |
| ***T5*** | Timepoint 5 |
| ***TGA*** | Therapeutic Goods Administration |
| ***uL*** | Microlitre |
| ***US FDA*** | US Food and Drugs Administration |
| ***UV*** | Ultraviolet |
| ***WA*** | Western Australia |
| ***WHO*** | World Health Organisation |
| ***XDH*** | Xanthine Dehydrogenase/Oxidase |

# Chapter 1 Introduction

The World Health Organisation (WHO) and the National Health and Medical Research Council (NHMRC) recommend exclusive breastfeeding for the first six months of an infant's life and in combination with nutritious solid food thereafter (1, 2). This recommendation was introduced based on research showing numerous health benefits of breastfeeding for both the mother and the infant (3). Breastfeeding protects infants from many childhood infectious diseases such as respiratory infections, otitis media, diarrhoea, pneumonia and necrotising enterocolitis, and reduces infant mortality from sudden infant death syndrome and childhood leukaemia (4). Breastfed infants also have enhanced cognitive development (5, 6). The benefits of breastfeeding for the mother include better mental and physical health outcomes such as reduced risk of ovarian cancer, breast cancer and type 2 diabetes mellitus (7, 8). It is widely accepted that breastfeeding is the best way of ensuring a good start in an infant's life as breastmilk not only has a favourable nutrient content but also provides passive immunity and various growth hormones to the breastfed infant (9-13). Simulation modelling places the global economic benefits of breastfeeding in the hundreds of billions of dollars through reduced morbidity and mortality for both the mother and the baby, better health outcomes for the breastfed child and economic gains through better cognitive function which results from breastfeeding (14, 15).

Suboptimal breastfeeding rates are estimated to cost the global economy over $300 billion dollars. These avoidable costs should encourage government policy makers to increase effective promotion of breastfeeding and support strategies (14, 16). The Australian National Infant Feeding Survey statistics have revealed that in children aged 0-24 months in Australia in 2010, 90% were initiated on exclusive breastfeeding but only 15.4% of these were exclusively breastfed at 5 months (17). Another Australian study also confirmed suboptimal rates of exclusive breastfeeding in the early post-partum period, providing evidence of the need for a supportive government policy to promote breastfeeding (18). The Australian National Breastfeeding Strategy: 2019 and beyond (The Strategy) seeks to establish an enduring policy framework for all Australian governments to provide a supportive

and enabling environment for breastfeeding, with the ultimate aim of increasing breastfeeding rates in Australia (19).

Whilst breastfeeding has many benefits, it is also important to acknowledge that nursing mothers often require medicines in the post-partum period and during the course of lactation. Medicine use during breastfeeding carries the concerns of inadvertent exposure through medicine transfer from the maternal plasma to the breastfed infant via breastmilk. It is also important to acknowledge that maternal age is increasing, and associated with this is a likelihood of increased medicine use for both acute and chronic conditions (20). Although most drugs are considered compatible with breastfeeding, and there is little concern for the safety of the breastfed infant, cases of toxic drug exposure have been reported (21-23). More importantly, there is often a lack of clear and conclusive information about the safety of medicines in breastfeeding, leading to unnecessary discontinuation of breastfeeding or suboptimal treatment of maternal medical condition(s) with a less effective alternative (22).

Most medicines are excreted into breastmilk to some extent. However, very low levels of a drug in breastmilk is often not cause for concern, and with many drugs this amount is considered to be sub-clinical and safe for the breastfed infant. Infant medicine exposure of equivalent to 10% of the mother's dose is usually considered as the threshold for concern (13). However, the ability of the infant to eliminate a drug based on their physiologic characteristics and the pharmacokinetic/pharmacodynamics properties of the drug is also an important consideration - not just the amount of drug present in the breastmilk (24). Calculation of infant drug exposure is usually based on mathematical modelling using the drugs' pharmacokinetic parameters supported by studies involving human and animal models. Despite some drugs demonstrating low predicted transferability into breastmilk, adverse drug reactions have been reported and, in some cases, significant adverse effects that did not match with such modelled drug levels in breastmilk (21, 25). This phenomenon has been observed with drugs such as acyclovir, cimetidine and nitrofurantoin which are actively transported across the mammary epithelial membrane, thereby linking the transfer of these substances to the function of transport proteins (23, 26).

Transporters are cell surface proteins that allow endogenous molecules and xenobiotics to enter and exit cells via carrier mediated mechanisms (27). Active transporters utilise the energy generated from ATP hydrolysis to move molecules across a cell membrane (28). The expression of transport proteins such as those belonging to the ABC (ATPase Binding Cassette) and Solute Carrier (SLC) superfamilies of transporters have been shown to vary greatly between lactating and non-lactating tissues in humans (29). Furthermore, animal studies indicate that their expression is lactation stage dependent (26, 30, 31). If the expression of efflux transporters is lactation-stage dependent in humans i.e. their expression changes as lactation duration increases, then the excretion of their various substrates (which includes many commonly used medicines as well as other xenobiotics) will vary greatly between the different phases of breastfeeding. Thus, a breastfed infant may be exposed to variable and possibly toxic amounts of the drug substrate through breastmilk, potentially causing adverse effects in the breastfed infant.

The composition of breastmilk is known to vary significantly between women. This variability has been attributed to numerous factors such as maternal characteristics (e.g. age, genetics, body mass index, lifestyle, health, diet), environmental exposures (chemical exposure, pollution), stage of lactation (colostrum vs mature milk), time frames (season, circadian rhythms), and time relative to maternal exposure (32-35). Human breastmilk has a variety of nutrients and a dynamic metabolome with many bioactive components and microbiota that is constantly changing to meet the needs of the rapidly growing infant. These include vitamins, neuroactive compounds, peptides and hormones which are all thought to be transported into milk via transporter proteins (36). While these nutrients are crucial to the development of the neonate, milk may also contain some undesirable substances such as environmental pollutants, carcinogens, allergens, medicines (taken by the mother) (37).

## 1.1 Composition of breastmilk

Breastmilk is considered a complete food for infants as it contains macronutrients such as amino acids, lipids, proteins and carbohydrates, and a myriad of nutritive components such as minerals, vitamins, enzymes, immune cells, immunomodulatory factors, glycoproteins, hormones, nitrates/nitrites and nucleotides (13, 33, 38-40).

There is an increasing body of research that shows that breastmilk also contains mesenchymal stem cells and various growth factors which can be beneficial for the infant through mechanisms yet unknown (40-42). The composition of breastmilk is known to be influenced by many factors including maternal health, nutrition and environmental exposure to chemicals from air, soil food and personal care products (43-45).Other factors such as lactation period, time since last breast emptying and lactation stage also affect the composition of breastmilk (46).

## 1.2 Functional anatomy of the lactating breast

The development of the mammary gland follows a distinct cycle of growth and development starting with the formation of the mammary crest and buds at an embryonic stage (47). The mammary gland remains relatively unchanged in the early years of life until puberty when exposure to oestrogen results in its rapid differentiation (48). In addition, the female mammary gland undergoes significant changes during pregnancy (and lactation) and finally around menopause (47). Of these developmental stages, the most significant changes, from a physical as well as functional perspective occurs during pregnancy when the mammary gland undergoes complete remodelling in various stages (48, 49). The lactating breast is comprised of a lobular alveolar system which has a ductal network and secretory alveoli. Several lobules consisting of alveoli join to form a lobe (Figure. 1.1). There are approximately 15 to 20 lobes present in each mammary gland, which are connected or served by mammary ducts (parenchyma) (50). In pregnant and lactating women, the lobules are supported and separated mostly by connective tissue, instead of the mainly adipose tissue present in the non-lactating gland. As shown in Figure 1.2, the alveoli which have a secretory role are lined by epithelial cells also known as lactocytes. The alveolus is defined by a monolayer of polarized alveolar mammary epithelial cells (MEC) arranged around a lumen, where milk is secreted under the influence of various hormones such as oxytocin and prolactin (51).

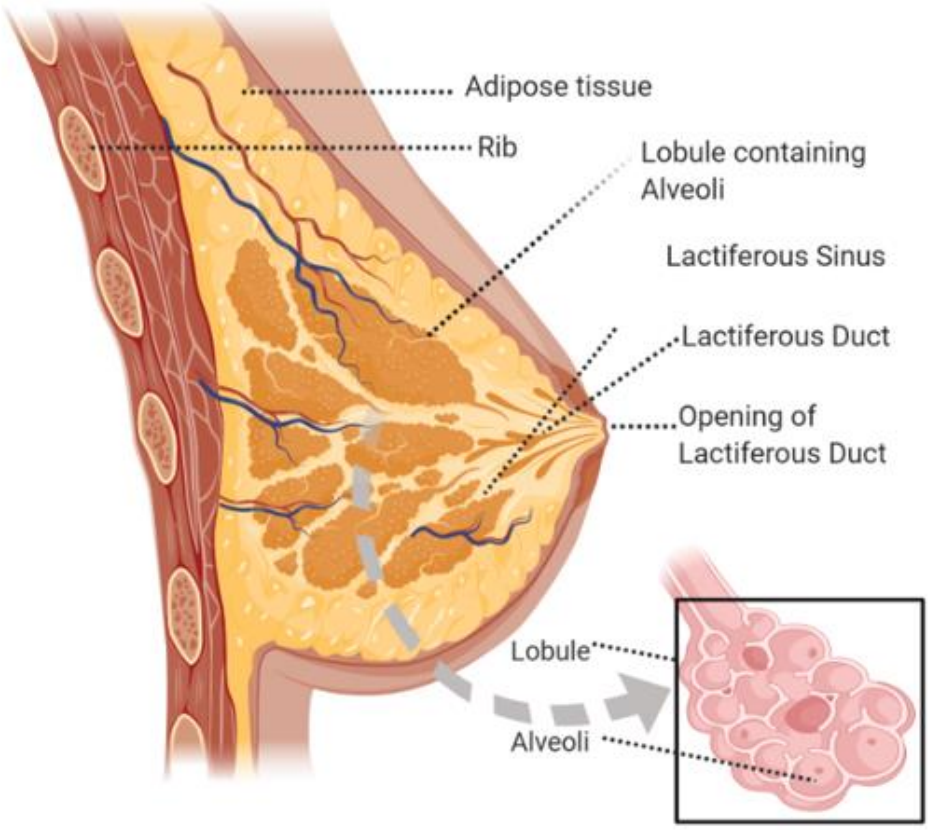



**Figure 1.1 Illustration depicting the structure of the lactating mammary gland. The lactating mammary gland is comprised of a lobular alveolar system which has a ductal network and secretory alveoli. Several lobules consisting of alveoli join to form a lobe. Each mammary gland is comprised of approximately 15 to 20 lobes supported mainly by connective and some adipose tissue. These lobes are connected by lactiferous ducts which collect the milk from the lobules within ach lobe and carry it to the nipple. (Figure created with Biorender.com)**

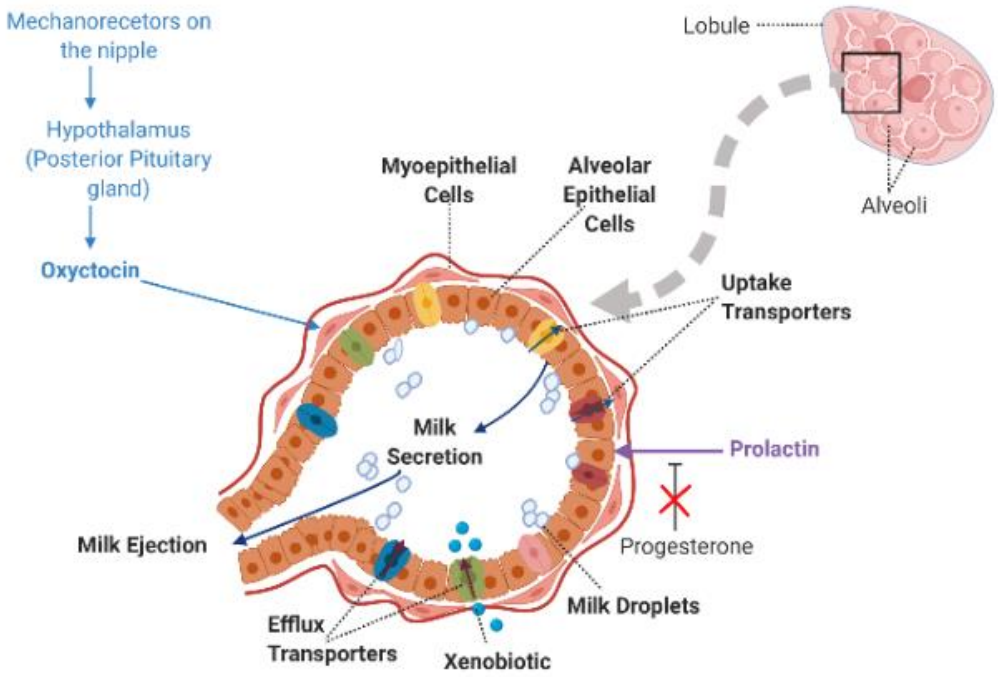



**Figure 1.2 Illustration depicting the structure of the mammary gland alveolus. The alveoli are lined by a monolayer of secretory epithelial cells arranged around a lumen. Milk is secreted by these epithelial cells under the influence of hormones such as oxytocin and prolactin. Prolactin stimulates the growth and development of the mammary tissue during pregnancy. However, pregnancy hormones such as estrogen and progesterone block the action of prolactin preventing milk from being secreted. The sudden drop in progesterone levels after delivery means that prolactin is no longer inhibited. The sucking motion from a breastfed baby activates mechanoreceptors on the nipple which stimulate the secretion of oxytocin from the posterior pituitary gland. Oxytocin makes the myoepithelial cells around the alveoli contract, which helps the milk to flow and fill the ducts. (Figure created with Biorender.com)**

## 1.3 Endocrine and autocrine control of lactation

### 1.3.1 Endocrine control

There are three main phases of lactation which include mammogenesis (mammary growth), lactogenesis (a process of initiation of milk supply) and galactopoiesis

(maintenance of milk secretion) (52). Although prolactin is the key hormone of lactation, many other hormones such as progesterone, oestrogen, growth hormone, insulin and glucocorticoids (commonly referred to as the lactogenic hormone complex) play a crucial role in the differentiation of the mammary gland into a secretory gland (53, 54).

Mammogenesis or mammary growth begins during puberty when oestrogen stimulates proliferation of ducts and causes deposition of fat while progesterone stimulates development of lobules. As shown in Figure 1.3, during pregnancy oestrogen and progesterone from the placenta stimulate the growth of the mammary gland whereby oestrogen specifically enhances the growth and branching of the ductal system and fat deposition in the stroma while progesterone enhances the growth of the lobule alveolar system which results in the budding of the alveoli and secretory changes in the epithelial cells (52, 55, 56). Although oestrogen and progesterone are essential for the development of the breasts, they inhibit secretion of milk (53). Other hormones such as human growth hormone, glucocorticoids, human placental lactogens and insulin also have important roles in the development of the mammary gland during pregnancy (52).

Lactogenesis is a process whereby cellular changes in the mammary gland allow its transformation from non-secretory to secretory tissue and cause the initiation of milk supply without increasing tissue mass (57). Lactogenesis can be subdivided into two stages, namely, initiation of lactation (lactogenesis I) and activation of lactation (lactogenesis II) (13, 53, 57). Lactogenesis commences mid pregnancy and is characterised by the expression of many genes which are involved in the synthesis of milk components (57). The alveolar epithelial cells undergo cellular and enzymatic differentiation and there is an increase in the expression of uptake transporters such as those required for amino acids, glucose and calcium, which are all essential for milk synthesis. During pregnancy, the endocrine regulation of lactogenesis is controlled by progesterone, which promotes mammary growth and blocks epithelial secretion. Prolactin, one of the key lactation hormones, is released from the anterior pituitary gland (58). Prolactin steadily rises up to 10-20 times its baseline level from the fifth week of pregnancy until birth (59). Prolactin stimulates mammary gland ductal growth and proliferation of the alveolar epithelial cells which induce milk protein synthesis. As demonstrated in Figure 1.3, a sudden change in the levels of

oestrogen and, more importantly, progesterone after delivery “activates” milk production. After delivery, reduction of progesterone and high levels of prolactin results in an increase in the expression of milk protein genes (52). The mammary gland also absorbs large quantities of metabolic substrates from the blood. At this stage the cytoplasmic lipid droplets and casein also move into the alveolar lumen. Immunoglobulins from the mother’s plasma are also transferred into milk. The initial secretion, colostrum, which is very rich in immunoglobulins is followed by milk. Suckling of the breastfed infant increases prolactin levels which in turn further increase the expression of genes involved in milk secretion and expansion of the alveolar epithelium. Lactation is maintained by the removal of milk from the breast (60).

Galactopoiesis is the maintenance of lactation once it has been established, usually occurring at around 9—15 days postpartum (61). During the postpartum period, hormones such as prolactin cause the milk to be secreted into the alveoli by the alveolar epithelial cells (62). Other hormones such as growth hormone (which supports an increase in the synthesis of lactose, protein and fat in the mammary gland), glucocorticoids, the thyroid and parathyroid hormones, and insulin also play an important role in the maintenance of lactation (63). Tactile stimulation by the suckling infant causes oxytocin to be released from the posterior pituitary gland which then acts upon lactocytes that surround the alveoli to contract and expel milk through the ducts into the mouth of the suckling infant (63, 64).

### 1.3.2 Autocrine control

There are many other local factors that influence the breast (65). Mammary epithelial cells are locally controlled via negative feedback mechanisms through pressure and stretching in addition to inhibition from bioactive factors, thereby regulating milk secretion within the alveoli (65). The volume of milk produced is not only governed by the concentrations of maternal hormones, but the efficiency of milk removal also has a key role (66). A protein factor called feedback inhibitor of lactation (FIL) is secreted with other milk components into the alveolar lumen. The FIL is insensitive to prolactin and therefore can result in a reduction in the amount of milk produced. If the breast remains full of milk, FIL inhibits milk production and thus milk secretion (67).

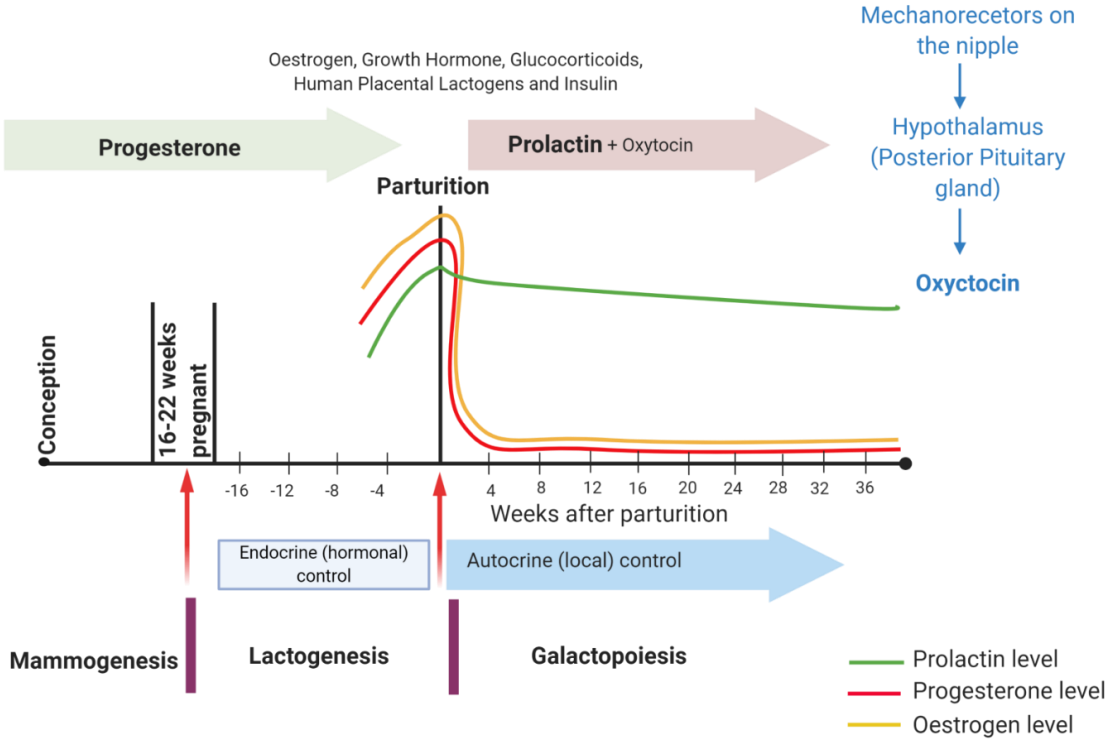



**Figure 1.3 Development of the mammary gland under hormonal influence during pregnancy and post-partum. During pregnancy oestrogen and progesterone from the placenta stimulate the growth of the mammary gland. After delivery, reduction of progesterone and high levels of prolactin facilitates milk production. (Figure created with Biorender.com)**

## 1.4 Passage of molecules into breastmilk

The mammary gland undergoes many changes during pregnancy and lactation. As Anderson (68) explains, the mammary epithelial cells in the alveolus form a semipermeable membrane which separates milk from plasma (Figure 1.4). In the early post-partum period (3-4 days) wide spaces exist between these epithelial cells which facilitates the passage of large molecules such as maternal immunoglobulins into breastmilk. After approximately a week, these pores begin to close off, only allowing smaller molecules (molecular weight < 200 Daltons) to pass through this membrane down their concentration gradient via passive diffusion. Large molecules such as heparin and warfarin are unable to pass through these junctions. However, mastitis can compromise the integrity of this membrane and transiently allow the passage of small amounts of large molecules into the breastmilk.

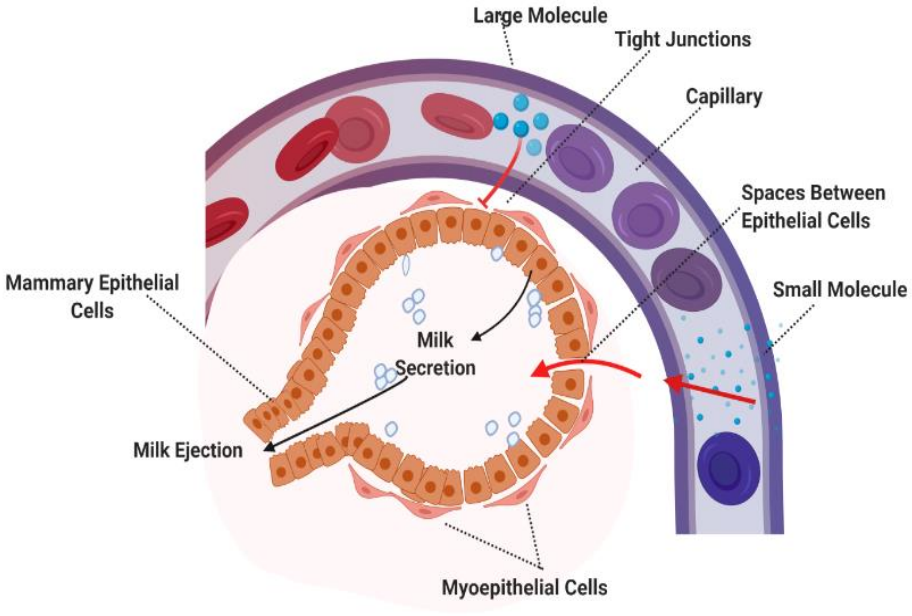



**Figure 1.4 Passage of molecules from mother's circulation into breastmilk. The alveolar mammary epithelial cells separates milk from plasma by forming a semi permeable membrane. Wide spaces between the epithelial cells in the early post-partum period facilitates the passage of large molecules into the breastmilk. As theses pores begin to close off, only smaller molecules are allowed to pass through these pores via passive diffusion. (Figure created with Biorender.com)**

## 1.5 Mechanisms of drug transfer into breastmilk

### 1.5.1 Passive diffusion

Most drugs transfer from maternal plasma to breastmilk primarily via passive mechanisms (69, 70). The critical determinants of passive transfer include drug protein binding, drug ionisation and fat partitioning (12, 69). These factors can be used to predict milk to plasma ratio (M: P) where passive diffusion is thought to predominate. Other pharmacokinetic parameters such as half-life of the drug, water and lipid solubility, route of drug administration, bioavailability, dissociation constant, volume of distribution, molecular size and ionisation potential can further help to determine the transfer of drugs from the mother’s plasma into breastmilk (71). Drugs with the shortest half-life, highest protein binding and lowest lipid

solubility usually have the lowest ductal milk transport (13). The drug dose received by an infant during breastfeeding is a function of the amount excreted into the breastmilk, the daily volume of the milk ingested and the average plasma concentration of the mother. Thus, M:P ratio has large inter-subject variability (72).

### 1.5.2 Active transport mechanisms

Although the transfer of most drugs into breastmilk can be explained by passive diffusion, a review of the literature shows that there are several drugs where the actual measured M:P ratio is significantly greater than predicted (23, 73-75). Lead, amisulpride, nitrofurantoin, acyclovir and cimetidine are some such toxicants/drugs which exhibited a significantly higher observed M:P ratio than predicted (65, 73, 75-79). In one study nitrofurantoin had an observed M:P ratio of 6 as opposed to the predicted 0.28 (74). Although the exact mechanism for this disparity between the actual and predicted M:P ratios is not known, it is attributed to active transport mechanisms whereby transport proteins such as ABC-Binding Cassette transporters on the membrane of mammary epithelial cells actively pump these drugs into milk, utilising ATP in the process as demonstrated in Figure. 1.5.

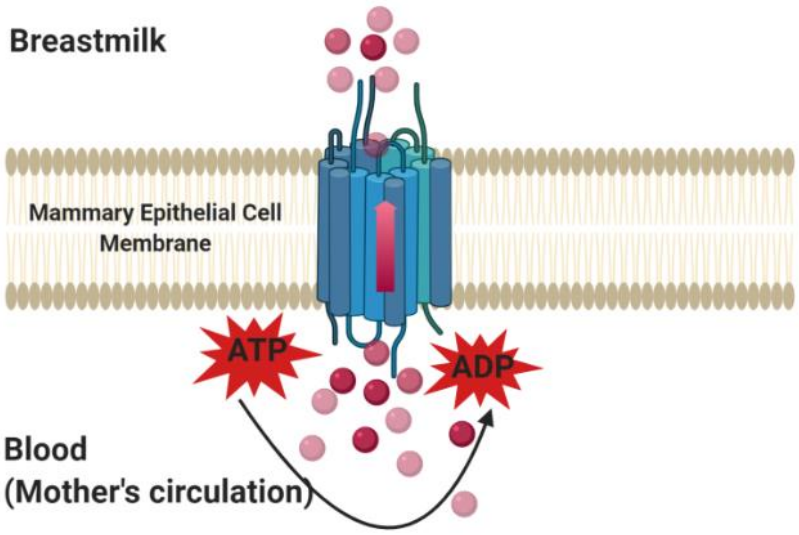



**Figure 1.5 Transport mechanism of active ABC transporter proteins in lactating mammary gland. ABC transporter proteins couple the transport of a diverse range of their substrates across the mammary epithelial cellular membrane to the hydrolysis of ATP. (Figure created with Biorender.com)**

While ABC transporters utilise direct hydrolysis of ATP for the transmembrane transportation of substances against their concentration gradient, other transporters such as the Solute Carrier (SLC) use alternative sources of energy such as ion gradients for this purpose (80). Both SLC and ATPase Binding Cassette ABC transporters are known to be expressed in the lactating mammary epithelial cells, playing a role in the transfer of various crucial nutrients, xenobiotics and drugs into the breastmilk (29, 81, 82). ABCB1 (MDR1/P-gp), ABCG2 (BCRP) and ABCC2 (MRP2) are some of the important efflux transporters expressed primarily in the liver, the blood brain barrier (BBB), the gut and in mammary glands. These transporters have been implicated in drug interactions and drug resistance.

Efflux transporters usually serve a protective role in the body as they reduce the body's exposure to xenobiotics by facilitating the removal of their various substrates from the body such as in the liver and kidney. They protect sensitive sites such as the brain (blood-brain barrier), foetus (blood-placental barrier) and testes (blood-testes barrier) by limiting access. Active transporters are also responsible for drug resistance in cancer treatment. They limit the access of chemotherapeutic drugs to the cancer, where they are usually overexpressed (83). As depicted in Figure 1.6, in the mammary gland the transporters that are located in the apical membrane of the alveolar epithelial cells function as an avenue to reduce maternal exposure similarly to the liver and the kidney (77). However, in doing so they can contaminate the breastmilk and expose the breastfed infant to potentially toxic substances.

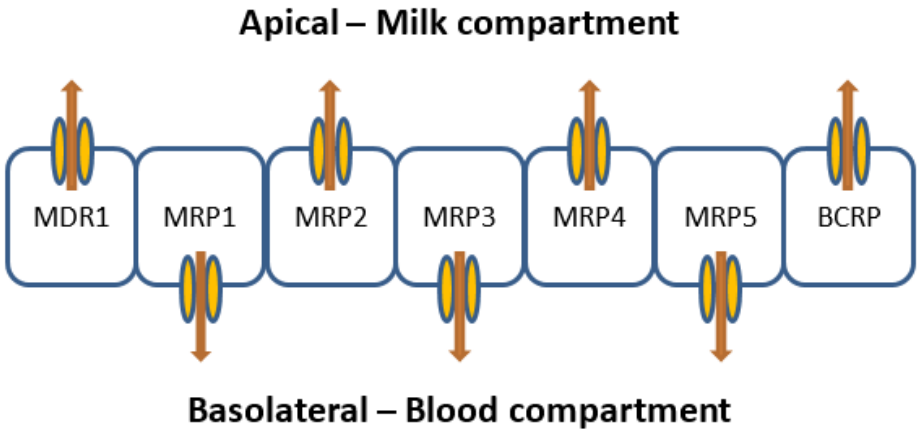



**Figure 1.6 Schematic representation of expected localisation of drug efflux transporters in the human mammary epithelial cells. MDR1, MRP2, MRP4 and BCRP are located in the apical membrane of the mammary epithelial cells and are therefore thought to contribute to the composition of breastmilk by pumping their substrates into breastmilk.**

Alcorn and colleagues (29) demonstrated that RNA expression of various transporters in the mammary epithelial cells (MEC) of the lactating breast varies quite significantly from the non-lactating gland, indicating a graded expression change during induction of the lactation process. This can result in significant changes in substrate transport during lactation. In the mammary gland, ABC efflux transporters have been implicated in the disposition of the quinolone group of drugs, thereby increasing their secretion into breastmilk (74, 75). Breast Cancer Resistance Protein (BCRP), another ABC transporter is strongly induced during pregnancy and lactation, and is thought to have an important role in regulating the composition of breastmilk (84). An overexpression of this transporter could result in contamination of milk with the substrates of this transporter, potentially placing the suckling infant at risk of toxicity. However, the extent of involvement of these ABC transporters in the transfer of nutrients and drugs into breastmilk has been only relatively recently reported and is still not fully understood (85). It has also been shown in murine models that the expression of these transporters is stage-dependent and changes as lactation progresses (30). This finding is supported by studies which show that the excretion of actively transported drugs such as cefepime changes significantly as

lactation progresses (86). However, no longitudinal human studies have been undertaken to date that confirm this finding.

## 1.6 Safety of drugs in breastfeeding

Drug safety authorities such as the US Food and Drug Administration (FDA), the Therapeutic Goods Administration (TGA) and the European Medicines Agency (EMA) determine the safety of medicines in lactation as "compatible" or "non-compatible", based mainly on pharmacokinetic studies considering the drug's physical and chemical properties, and its interaction with breastmilk, and they recommend PBPK modelling studies for New Drug Entities (NDE) (87). When infant exposure is expected to be low, usually accepted to be around 10% of the maternal dose, a drug is deemed safe or compatible in breastfeeding. However, over half of the drugs available on the market currently lack conclusive information relating to safety in breastfeeding (further discussed in chapter 2 of this thesis). The majority of drugs are therefore not recommended for use during breastfeeding, primarily due to a lack of conclusive and definitive evidence. Consequently, prescribing of such drugs occur "off-label" due to a lack of data.

Some drugs such as opioids are deemed "compatible" with breastfeeding, yet they have been implicated in serious adverse drug reactions and placed breastfed infants at risk of toxicity and even death (21). Another such group of drugs is antibiotics, where short-term adverse effects such as diarrhoea, vomiting and rashes are well-known and contribute greatly to the recorded ADR reports. However, recent studies show that adverse effects of antibiotics in children exposed in infancy continues beyond the exposure period and may result in significant neurobehavioural adverse outcomes later in life (88, 89). Similarly, antidepressants, antipsychotics and other central nervous system acting drugs lack the long-term follow-up necessary to determine absolute safety in breastfeeding (90, 91). This highlights the need not only for better medicine safety data in lactation but also a more robust pharmacovigilance system where clinicians are adequately trained in identification, management and reporting of breastfeeding related infant ADRs and toxicity.

The ultimate objective of this thesis is to enhance the quality of medication safety data in breastfeeding to support lactating women in making informed decisions about their usage of medicines during breastfeeding. In particular, this study examines the role of active efflux transporters in the lactating mammary gland and how they change during the course of lactation. This study will have implications for use of substrates of these transporters during lactation.

The first objective of this study is to ascertain the occurrence, reporting rate and nature of infant ADRs attributed to maternal medication use. This data will be collected via an online survey where a self-reported account of lactating women with respect to infant ADR will be evaluated. This study also aims to document an account of the impact of the perceived infant ADRs on their breastfeeding experience.

The second objective of this study is to investigate the expression profile of four efflux transporters namely MDR1, MRP2, BCRP and MRP1 in the human mammary gland during different lactation stages, and to ascertain if these could impact the transfer of xenobiotics, drugs and toxins that are substrates of these transporters into breastmilk.

The third and final objective of this study is to develop a personalised, non-invasive, reliable and cost-effective model to test drug safety of actively transported drugs using breastmilk. This will be achieved by optimising the growth conditions for human breastmilk derived mammary epithelial cells (HMEC) to create a reliable model.

# Chapter 2 Survey

**A cross-sectional population-based study of breastfeeding mothers on the appearance of perceived adverse reactions in their breastfed infant attributed to maternal medicine use.**

## 2.1 Background

Breastfeeding women frequently take medicines for common post-partum conditions such as caesarean wound pain, urinary incontinence, constipation, haemorrhoids, coughs/colds/ minor illnesses, backache, infections, mastitis, stimulation of lactation, depression and other neuropsychiatric illnesses (92, 93). The extent of medicine use in post-partum women varies significantly according to reports, ranging from 34% to 100%. This is largely due to a lack of uniformity in medicine use reporting systems, and differences in study designs (93-95). Some studies suggest that women are more likely to take medicines when breastfeeding compared to while pregnant (95). While most medicines are considered safe and compatible with breastfeeding, ADRs do occur in breastfed babies that are attributed to transfer via breastmilk. The nature and scope of ADRs in infants and children are not predictable based on post market surveillance reports that rely heavily on adult drug experience. Globally, breastfeeding related ADRs are infrequently reported and are thought to be uncommon (21, 96). However, there is an inherent difficulty in establishing causal relationships between a clinical presentation in an infant and their exposure to medicines or xenobiotics via breastmilk, particularly when the signs and symptoms are not obvious. Due to their nonspecific nature, subtle signs and symptoms such as crying, discomfort, sleep disturbance, poor feeding and irritability in an infant can be easily associated with other potential causes e.g. viral infections. In cases where signs are more noticeable such as skin rashes, vomiting or diarrhoea, infant drug exposure via breastmilk may be suspected but not always investigated (as described in Chapter 3 of this thesis) as health professionals may not always have the lactation training to consider breastfeeding as a potential cause. The lack of training and education of health care professionals in breastfeeding related matters has been highlighted in numerous studies (97-103).

The greatest potential risk to a baby from exposure to medicines via breastmilk is drug toxicity and undetermined long-term effects on neurobehavioural development. The infant risk from exposure to medicines via breastmilk can range from mild adverse reactions to death, with the majority of cases likely to be mild and transient. While a rash, gastrointestinal upset or increased crying may not cause any real long-term harm to the baby, it can result in unnecessary anxiety and worry for parents and potentially significant economic burden on the health system if further investigations are unnecessarily carried out. Counselling parents on the symptoms of a possible ADR may help relieve anxiety but the potential long term effects of drugs used for chronic conditions such as antidepressants, antipsychotics and anti-epileptics necessitates better pharmacovigilance, especially since this does not currently exist (90). The Australian Institute of Health and Welfare (AIHW) report that maternal age has risen over the years and this coincides with a rise in the incidence of chronic diseases such as diabetes, hypertension and neuropsychiatric conditions among new mothers, with 13% of pregnant women suffering from gestational diabetes. (104).

Studies show that breastmilk related ADRs are worst in the first two months of life when an infant's metabolic and excretory organs are underdeveloped, but can occur at any time during breastfeeding (21, 25, 105). Usually an infant exposure of 10% of the maternal dosage is considered to be an acceptable and safe level (13). However, the mere presence of a drug in an infant's serum may not be a cause for concern nor does an undetectable drug level necessarily endorse safety. Breastfeeding "compatibility" may be too broad a term to include all the necessary caveats that must be considered before drugs are prescribed to breastfeeding mothers. Therefore, it is important to have an appropriate pharmacovigilance system whereby breastfeeding related ADRs are reported, investigated and long term follow up is facilitated where necessary. Given that the composition of breastmilk changes over time and is influenced by many maternal and environmental factors, a far more nuanced approach to recommendations around the use of drugs while breastfeeding may be safer and more appropriate.

The clinical and economic burden of adverse drug reactions (in the general population) is well known and is estimated to cost the public health system greatly, both as a direct consequence and indirectly by increasing morbidity and contributing to mortality (106). However, the impact of breastfeeding related ADRs has not been

previously determined, largely due to the low number of breastfeeding related ADRs reported.

## 2.2 Clinical implications of medicine use in breastfeeding

Not all drugs are excreted in clinically significant amounts into human milk, and the mere presence of a drug in human milk may not pose a risk for the infant. To weigh the risks and benefits of breastfeeding, physicians need to consider multiple factors which include the mother's clinical need for the drug, the potential effects of the drug on milk production, the amount of the drug excreted into breastmilk, the extent of oral absorption and potential adverse effects on the breastfeeding infant. The age of the infant is also an important factor in the decision-making process, because adverse events associated with drug exposure via breastmilk occur most often in neonates younger than 2 months and rarely in infants older than 6 months which is confirmed by our own review (96, 107). Developmental changes in physiology and consequently, in pharmacology influence the efficacy, toxicity and dosing regimen of medicines (108).

## 2.3 Lactation related medicines information

Most drugs are excreted in breastmilk and may therefore be ingested by breastfed infants. Generally, data regarding the safety of drugs taken by breastfeeding women is sparse, and little is known about breastmilk transfer for many drugs (22, 92). Concerned breastfeeding mothers who take medicines often resort to their primary care providers such as their midwife, community pharmacist or general practitioner to obtain information on the safety of their prescribed medicine. It is crucial for the primary care providers to have access to conclusive, reliable and easily accessible information. A survey of Australian community pharmacists showed that they commonly used references such as the drug company product information, the Australian Medicines Handbook and the Royal Women's Pregnancy and Breastfeeding Medicine Guide to obtain lactation related medicine safety information (109).

In a bid to assist clinicians with better prescribing guidance, the FDA mandates that pharmaceutical companies are to report lactation safety data in their product information but for a great majority of currently available drugs this crucial data are lacking and unspecified (87). Similarly, the evidence base for the use of complementary medicines during lactation is largely lacking despite their widespread use (110). Product information (PI) and consumer medicine information (CMI) rarely provide conclusive lactation safety data, most commonly citing "no lactation data available" (22, 92). Table 2.1 lists some examples of lactation safety data taken from MIMS Online which demonstrate the conflicting and rather inconclusive nature of published lactation safety data for some commonly used medicines during lactation (111-117).

**Table 2.1 eMIMS listed product information on lactation safety for selected drugs.**

| Drug | Product Information on use of drug in lactation |
|---|---|
| Cephalexin | Cephalexin is excreted in the milk. Caution should be exercised when cephalexin is administered to a nursing woman. Alternative feeding arrangements for the infant should be considered. |
| Amoxicilin/Clavulanic Acid | Amoxicillin is excreted in the milk; there are no data on the excretion of clavulanic acid in human milk. Therefore, caution should be exercised when amoxicillin/clavulanic acid is given to a breastfeeding woman. |
| Ceftriaxone | Low concentrations of ceftriaxone are excreted in human milk. Caution should be exercised when ceftriaxone is administered to a breastfeeding woman. |
| Sertraline | Only limited data concerning sertraline levels in breastmilk are available. However, in breastfed infants whose mothers were taking sertraline there have been reports of adverse effects. Because sertraline is excreted in human milk, breastfeeding while on sertraline is not recommended. If sertraline is used during lactation, the physician should be aware that withdrawal reactions have been reported in some neonates whose mothers had been on SSRI antidepressants, including sertraline. |
| Olanzapine | In a study in lactating, healthy women olanzapine was excreted in breastmilk. Mean infant exposure (mg/kg) at steady state was estimated to be 1.8% of the maternal olanzapine dose (mg/kg). Patients should be advised not to breastfeed if they are taking olanzapine. |
| Dexchlorpheniramine | This medicine is excreted in breastmilk. Therefore, caution should be exercised when administered to nursing mothers. |
| Levocabastine (Zyrtec® eye drops and nasal spray) | Based on determinations of levocabastine concentrations in saliva and breastmilk in a nursing woman, who received a single oral dose of 0.5 mg levocabastine, it is expected that approximately 0.6% of the total intranasally and approximately 0.3% of the total ophthalmically administered dose of levocabastine may be transferred to a nursing infant. However, due to the limited nature of the clinical and experimental data, it is recommended that Zyrtec® nasal spray or eye drops be avoided in breastfeeding mothers. |

The perceived ethical and financial risks associated with testing of drugs in pregnant and breastfeeding mothers is a disincentive to pharmaceutical companies from conducting drug safety tests in this population. Consequently this data is unavailable for most drugs and pharmaceutical companies recommend their products not be used in this population (118). Similarly, independent research in this cohort is hampered

by strict ethical codes, making it difficult for public health researchers to conduct large scale independent studies and clinical trials. Therefore, when lactating mothers require medicines, clinicians are forced to prescribe medicines “off-label” without the usual guidance or evidence base used in prescribing for other populations (119).

While several resources such as the electronic Therapeutic Guidelines (eTG) and Lactmed website/database are also readily accessible by healthcare professionals, there is a concerning lack of quality information in these references, forcing clinicians to resort to small observational studies or case-studies to obtain their lactation safety data. While case-reports are valuable, they often lack robust methodology and study design, which when added to the inter-individual differences seen with breastmilk composition, makes it even more difficult to provide high quality evidence-based information to women. For some drugs, safety information is limited to data from animal studies which may not correlate well with human physiology (120). Although rare, case reports of toxic drug exposure resulting in adverse effects in the breastfed infant may encourage overly cautious healthcare professionals to recommend discontinuation of breastfeeding or prescribe a less effective alternative drug for fear of potential medico-legal consequences, resulting in suboptimal health outcomes for the mother and/or deprivation of breastfeeding benefits for the baby (100).

In order to evaluate the accessibility of reliable and readily accessible lactation medicines information other than the PI, we explored the eTG 2017 (electronic Therapeutic Guidelines), considered one of the gold standard electronic references in clinical practice (121). The eTG classifies lactation safety of drugs into three broad categories namely “compatible”, “caution” and “avoid”. As shown in Figure 2.1, 46% from a total of 775 drugs are deemed compatible with breastfeeding but for the majority (54%) the recommendation is to exercise caution or avoid use due to lack of data (i.e. err on the side of caution) (121). According to the eTG, many drugs such as antibiotics are deemed compatible with breastfeeding despite antibiotics being implicated in the majority of infant ADRs (21).

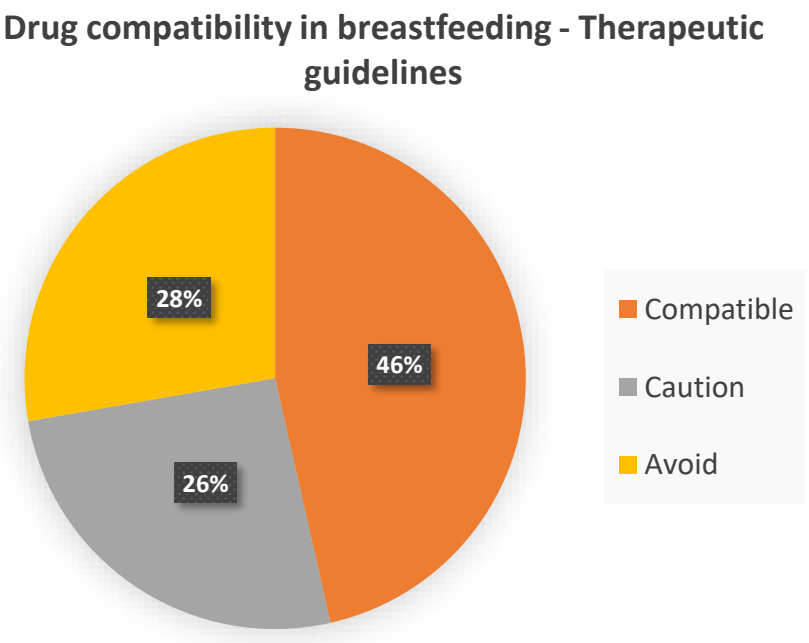



**Figure 2.1 Drug compatibility in breastfeeding as per the Australian electronic Therapeutic Guidelines (eTG). From a total of 775 drugs analysed, the eTG deemed 46% of the drugs compatible with lactation while advising caution or avoidance for the remaining 54% predominantly due to a lack of lactation safety data.**

## 2.4 Reporting of breastfeeding related ADRs

Although infant adverse reactions from drugs in breastmilk are possible, these reactions seem to be notoriously underreported (21, 25, 96). However, whether these reports reflect the true occurrence of infant ADRs remain undetermined as identification of infant ADRs may be inherently difficult and consequently may not be reported. According to a review conducted by Anderson and colleagues, serious adverse drug effects appear to be uncommon in breastfed infants (25). Some drugs such as those affecting the central nervous system are found to be causing more infant ADRs than others (21, 94). Any drug that is designed to cross the BBB is likely to also have good transport through other tissues including breast tissue and subsequently be accessible to the breastfed infant (122).

### 2.4.1 Reporting of breastfeeding related ADRs in France

In France, reporting of ADRs is mandatory and every health practitioner is required to report 'serious' or 'unexpected' ADRs to their Regional Pharmacovigilance Centre (31 centres in France) (21). On the other hand, in Australia, the reporting of ADRs including those that may result from passage of the drug through breastmilk is a voluntary process which relies on healthcare professionals and members of the public to report to the TGA. 'Serious' adverse drug reactions are defined as "any untoward medical occurrence that, at any dose results in death, requires hospital admission or leads to prolongation of hospitalisation for patients already in hospital, results in persistent or significant disability/incapacity, is life threatening, results in cancer, congenital abnormalities or birth defects or any medical event that would be regarded as serious if it had not responded to acute treatment" (21, 120). For each ADR report in the France Pharmacovigilance Database (FPVD), information about the patient (age, sex, medical history), drug exposure (to the suspected drug and other associated non-suspected drugs) and ADR characteristics ('serious' or 'non-serious', 'expected' or 'unexpected', causality score, outcome) are recorded. A detailed summary of the clinical description of the patient is added to the end of each pharmacovigilance case report. The route of administration, including breastfeeding, is specified. In the FPVD, ADRs are encoded as per the Medical Dictionary for Regulatory Activities (MedDRA) classification.

According to the FPVD data from 1985 to 2011, there were only 174 reports (average of 6.7 reports per year) of ADRs in breastfed children, of which 37% (n=64) were considered to be serious ADRs. The most frequently reported ADRs were those concerning the central nervous system (behavioural problems, sedation and insomnia) followed by digestive problems (diarrhoea, vomiting) (21). Resolution of the ADRs was reported in almost eighty percent of the cases with the outcome unknown in the remaining 20%.

### 2.4.2 Reporting of breastfeeding related ADRs to the Therapeutic Goods Administration (TGA) in Australia

In Australia, ADR reporting (in general and in lactation) is a voluntary process and relies on consumers, pharmaceutical companies and healthcare professionals to report any untoward reactions to the TGA, the authority responsible for regulating medicines, medicinal substances and devices. Whilst reporting is mandatory for sponsors of drugs, patients experiencing ADRs or their healthcare professionals are strongly encouraged to do so. This process involves the completion of a report as outlined in the TGA website (123). The TGA website states *"Each adverse event report the TGA receives is entered into a database, which is continually analysed by TGA staff to identify potential emerging problems for detailed investigation. If the TGA identifies a safety concern relating to a medicine or vaccine, they take regulatory action that may involve further investigations, product recall or in extreme cases suspending or cancelling products" (123).*

The TGA reporting has become electronic in the last decade or so, prior to which reporting occurred through the completion of a form which was mailed out to healthcare professionals such as general practitioners and pharmacists. Although the system has become more accessible, it is still a voluntary practice and the reports made to the TGA may be an underestimation of the actual ADR cases. ADRs in breastfed infants due to potential drug transfer in breastmilk is expected to be even lower due to the previously discussed issues.

In order to gain a better understanding of the Australian data on breastfeeding related ADR reporting, the TGA was contacted in May 2016 to obtain reports of ADRs in breastfed infants. The TGA provided the requested information under the Freedom of Information Act and did not require an ethics application. The TGA employ MedDRA terminology in their reports hence it was identified that "exposure during breastfeeding" would be the key search term for the requested reports. Other key words such as breastfeeding, lactation, human milk, nursing, infant, breastmilk, breastmilk, human milk could not be used to conduct additional searches due to not being MedDRA terms. The TGA provided two documents containing the Case Line

Listing Report (providing a basic account of each adverse event) and a Public Case Detail Report (providing further information on each report including a description of the adverse event). The data included ADRs from 2003 until 2016. There were a total of 65 cases that met the requested search criteria i.e. “exposure to breastfeeding”. Of these 65 reports, it was found that 5 reports were duplicated. Hence, the final number of reports analysed was 60 during the thirteen year period from 2003 until 2016 (Appendix C).

Each Public Case Detail Report was further analysed and documented in an excel spreadsheet recording all the important aspects of the report which included the following:

- The year the data was reported/entered
- Source that reported the ADRs
- Offending drug
- Description of the reaction
- Outcome of the case
- Impact of this ADR on breastfeeding or pharmacotherapy
- And whether the ADR was considered subjectively “major” or “minor”

Analysis of case reports provided by the TGA found that the data recorded by the TGA lacked consistency, uniformity and vital information. The infant age at the time of reporting, a crucial factor, was not stated in the majority of the reports and it was difficult to confidently know the reporter/source in most cases. According to the studied reports, medicine use by mother was discontinued or breastfeeding was ceased in the majority of cases. Currently, no long-term follow up of these cases is conducted (as confirmed with TGA) to enable assessment of long-term effects of the ADRs on the infant. Central nervous system acting drugs such as antidepressants and antipsychotics, and antimicrobial agents were implicated in the majority of the cases as demonstrated in Figure 2.2. However, the reliability of these cases is questionable at best. What is clear is that regardless of whether a country has mandatory ADR

reporting like France or voluntary reporting like Australia, it appears that overall the numbers of reported breastfeeding related ADRs are low.

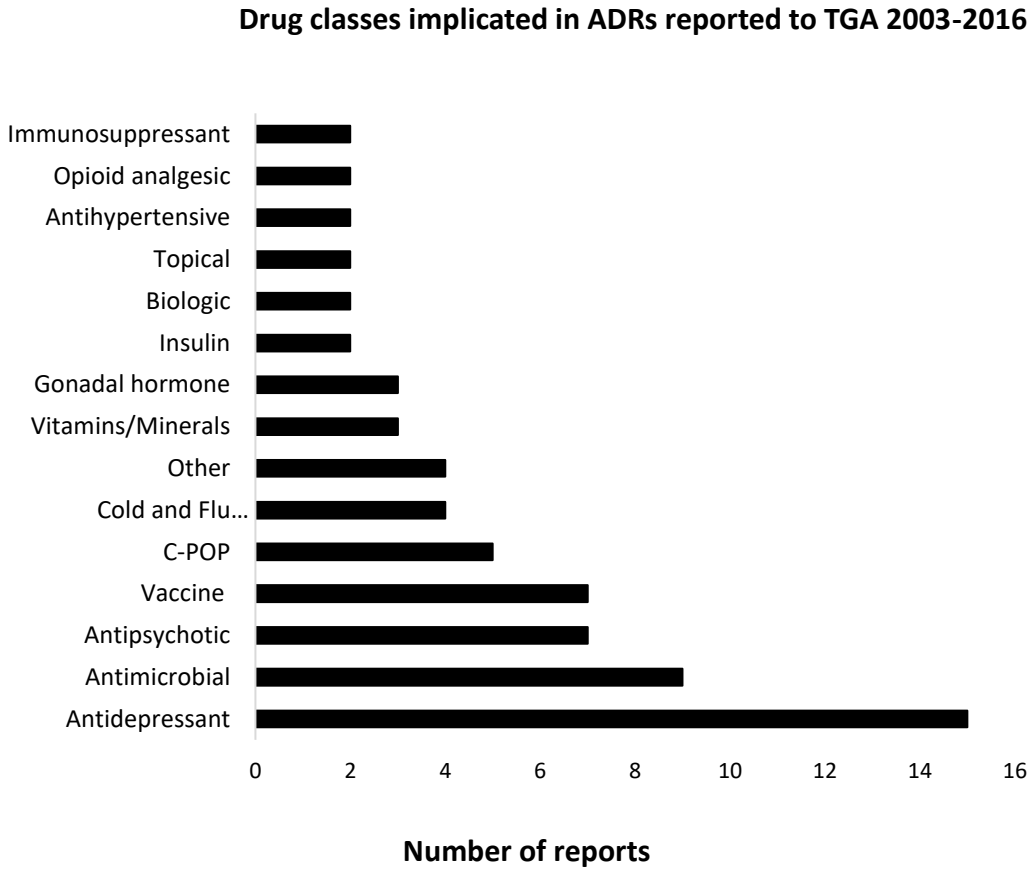



**Figure 2.2 Drug classes implicated in breastfeeding related ADRs reported to the TGA between 2003 and 2016. Antidepressant, antibiotic and antipsychotic medications were found to be the three top drug classes implicated in TGA Adverse Drug Reaction reports comprising almost half of all the ADRs reported during this period.**

## 2.5 Barriers to reporting of breastfeeding related ADRs

One possible explanation for the low reporting of breastfeeding related ADRs could be that the types of ADRs experienced by breastfed infants have not been well

defined. Secondly, little is known about the passage of drugs from plasma to breastmilk for almost half the drugs currently on the market. Pharmacokinetic and follow-up data for breastfed children are even rarer due to complexities of therapeutic drug monitoring, inconvenience, ethics and the cost involved. Thirdly, there is an inherent difficulty in establishing a causal relationship between the appearance of a suspected ADR and maternal medicine intake, particularly when pharmacokinetic data is limited and infant signs and symptoms are non-specific. Finally, healthcare professionals are undertrained in lactation and management of lactation related issues which may affect confidence and the ability to recognise and report infant ADRs (99, 101).

## 2.6 Aims of this study

The TGA data lacks crucial information, making it difficult to draw accurate inferences about the occurrence of breastfeeding related ADRs in Australia. The aforementioned potential barriers to the reporting of ADRs further reduces the reliability of such data relating to the hypothesis that breastfeeding related ADRs are greater than reported. It was therefore deemed necessary to conduct research into the occurrence of breastfeeding related ADRs as perceived by the mothers of breastfed infants as these mothers had first-hand experience of any untoward reaction in their baby regardless of whether it was reported or not. This study aimed to ascertain the occurrence, reporting rate and nature of ADRs experienced by breastfed infants whose mothers took medicines during lactation. This study also aimed to document an account of the impact of the perceived infant ADRs on their breastfeeding experience.

## 2.7 Survey design and methodology

### 2.7.1 Sample size calculation

*A priori* sample size estimation was based on an average of 300, 000 births per year in Australia (124). With a margin of error of ± 5% (95% CI), the sample size needed for an adequately powered study was determined to be 385 participants.

### 2.7.2 Survey questions

This study was conducted using a self-administered online questionnaire validated through several pilot studies. The survey was presented on a Microsoft Azure supported website, fully secured with a username and password (www.breastfeedingresearch.com.au). Participants were required to be 18 years or older, breastfeeding or had breastfed in the past 12 months. Participants were recruited from various community and child health centers through the display of posters at West Australian Child health clinics and Ngala, and advertising on the Australian Breastfeeding Association (ABA) website. The survey was online and anonymous. Participants were asked to provide an email address to ensure the integrity of data by preventing duplications. Duplicate responses identified through email address and incomplete responses where section 1 was not done finished were excluded from the analysis of results. The online survey was comprised of 42 questions. Refer to Appendix C for screenshots from the website and a copy of the questionnaire.

The survey was comprised of six sections, with each section represented as a page on the website. Each section was designed to obtain an in-depth overview of the mother's health and perceptions regarding breastfeeding. The following were the page titles:

Section 1: General details (6 questions)

Section 2: Mother's general health and wellbeing (4 questions)

Section 3: Medicine use during breastfeeding (10 questions)

Section 4: Perceived reactions to food products (4 questions)

Section 5: Adverse drug reaction probability (14 questions)

Section 6: Impact on breastfeeding (4 questions)

The survey was piloted and validated 5 times (n=25) on breastfeeding and non-breastfeeding women to ensure that there were no issues with the content of the survey, the web design and the interpretation of the Naranjo questions. After each pilot, the questions were revised and issues identified were rectified before the amended questions were re-piloted.

Section 4 (Perceived reactions to food products) was not directly related to the aims of this study. However, it was included as food products are anecdotally associated with perceptions of untoward reactions in breastfed babies resulting in avoidance of certain foods by lactating mothers. Section 5 of the survey titled "adverse drug reaction probability" was based on the Naranjo Algorithm (125, 126). This algorithm assesses the likelihood of the adverse reaction being due to the drug as opposed to other factors. The Naranjo Algorithm aims to assess whether there is a causal relationship between an identified untoward clinical event and a suspected medicine (drug) by employing critical causation variables (125). A Naranjo total score of 1-4 indicates a possible causal relationship between the suspected drug and the ADRs, a total score of 5-8 indicates a probable causal relationship and a total score greater than 9 indicates a definite causal relationship between the drug and the ADRs. The Naranjo algorithm is a reliable and validated tool in terms of content as well as ease of use (126). The Naranjo algorithm has been used successfully in evaluating the probability of suspected ADRs with a high likelihood in daily clinical practice (126, 127). While the Naranjo algorithm is a reliable way of establishing causal relationships between an ADR and a suspected drug, it is aimed for use by healthcare professionals. In order to make the questions more understandable and applicable to our target audience (survey participants), questions were rephrased, simplified and the order rearranged. In order to maintain the flow of the questions while keeping the focus and the content unaltered, an additional four questions were added. The additional questions were not scored. Table 2.2 contains the original and the corresponding modified Naranjo questionnaire (126).

**Table 2.2 Adverse Drug Reaction probability scoring (Naranjo) questions**

| **Naranjo Questions** | **Modified Questions** | **Scoring** |
|---|---|---|
| Q1: Did the Adverse reaction/ event appear after the suspected medicine was administered? | Q1: Did you notice any untoward or adverse reaction in your baby after you breastfed him/her after you took your medicine? | *Yes (+2) No (-1) Do not know (0)* |
| Q2: Are you aware of any conclusive reports of reactions of this nature? | Q2: Are you aware of any similar reactions being reported in other babies? | *Yes (+1) No (0) Do not know (0)* |
| N/A | Q3: How did you resolve this problem? | Not scored |

| **Naranjo Questions** | **Modified Questions**<br>• Stopped breastfeeding<br>• Stopped medicine<br>• Changed the timing of medicine<br>• Other | **Scoring** |
|---|---|---|
| Q3: Did the adverse event improve when the suspected medicine was stopped or a counter drug given? | Q4: Did your baby's symptoms improve when you did the above? | *Yes (+1) No (0) Do not know or not done (0)* |
| Q4: Did the adverse drug reaction reappear when the suspected medicine was administered? | Q5: Did you try to expose your baby to the suspected medicine again by giving them more breastmilk while you were taking this drug? | YES(go to Q6)<br>NO (go to Q7)<br>NO (0)<br>*(If the answer is NO, a score of zero will be allocated as it will be same as NOT DONE. The survey will then go to Q7)* |
| N/A | Q6: (If above is YES) Did the problem reappear when the baby was re-exposed as you breastfed again? | *Yes (+2) No (-1) Do not know or not done (0)* |
| Q5: Are there any alternative causes that could on their own have caused this reaction? | Q7: Could there be anything else that you think may have caused this reaction in your baby?? | *Yes (-1) No (+2) Do not know or not done (0)* |
| Q6: Did the reaction reappear when a placebo was given? | Q8: Did you try an alternative non-medicated substance or a placebo where your baby reacted the same way as before? | *Yes (-1) No (+1) Do not know or not done (0)* |
| Q7: Was the drug detected in blood or other fluids in concentrations known to be toxic? | Q9: Were there any medical tests e.g. blood tests or breastmilk tests done? | YES (go to Q10)<br>NO (go to Q11)<br>DON'T KNOW(go to Q10)<br>NOT DONE (go to Q11)<br>*(Q10 is a scored follow-up question)* |
| N/A | Q10: Were you made aware of any concerns regarding the blood test, for example high levels of the drug in the baby's blood or your milk sample | *Yes (+1) No (0) Do not know or not done (0)* |
| Q8: Was the reaction more severe when the dose was increased or less severe | Q11: Did you trial changing the dose of your medicine? | YES (go to Q12)<br>NO(go to Q13)<br>NOT DONE (go to Q13) |

| **Naranjo Questions** when the dose was decreased? | **Modified Questions** | **Scoring** *(Q12 is a scored follow-up question)* |
|---|---|---|
| N/A | Q12: (If YES) Was the reaction more severe when the dose was increased or less severe when the dose was decreased? | *Yes, More severe when dose increased Yes (+1)*<br>*Yes, less severe when dose decreased Yes (+1)*<br>*No change at all No (0)*<br>*Not done not done (0)* |
| Q9: Did the patient have a similar reaction to the same or similar drugs in any previous exposure? | Q13: Are you aware of your baby reacting to another drug in a similar way at a different time? | *Yes (+1) No (0) Do not know or not done (0)* |
| Q10: Was the adverse event confirmed by any objective evidence? | Q14: Were there any conclusive tests or diagnosis made by the treating medical professional that confirmed this adverse reaction in your baby was due to transfer of the drug in your breastmilk? | *Yes (+1) No (0) Do not know or not done (0)* |

### 2.7.3 Ethics

This project had ethics approval from Curtin University Human Research Ethics Committee (HR2012/110). The Child and Adolescent Health Services of WA also provided ethics clearance through the office of research and governance. The Australian Breastfeeding Association and Ngala had their organization specific ethics clearance avenues that were also obtained prior to advertising through these platforms. Ethics approval letters can be found in Appendix D.

### 2.7.4 Recruitment of participants

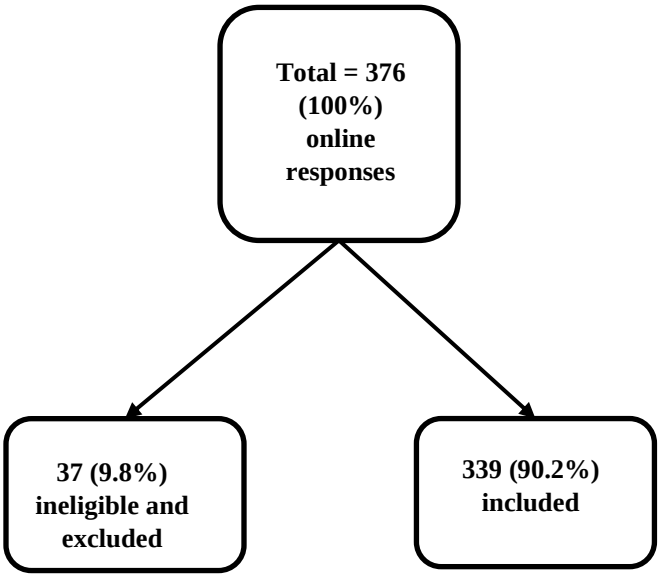
Participants were recruited by advertising on the Australian Breastfeeding Association (ABA) website, through word of mouth and via poster displays at the Ngala Association office in Perth, child health centres in Western Australia seeking participation by women currently breastfeeding or having breastfed in the last 12 months. The ABA and Ngala were selected as these organisations are frequented by breastfeeding women. The ABA is a national organisation that promotes breastfeeding and provides many useful breastfeeding related resources through their website. Ngala is a West Australian organisation that delivers services such as parenting support, breastfeeding support and childcare centres for Western

Australian families. Therefore, the ABA and Ngala were deemed as appropriate and targeted advertising platforms for this survey. West Australian metropolitan and regional Child Health Centres were also utilised for this purpose as these centres provide care and support to infants in early life including post-birth appointments, baby weighing facilities, breastfeeding and general infant advice, and childhood immunisations starting at 2 months of age. The use of maternity hospital as a recruitment platform was considered. However, it was not pursued due to ethical challenges. As an incentive, participants were offered a chance to enter a draw for one of five $50 ColesMyer® gift vouchers upon completion of the survey.

## 2.8 Results

### 2.8.1 Demographic and general details of the participants

This online survey was made available for seven months from November 2016 to June 2017 and was completed by a total of 376 participants. Of these, 37 surveys were deemed ineligible for inclusion due to duplication and/or incompleteness. A total of 339 responses (95% CI; ± 5.3% margin of error) were included in the analysis of this study.




As demonstrated in Table 2.3, the majority (83.8%) of the 339 participants (95% CI) were from the state of Western Australia. Participants who were breastfeeding at the time of the survey formed 78.2% of the respondents. The average breastfeeding duration was 45 weeks (range 0.86-312 weeks; SD±42 weeks). The education level of the respondents was generally high with 62% stating that they had at least an

undergraduate degree and over a quarter of total respondents having a postgraduate degree.

Table 2.3 Demographic data of the survey participants (n=339)

| | |
|---|---|
| **Breastfeeding status** | |
| Not breastfeeding at the time of survey | 74 (22%) |
| Time since breastfeeding stopped | 4.25 months (range 0.3-14) |
| Mean breastfeeding duration | 61.4 weeks (range 4.29-312.86) |
| **Education Level** | |
| Secondary school | 37 (10.3%) |
| Graduate diploma/TAFE/college | 78 (21.7%) |
| Undergraduate degree | 131 (36.4%) |
| Postgraduate degree | 92 (25.6%) |
| Undisclosed | 22 (6.1%) |
| **State of residence** | |
| WA | 284 (83.8%) |
| NSW | 49 (14.5%) |
| ACT | 1 (0.3%) |
| VIC | 1 (0.3%) |
| Not Specified | 25 (7.4%) |

### 2.8.2 Mother's general health and medicine use during pregnancy and lactation

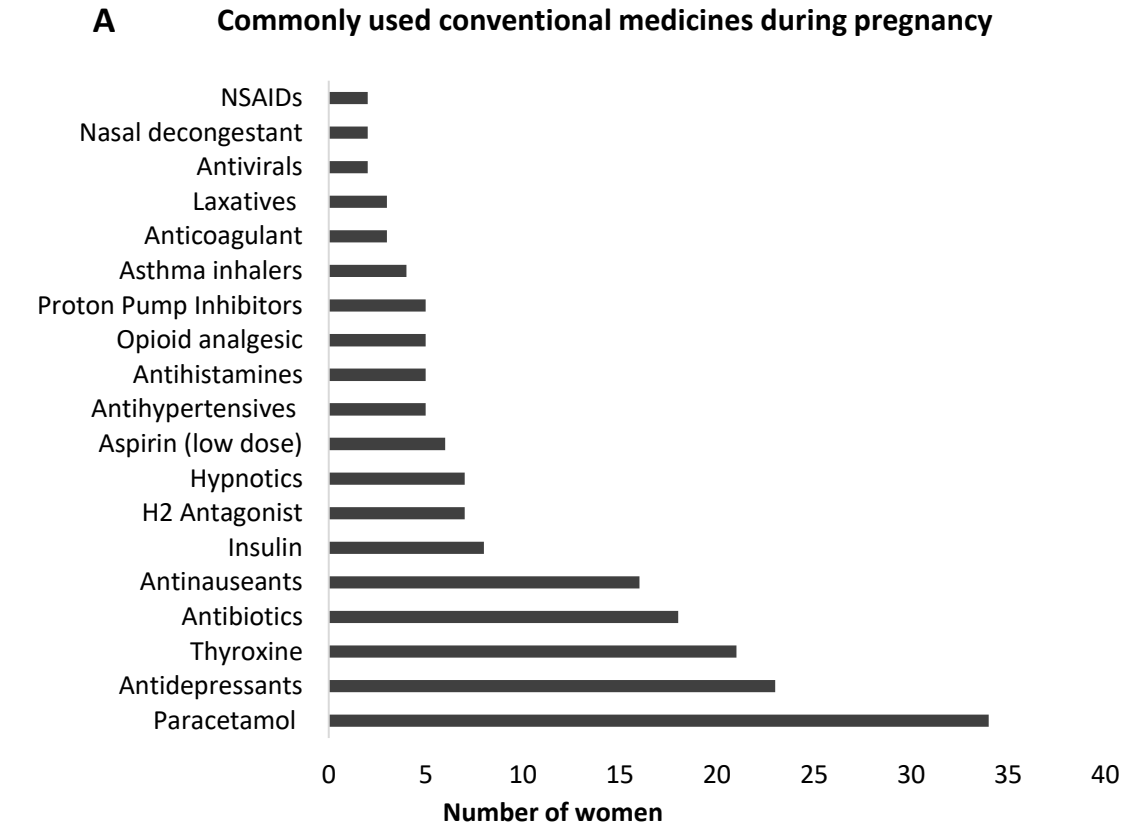
Approximately one-third of the survey respondents (29.2%; n=99) indicated that they had a medical condition but only a quarter of these participants disclosed the nature of their condition as demonstrated in Table 2.4. Gastrointestinal disorders, depression and migraines were the most reported medical conditions. Notably, a large proportion of respondents with a reported medical condition indicated that their condition did not fall into the stated common conditions and the "other" category was subsequently selected but further information was not provided.

**Table 2.4 Medical conditions and medicine use during pregnancy and breastfeeding**

| **Medical conditions** | ***n*= 339 (%)** |
|---|---|
| Depression | 3 (0.9) |
| Gastrointestinal Disorders | 4 (1.2) |
| Migraines | 4 (1.2) |
| Mood disorders | 1 (0.3) |
| Skin conditions | 2 (0.6) |
| Other | 11 (3.2) |
| **Medicine use** | |
| Used one or more medicine(s) during pregnancy | 210 (60) |
| Started one or more NEW medicine(s) post-partum not taken during pregnancy | 108 (30) |
| Took one or more medicines while breastfeeding | 142 (42) |

### 2.8.2.1 Medicine use during pregnancy

Overall, 210 (60%) participants reported using one or more medicines (prescribed, complementary and over the counter) during pregnancy. A total of 351 medicines were used by these women (1.35 medicines per participant). As shown in Figure 2.3, the most commonly used medicines during pregnancy were supplements (collectively) such as multivitamins, iron, magnesium, vitamin D, calcium, fish oil, folic acid and probiotics. Amongst prescribed and over the counter medicines, paracetamol, antidepressants, thyroxine, antibiotics and antinauseants were most commonly used.



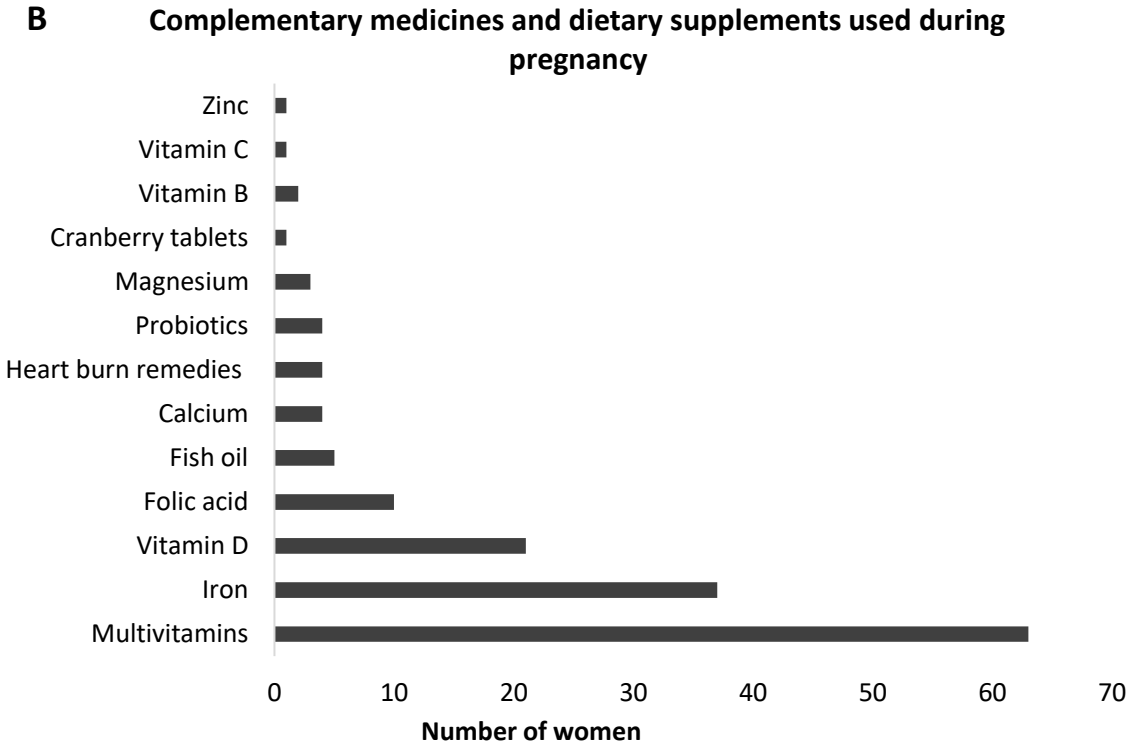




**Figure 2.3 (A) Commonly used conventional medicines during pregnancy; (B) Commonly used complementary medicines and dietary supplements during pregnancy**

#### 2.8.2.2 Medicine use during lactation

The use of prescribed and over the counter medicines (including complementary medicines) was high amongst breastfeeding mothers with a total of 142 (42%) women taking one or more medicines at some stage while breastfeeding (average=1.42 medicines/supplements per person). 108 participants stated that they had started a new medicine after the birth of their baby, which they were not exposed to or had taken during pregnancy while the remaining 34 had continued taking the same medicine that they were taking during pregnancy. 27.8% (n=30) of the mothers who took medicines during breastfeeding had not taken any medicines during pregnancy at all.

As shown in Table 2.5, supplements and vitamins that are considered safe and often recommended to be used, were the most commonly used medicine group in breastfeeding. Antidepressants, hormonal contraceptive (progesterone only), thyroid hormone replacement (thyroxine), inhaled corticosteroids and galactogogues such as domperidone were also commonly used. Although uncommon, some medicines with limited lactation safety data and potential adverse effects on breastfeeding or breastfed infant such as olanzapine, quetiapine, pregabalin and garcinia cambogia were also used.

**Table 2.5 Medicines (prescribed and over the counter) and supplements used by survey participants while breastfeeding**

| ***Drug Class*** | ***n*** |
|---|---|
| ***Antidepressants*** | **26** |
| *Sertraline* | 6 |
| *Desvenlafaxine* | 3 |
| *Citalopram* | 2 |
| *Fluoxetine* | 2 |
| *Paroxetine* | 2 |
| *Venlafaxine* | 2 |
| *Not disclosed* | 5 |
| *Fluvoxamine* | 1 |
| ***Contraceptives*** | **21** |
| *Mirena® IUD* | 3 |
| *Progesterone only pill* | 18 |
| ***Thyroxine*** | **12** |
| ***Inhaled corticosteroids*** | **8** |
| *Bricanyl®* | 1 |
| *Symbicort®* | 1 |
| *Flixotide®* | 1 |
| *Nasonex®* | 1 |
| *Seretide®* | 2 |
| *Not disclosed* | 2 |
| ***Domperidone*** | **7** |
| ***Paracetamol*** | **7** |
| ***Proton Pump Inhibitors*** | 4 |
| *Esomeprazole* | 1 |
| *Omeprazole* | 2 |
| *Rabeprazole* | 1 |
| ***Non-steroidal anti-inflammatory drugs*** | **3** |
| *Ibuprofen* | 2 |
| *Naproxen* | 1 |
| ***Antihistamines*** | **3** |
| *Dexchlorpheniramine* | 1 |
| *Fexofenadine* | 1 |
| *Loratidine* | 1 |
| ***Antihypertensives*** | **3** |
| *Methyldopa* | 1 |
| *Atenolol* | 1 |
| *Labetalol* | 1 |

| ***Drug Class*** | ***n*** |
|---|---|
| ***Opioid analgesics*** | **3** |
| *Codeine* | 1 |
| *Tramadol* | 2 |
| ***Antipsychotics*** | **2** |
| *Olanzapine* | 1 |
| *Quetiapine* | 1 |
| ***Osmotic laxatives*** | **2** |
| ***Metformin*** | **2** |
| ***Others*** | |
| *Temazepam* | **1** |
| *Pregabalin* | **1** |
| *Oral prednisolone* | **1** |
| *Insulin* | **1** |
| *Ranitidine* | **1** |
| *Low dose Aspirin* | **1** |
| *Anti-thyroid(propylthiouracil)* | **1** |
| *Antibiotics* | **1** |
| *Analgesics - not specified* | **1** |
| *5-aminosalicyclic acid* | **1** |
| *Topical steroid (cream)* | **1** |
| *Haemohorroid cream* | **1** |
| ***Supplements*** | |
| *Multivitamins* | **38** |
| *Vitamin D* | **10** |
| *Iron* | **8** |
| *Probiotics* | **8** |
| *Fish oil* | **5** |
| *Calcium* | **4** |
| *Folic acid* | **3** |
| *Magnesium* | **3** |
| *Vitamin B* | **2** |
| *Garcinia Cambogia* | **1** |
| *Vitamin C* | **1** |
| *Cranberry* | **1** |

### 2.8.2.3 Short-term medicines in the immediate post-partum period

The use of short-term medicines post-partum was reported in 56 (16.5%) participants. As shown in Table 2.6, the most common short-term medicines were antibiotics and analgesics including Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and opioids. Antibiotics are prescribed to breastfeeding women for short durations to treat various infections such as mastitis and prophylactically following a caesarean section. Analgesics are commonly prescribed for post-partum pain relief. Hypertension is a common disorder encountered in up to 8% of pregnancies, mostly resolving spontaneously in the early post-partum period which explains the short-term use of antihypertensives such as beta blockers (128, 129).

**Table 2.6 Commonly used short-term medicines in the post-partum period**

| **Drug class** | ***n*** | **Drug class** | ***n*** |
|---|---|---|---|
| **Antibiotics** | **21** | **Analgesics - Unspecified** | **3** |
| **Anticoagulants** | **2** | **Analgesics - NSAIDs** | **11** |
| **Antihypertensives** | **6** | Celecoxib | 2 |
| Beta blocker(unspecified) | 1 | Diclofenac | 5 |
| Labetolol | 2 | Ibuprofen | 3 |
| Methyldopa | 1 | Naproxen | 1 |
| Nifedipine | 1 | **Analgesics - Opioids** | **4** |
| Propranolol | 1 | Oxycodone & Naloxone | 1 |
| **Galactogogues** | **9** | **Analgesics - Paracetamol** | **3** |
| Domperidone | 8 | | |
| Fenugreek | 1 | | |

## 2.8.3 Concern regarding transfer of medicines

Of the 339 participants, 134 (39.5%) stated that they were generally concerned about the transfer into their breastmilk of both long and short-term medicines. A great majority of these women (n=130) sought professional advice regarding their concerns. It is not known whether the professional advice sought, or the information obtained by these mothers affected their choice of treatment. As shown in Table 2.7, 48 (37%) of those who sought professional advice stated the source of their advice. The Internet (n=10) was reported to be the most commonly used resource to obtain this information, followed by hospital (n=7), pharmacist (n=7) and a specialist doctor (n=7).

**Table 2.7 Sources of information consulted by breastfeeding women to obtain safety information about medicines**

| Source of professional advice | Number (n=48) |
|---|---|
| Internet | 10 |
| Hospital | 7 |
| Pharmacist | 7 |
| Specialist Doctor | 7 |
| General Practitioner | 6 |
| Midwife | 3 |
| Lactation consultant | 2 |
| Friends | 2 |
| ABA website/hotline | 2 |
| Community nurse | 1 |
| Books | 1 |

## 2.9 Perceived adverse reactions of breastfed infant to food products

Untoward infant reactions attributed to passage through breastmilk of common food consumed by the mother was reported in 75 (22%) of the survey participants. These women indicated that they noticed an untoward reaction(s) in their breastfed baby after they consumed a common food product. Dairy and/or milk products were implicated as the offending food in the majority of cases, followed by chocolate and onions as shown in Figure 2.4 (A). Mothers reported behavioral changes such as increased crying, unsettled behaviors and loss of sleep as the most common perceived adverse effect of food products on breastfed infants as shown in Figure 2.4 (B).

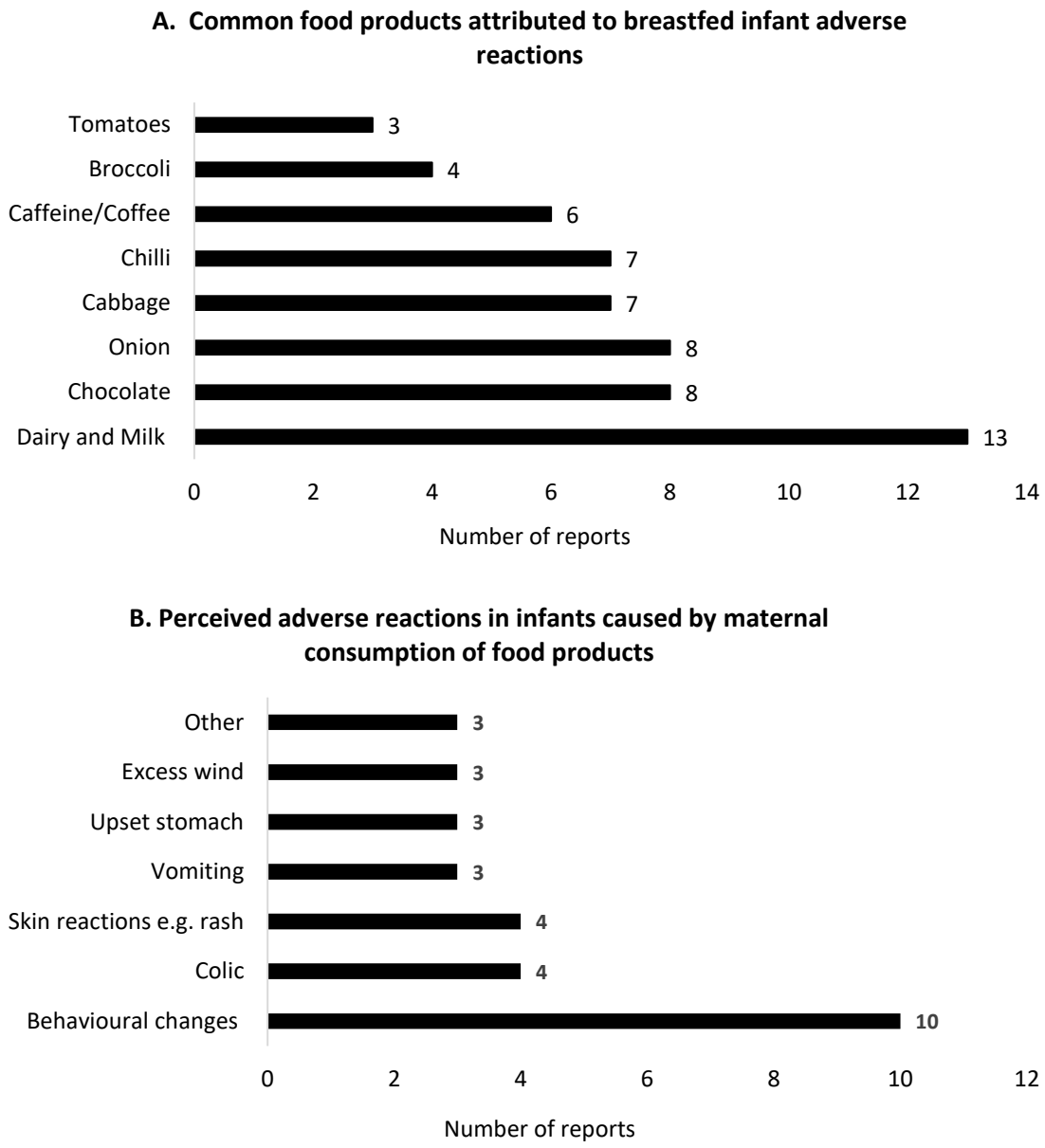



**Figure 2.4 (A) Food products consumed by mother perceived to have contributed to an adverse reaction in the breastfed infant. (B) Type of adverse reaction in the breastfed infant attributed to a food product consumed by the mother**

## 2.10 Establishing causal relationship between the offending drug and the appearance of the perceived ADRs using Naranjo Algorithm

Of the 339 survey respondents, 23 participants (6.78%) indicated that they noticed or suspected an ADR in their breastfed infant after they took a medicine. Of these participants with a suspected ADR, 16 (4.7%) had a positive Naranjo score, ranging

from 1 to 6, associating the offending drug with the suspected ADR. The average age of infants at the time of the perceived ADRS was 25.6 days (95% CI; 4 – 85 days). Antibiotics caused 12 of these suspected ADRs, followed by opioid analgesics including tramadol and oxycodone. Atenolol and Juice Plus® were thought to have caused the remaining two ADRs. ADRs attributed to the offending antibiotics included gastrointestinal upset (vomiting/diarrhoea) (6), colic (2), skin rashes (1) and behavioral changes (1). In the remaining 2 cases, symptoms were not specified. 14 (87%) of these participants stated that they were concerned about the transfer of their medicine into their breastmilk and sought professional advice regarding this from various sources including their specialist doctor, midwife, lactation consultant and pharmacist. Six of the participants reporting ADRs also reported adverse reactions to food products. The offending foods included broccoli, cabbage, garlic, capsicum, potatoes, milk, chocolate, lentils and chilies.

**Table 2.8 Medicines suspected to have possibly or probably caused infant ADRs in the study population (Naranjo score >0)**

| Offending drug | Naranjo Score | Action |
|---|---|---|
| Antibiotic | 1 | Other- "*dealt with side effects*" |
| Antibiotic | 1 | Stopped medication |
| Antibiotic | 2 | Changed the timing of the feed/medication to reduce baby's exposure |
| Oxycodone and naloxone(Targin®) | 2 | Stopped medication |
| Antibiotic | 3 | Stopped medication |
| Antibiotic | 3 | Changed the timing of the feed/medication to reduce baby's exposure |
| Antibiotic | 3 | Stopped breastfeeding |
| Antibiotic | 3 | Other- "Stopped medication and commenced another antibiotic" |
| Tramadol | 3 | Changed time of feed |
| Juice Plus | 3 | Stopped medication |
| Antibiotic | 4 | Other- finished course despite ADR |
| Antibiotic | 4 | Stopped medication |
| Antibiotic | 4 | Other- *"made sure to space dose 4 times a day rather than same dose twice a day, seemed to work fine"* |
| Atenolol | 5 | Stopped medication |
| Antibiotic | 6 | Stopped medication |
| Antibiotic | 6 | Other- "continued taking the medicine despite infant having severe diarrhoea. Symptoms resolved upon cessation of the medicine." |

## 2.11 Impact of ADRs on continuation of breastfeeding and maternal treatment

Of the 16 participants with a positive Naranjo score for the reported ADR:

- Only one participant reported that they stopped breastfeeding due to the perceived ADR (antibiotics for mastitis).

- Three participants stated that they changed the timing of the medicine/feed to reduce baby's exposure to the medicine (antibiotic).
- A further two participants changed the timing of their feeds pre-emptively, presumably to reduce their baby's exposure.
- One participant reported discontinuing the prescribed medicine (an antibiotic) but then commenced a different antibiotic, to which their baby had a similar reaction.
- One participant stated that they split the dose from twice daily to half the dose four times daily "*Made sure to space dose 4 times a day rather than same dose twice a day, seemed to work fine".*
- Another participant stated that they "*continued taking the medicine despite their infant having severe diarrhoea* which *settled upon cessation of the medicine*".
- Two participants stated that they finished the course of their antibiotics despite the side effects. It appears that the infants' symptoms resolved upon cessation of the medicine. "*Took the course of antibiotics until all finished. Diarrhoea stopped after medicines ceased*".
- Five participants taking antibiotics indicated that they stopped taking the suspected offending drugs. However, it is unclear as to whether they replaced it with a different medicine or stopped after the course was completed. It is unusual for patients to discontinue antibiotics when there is unresolved infection.
- Ten participants (62.5%) stated that they reported the perceived ADR to a healthcare professional. However, it is not known whether the healthcare professional went on to report this ADR to the TGA in any of the cases.

## 2.12 Discussion

Adverse drug reactions are common and can occur in anyone taking medicines. Medicines are known to pass through placenta and breastmilk, and these can serve as

indirect routes of infant exposure to xenobiotics. However, breastfeeding related internet

ADRs are thought to be uncommon as the globally reported cases of breastfeeding related ADRs is low (25, 96, 130). Australian reports of infant ADRs was also found to be very low as only 60 cases of infant ADRs were reported over a 13 year period (2003-2016). This study hypothesised that breastfeeding related infant ADRs are more frequent than reported but due to the non-specific nature of some ADRs, a lack of awareness and identification of ADRs by mothers and clinicians, a significant number of them are potentially dismissed and not reported. Furthermore, the difficulties associated with establishing a causal relationship between a drug and an ADR in a breastfed infant coupled with a lack of healthcare professional training was thought to be a contributing factor. This inherent filtering of information is thought to have led to the underreporting as opposed to a genuinely low occurrence of infant ADRs. The other problem with the underreporting of breastfeeding ADRs is the potential unnecessary burden on the health system that may occur as a result of misdiagnosis (this is further discussed in Chapter 3) or long term economic losses resulting from discontinuation of breastfeeding. Additionally, mothers choosing not to treat their medical condition for the fear of inadvertently exposing their breastfed infant to a medication can have detrimental enduring effects on the health and wellbeing of both mother and the infant alike. Not having an accurate account of infant ADRs due to exposure from breastfeeding makes it difficult to determine the magnitude of this effect. Hence, this survey was conducted to obtain a first-hand account of mothers who were breastfeeding their children while taking medicines to evaluate the real occurrence of ADRs, reported or otherwise. This study also aimed to evaluate the impact of the ADRs on the continuation (or otherwise) of breastfeeding and maternal treatment.

The healthcare system in Australia is largely uniform across the states and territories. The availability of health services and information is also deemed to be of a similar standard across the states and territories. Therefore, the state of residence of the participants was not thought to have resulted in the skewing of the data. However, it was deemed important to highlight this as a limitation of the participant recruitment process. While in WA the recruitment was multichannel through child health clinics, Ngala parenting centres and the ABA website, the sources of participant recruitment

for the rest of the country was through the ABA website only. Thereby providing justification for a majority West Australian participation. Higher levels of maternal education is associated with better health literacy (131). Greater than 60% of survey respondents possessed a graduate degree or higher which is indicative of a high level of health literacy suggesting that their responses and reports of perceived infant ADRs would be a realistic representation of infant ADRs in the general population.

Sixty percent of women reported taking a medicine during pregnancy while forty two percent reported taking at least one or more medicines during breastfeeding. One third of the respondents reported suffering from a medical condition but the majority did not reveal the nature of their medical condition. The most common medicines used in both pregnant and lactating women were supplementary medicines including multivitamins and minerals such as iron, folic acid, calcium and magnesium. During pregnancy other common medicines included antidepressants, antibiotics, antihypertensives, thyroid hormone replacement, analgesics, insulin and anti-hyperglycemics. In lactating women, antidepressants, hormonal contraception, galactagogues, analgesics (paracetamol, non-steroidal anti-inflammatories and opioids) and antibiotics formed the most commonly used medicines after supplements. Although used relatively infrequently, breastfeeding women also reported using antipsychotics (olanzapine and quetiapine), benzodiazepines (temazepam), hypnotics (doxylamine), anti-inflammatories (5-Aminosalicylic acid), high dose oral corticosteroids, propylthiouracil, pregabalin and garcinia cambogia. Most of these drugs are known to transfer into breastmilk. However, there are no studies evaluating the long term effects of these drugs on breastfed infants.

More than one-third of the survey respondents indicated that they were concerned about exposing their breastfed infant to medicines through breastfeeding. Almost all of these women sought professional advice regarding the safety of the medicine that they were taking. Most participants did not specify the source of advice but for those that did, they used reliable sources of information such as their healthcare professional. A further 10 participants stated that they used internet to obtain medicines information, but the exact website or online source was not explored. Given that the internet is easily accessible to most people, it is expected that women would consult online sources (132). It is important that they use reliable and appropriate websites for this purpose as opposed to forums or chat groups where the

accuracy of the information may not be trustworthy. In this survey the source of the online information was not explored, and this should be considered in future studies as reliability and the source of data is an important factor not only in the correct decision making by the mother but also their ongoing relationship with their health care professional (132-134).

Overall, only a small number (n=23; 6.8 %) of women reported noticing an ADR in their breastfed infant. Out of these, a possible or probable causal relationship with maternal medicine use could be established in only 16 (4.7%) cases, 13 had a Naranjo total score of between 1 and 4 (possible causal relationship) while the remaining 3 had a total Naranjo score of between 5 and 8 (probable causal relationship). No ADRs were identified to have been definitely caused by maternal medicines (Naranjo score >9). Antibiotics (n=12) and opioids including tramadol and oxycodone (n=2) were identified as the most common ADR causing drugs. The average age of infants at the time of the perceived ADRs was 25.6 days (95% CI; 4 – 85 days), which aligns with evidence that infant ADRs mostly occur under 2 months of age (21). Ten participants reported the ADR to their clinician. However, it is unbeknown to the participants whether the clinician reported the perceived ADR further to the TGA.

In this study population, breastfeeding related ADRs appear to be low with only 23 ADR reports from 339 respondents over a 7-month period. However, when compared to the TGA reports of 60 ADRs over a 13-year period from 2003-2016, it is significantly (8.5 times) higher. With an average new births of 35,000 per year in Western Australia, this study was completed by an estimated 1% of women who gave birth in that year (135). Furthermore, due to the known decline in the number of women who continue breastfeeding over time and this study including participants at various stages of breastfeeding (including those who ceased breastfeeding in the preceding 12 months), this data lacks a denominator. Hence, the prevalence of breastfeeding related ADRs is likely an underestimation. Despite the limitations, this study confirmed the hypothesis that breastfeeding related ADRs are underreported. While it can be argued that some of the ADRs reported in this study are common side effects of these medications and do not warrant reporting to the TGA, in the absence of large clinical studies and prevalent off-label prescribing of medicines to

lactating women, increased pharmacovigilance through reporting is the best way of identifying safety concerns in breastfed children who are exposed to medicines.

The findings of this study are supported by studies published by Soussan *et al.* (1), Cliff Eribo *et al. (136)* and Ito *et al.* (19) which show anti-infectives to be one of the most common drug classes implicated in infant ADRs via breastmilk. The analysis of TGA reports (2003-2016) also found antibiotics to be the second most implicated drug class in infant ADRs in Australia. In this study, only one participant (0.29%) reported discontinuation of breastfeeding because of a suspected ADR to an antibiotic that she was taking for mastitis. It is not clear whether breastfeeding was discontinued due to the infection or side effects from the antibiotic. Other participants who took medicines while breastfeeding used strategies such as changing the time of feeds or the medicine to minimize infant exposure. Two participants stated that they tolerated the adverse effects (diarrhoea) as no suitable alternatives were available and that the side effects resolved upon cessation of the antibiotics. Being a commonly used medicine group amongst breastfeeding women, antibiotics are largely considered "compatible" with breastfeeding with some exceptions such as tetracyclines (137). Transient side-effects such as diarrhoea, rashes and vomiting are commonly seen in breastfed infants whose mothers use these drugs (138). However, recent studies have shown that antibiotic exposure in the early years of life has been associated with adverse neurodevelopmental and neurocognitive outcomes later in life (88, 89, 139). These associations were only made possible with long-term follow-up of those children. Therefore, a drug being compatible with breastfeeding does not equate to infant safety in the long run and is further reason for implementation of more thorough pharmacovigilance systems. If the TGA reports are only a proportion of actual ADRs, then the real numbers are expected to be much higher making it important to have long term follow-up of infant exposure to medicines via breastmilk. Furthermore, it is worth noting that breastfeeding women are likely to be non-compliant with anti-infective treatments due to safety concerns and have been shown to discontinue breastfeeding or not take their antibiotic as prescribed (140, 141). Non-compliance to prescribed medicines in pregnancy and breastfeeding due to safety concerns for the baby are commonly reported (142-144). In this study, only one participant reported taking her prescribed antibiotic four times per day instead of the prescribed twice daily. Making changes

such as this to prevent infant ADRs can have unfavorable effects in the mother including potential treatment failure and antibiotic resistance.

Compared to drug related adverse effects, significantly higher accounts of food related adverse effects were reported with 75 respondents (22%) reporting an ADR in their breastfed infant that they attributed to a food product. Of these, 84% (n=63) stated the name(s) of the food product(s) and about half of these stated the reaction that they noticed in their breastfed infant. In contrast, only three participants with a reported ADR to antibiotics specified the ingredient/brand name of the medicine. It is interesting to see that participants in this survey reported high rates of adverse reactions to common food products, with dairy/milk products, onions and chocolate having the highest prevalence of perceived offending foods. While these reactions could certainly have occurred, it begs the question as to whether this is a reflection of food reactions or whether a consequence of the mother's familiarity and awareness of possible reactions to food products. It is well known that many cultures prefer or avoid certain food products during breastfeeding for their perceived effects on the breastfed infant and milk supply, which can serve to influence a mother's perception of an adverse reaction. Food products such as fennel and fenugreek are being used in many cultures as natural galactagogues despite a lack of conclusive scientific evidence (145, 146). The relatively high number of reports of adverse food reactions may possibly be due to the participant's awareness and familiarity with foods, their preconceived ideas about certain foods or anecdotal reports of infant reaction to food products, making these women more willing to assign an association to their infant's reactions with the foods that they consumed. On the other hand, a possible lack of knowledge about medicines, and the reassurance provided by healthcare professionals could have influenced their perception of possible ADRs related to medicines over foods which if true could have potentially contributed to the low reported numbers of ADRs (147).

An ADR in a breastfed baby, particularly if causing acute distress to the infant, can have detrimental effects on the breastfeeding experience and may cause unnecessary discontinuation of maternal treatment in addition to causing anxiety and distress for new parents (147) . Breastfeeding related ADRs may not be considered by healthcare professionals when other more serious conditions need to be excluded, thereby missing an opportunity to add to the body of evidence relating to medicine safety in

breastfeeding. As demonstrated by this study, the general practitioners and healthcare professionals are less likely than the mother to report the infant ADR. This could potentially be due to a lack of training and awareness of breastfeeding related ADRs, lack of time and competing priorities especially when the ADR is considered as an expected side effect of the drugs (e.g. gastro intestinal upset with antibiotics).

The best approach to obtaining more definitive and relevant lactation related medicine information undoubtedly remains larger clinical studies involving direct measurement of drug levels in infant plasma samples, breastmilk samples and controlled clinical trials. However, this may not always be possible due to the ethics of conducting trials in this vulnerable population further highlighting the need for greater pharmacovigilance so that breastfeeding related ADRs are reported and these reports contribute to the body of evidence. However, a systematic infrastructure that facilitates the reporting of breastfeeding related ADRs is lacking potentially creating a circularity effect whereby the lack of ADR reporting may be interpreted as breastfeeding related ADRs being uncommon. Increasing the awareness of mothers about suspected breastfeeding related ADRs and encouraging them to report these to the TGA through provision of information leaflets at maternity hospitals may help with enhancing the pharmacovigilance. TGA reporting is voluntary and many women may be reluctant to report ADRs especially if their healthcare professional is not encouraging due to a lack of an established causal relationship. Empowering women through awareness that an ADR can also be reported if it is suspected but not proven may help with increased reporting. Information leaflets about awareness of breastfeeding related ADRs and the process to report suspected ADRs to the TGA should be considered for inclusion as part of the information leaflets given to new parents in maternity hospitals.

Increased pharmacovigilance is particularly important in drugs with potential long-term consequences. As previously mentioned, antibiotics, which are largely considered compatible with breastfeeding and have mild known adverse effects associated with their short-term use are now being investigated for long term negative consequences and outcomes on the neurobehavioural development of exposed children (88, 137, 139). This also calls into question the “blanket rule” approach taken by current references and guidelines where medicine safety in breastfeeding is classified into two to three broad categories of compatibility namely

"compatible and non-compatible" or "safe, caution and avoid". This survey highlights that "compatible" drugs such as antibiotics and opioids are commonly used by breastfeeding women and they account for a significant proportion of reported ADRs which seem to be mild in nature. However, due to a lack of long term follow-up and appropriate pharmacovigilance, their long term effects will not be fully known.

## 2.13 Limitations of the study

This survey was an online survey which was publicised through various avenues including Child Health centres in WA, ABA website and Ngala (parenting community support centres in WA). An email address was used to filter out duplicated surveys and ensure data integrity. Despite this, 10% of surveys were deemed ineligible due to being incomplete or duplicated. Being solely an online survey, the response rate was expected to be low (148, 149). However, this medium was thought to be the best for the target audience, all likely to be busy new mothers to allow them time and flexibility in completing the survey. Despite the targeted advertising and communications, it took 7 months to reach an adequately powered sample size (95% confidence interval) due to the low response rate. This could potentially be due to the voluntary nature of the survey and the limitations of the recruitment process. The target audience of this study were busy new mothers who may be reluctant to commit time to low priority activities such as completing a survey. The survey was relatively long with 42 questions to get an in-depth account of infant ADRs attributed to maternal medicine use. From the pilot studies, it was estimated that the survey would take 10-15 minutes to complete. This amount of time could have potentially been too long for new mothers leading to some respondents not completing all of the survey questions. Although the Naranjo algorithm questions were simplified significantly, not all questions were answered. This could have been due to the length of the survey or the lack of the participant's understanding of the questions. Incomplete responses to the Naranjo questions could result in lower scores where an ADR was suspected and consequently could have skewed our results through underreporting of ADRs.

There were also some responses where the participants indicated that they did not notice an ADR in their breastfed infant, yet they have indicated that they reported the

ADR to their healthcare professional. In these cases, a food related adverse reaction was reported by the participant. However, the intention of the question was to gather information related to medicine related adverse drug reactions. Therefore, only responses relating to ADRs were included in the final analyses and not the food related reactions. In order to keep the length of the survey short, certain questions such as the mother's age was deemed unnecessary at the time of survey development. However, this information may have provided additional valuable information about the participant's demographics. The survey did not explore where the participants first got to know about the survey. This information would have been useful in drawing associations between participant demographics and their survey responses, as participants actively looking on the ABA website or visiting Ngala may have been more inclined to participate potentially due to an "issue".

## 2.14 Conclusion

This study confirms that lactating mothers do commonly take medicines that they fear may pass to their offspring through breastmilk. Infant ADRs reported by mothers were generally mild and associated with antibiotic and opioid analgesic usage. This is supported by existing research which implicates these two drug classes in causing breastfed infant ADRs. The study showed that concerned mothers seek professional advice when taking medicines during lactation. It is estimated that this study was completed by less than 1% of women who gave birth in the year the survey was undertaken. Despite this, the study confirmed that infant ADRs reported are relatively uncommon and transient with only 4.7% of the respondents reporting an ADR with a possible or probable link to maternal medication use. However, when compared to official reports (TGA), suspected ADR reporting in this study was significantly greater which confirmed the hypothesis that breastfeeding related infant ADRs are grossly underreported. Furthermore, participants who discussed the ADR with their healthcare professional indicated a lack of knowledge of further reporting to the TGA showing that healthcare professionals are less likely than the mothers to report breastfeeding ADRs. It is suspected that these ADRs were potentially not reported as most were expected side-effects of antibiotics e.g. gastrointestinal upset. Due to emerging evidence which links antibiotic exposure in early life to long term

negative neurobehavioural outcomes, it is imperative that any drugs used during lactation be subject to stricter pharmacovigilance and any ADRs, perceived or real should be reported. This survey also showed that drugs with limited lactation safety data such as antipsychotics, pregabalin and garcinia cambogia were used during lactation but mothers did not report any associated ADRs in their nursing infant. Sertraline, an antidepressant which is not recommended for use in lactation by the drug manufacturers was one of the most commonly used medications with no associated ADRs reported (117). Drugs such as sertraline with limited and conflicting safety data available and often used long-term necessitates a more thorough evaluation and follow up of exposed children especially given the ethical complexities associated with randomised controlled trials in this subpopulation. Due to the current ethical challenges surrounding controlled trials, enhanced pharmacovigilance through better reporting is the second-best way of ensuring lactation medication safety data is improved. Provision of education and information to new mother to increase their awareness of reporting should be considered as part of the post-partum education provided to women in maternity hospitals while efforts to improve national infrastructures are continuing. Additionally, it is important to acknowledge that labelling of drugs as "compatible" or otherwise with breastfeeding does not assure absolute safety in breastfed infants as it does not evaluate long term safety as is evident with antibiotic use. This further highlights the need for improved pharmacovigilance and long-term follow-up of children exposed to medicines through breastmilk.

# Chapter 3 Is maternal therapeutic opioid use instigating misdiagnosis in breastfed infants? A case report.

## 3.1 Abstract

Despite the known risks associated with opioid use during breastfeeding, their place in therapy is established as part of a multimodal approach to treatment of pain in the early postpartum period. Opioids may be prescribed for post-caesarean analgesia without adequate patient education, resulting in adverse drug events in breastfed infants. We report the case of an exclusively breastfed 6-day-old infant who presented with symptoms of progressive drowsiness, somnolence and inability to feed. Maternal medicine use was discounted as a potential causative factor and it was not explored further despite the mother taking a long-acting opioid at the time. A series of invasive investigative tests were carried out and the infant was commenced on intravenous antibiotics for suspected sepsis. All test results were negative for infections and no causes were identified for the infant's symptoms who was discharged three days later with a formal diagnosis of a "*probable viral infection*". A lack of understanding of the impact of maternal medicine use (particularly drugs with known risks) by healthcare professionals in breastfed infants can result in infant ADRs, inappropriate prescribing, stress and anxiety for new parents and a lost opportunity to contribute to lactation-related medicines information by not conducting specimen testing of breastmilk and/or infant plasma.

## 3.2 Case presentation

A female baby was born at 38+6 weeks gestation (birth weight 2.6kg) by caesarean section on the background of oligohydramnios and intrauterine growth retardation. The APGAR scores at birth were 9 and 9 at 1 and 5 minutes respectively. The baby

had no post birth complications and was discharged home on day 5. Lactation was fully established on day 3. The mother was healthy with no prior medical conditions and no history of medicine use other than pregnancy multivitamins. For post-partum analgesia, the mother was prescribed diclofenac 50 mg, a non-steroidal anti-inflammatory drug three times daily and Di-gesic® (containing paracetamol/dextropropoxyphene) 650 mg/65 mg (equivalent to 2 tablets) six hourly. The mother took 13 doses of the analgesics as follows: Day 2: 3 doses | Day 3: 3 doses | Day 4: 3 doses | Day 5: 3 doses| Day 6: 1 dose.

On day 6 postpartum, the mother noticed her exclusively breastfed infant to be drowsy, falling asleep during feeds and being unresponsive to stimuli including pain (pinching of the toes). The infant was admitted to the local paediatric hospital for assessment. Although initial observations such as body temperature, oxygen saturation, heart rate and blood pressure were unremarkable, investigative tests such as full blood picture, blood cultures, lumbar puncture and urine microscopy culture and sensitivities were ordered. The infant was commenced on intravenous fluids for hydration, and empirical antibiotics (benzylpenicillin and cefotaxime) to cover for sepsis and meningitis.

Despite the initial blood test results not showing any abnormalities, the infant was closely monitored while waiting for the microscopy and culture results. Over the ensuing 24 hours, the infant's vital observations remained unremarkable. The mother had ceased taking Di-gesic® (last dose being on the morning of day 6 postpartum) and at this stage was taking paracetamol for pain relief. In addition to intravenous hydration, breastfeeding was continued, and the baby was observed to be more alert with noticeable improvements in feeding. The investigative tests returned normal results and did not show any abnormalities or infections in the infant as shown in Table 3.1. Vital observations also remained unremarkable during the entire admission. Antibiotics were ceased after 36 hours and the infant was discharged home after three days of hospitalisation with a formal diagnosis of a "probable viral infection".

The infant's mother came to know about the breastmilk research that was being undertaken by the researcher at the time through an acquaintance who was a participant in the longitudinal study (Chapter 4) of this research project. She

approached the researcher and requested further exploration of the case, and the possibility of publication of their case-study to raise awareness of maternal medication use in breastfeeding mothers. The researcher was involved in literature review, collation of data, parent interviews and the writing of the case study.

The mother stated that she volunteered information about her Di-gesic® use to the doctors on two occasions in the emergency ward and again in the inpatient ward as she was aware of the notion that foods and medicines taken by a mother can affect her breastfed infant. However, she was assured by junior and senior paediatric doctors that Di-gesic® was unlikely to have caused the baby's symptoms with the junior doctor declaring *"if the obstetrician prescribed it, it is safe.*"

**Table 3.1 Laboratory test results on admission and microbiological testing results 48 hours post admission**

| Test | Result | Normal Range |
|---|---|---|
| **Inflammatory Markers** | | |
| C-Reactive Protein | <5 mg/L | [<15 mg/L] |
| **Full Blood Picture** | | |
| Haemoglobin | 174 g/L | [135-195 g/L] |
| White Cell Count | 12.10 X10^9/L | [5.00-25.00 X10^9/L] |
| Platelet count | **472 X10^9/L** | [150-400 X10^9/L] |
| Neutrophils Absolute | 3.02 X10^9/L | [3.00-18.00 X10^9/L] |
| Lymphocytes Absolute | 7.02 X10^9/L | [2.00-10.00 X10^9/L] |
| Monocytes Absolute | 1.81 X10^9/L | [0.20-2.20 X10^9/L] |
| Eosinophils Absolute | 0.24 X10^9/L | [0.00-0.50 X10^9/L] |
| **Urea and Electrolytes** | | |
| Sodium-Plasma | 139 mmol/L | [132-147 mmol/L] |
| Potassium-Plasma | 5.1 mmol/L | [3.5-6.2 mmol/L] |
| Bicarbonate- Plasma | 19 mmol/L | [17-28 mmol/L] |
| Urea- Plasma | 2.4 mmol/L | [2.0-8.0 mmol/L] |
| Creatinine - Plasma | 37 µmol/L | [22-93 µmol/L] |
| **Cerebrospinal Fluid** | | |
| Glucose | **2.6mmol/L** | [2.7-4.4 mmol/L] |
| Protein | 1.04g/L | [0.3-1.10 g/L] |
| **Microbiology Results** | | |
| CSF Culture | No growth | |
| Blood Culture | No growth | |
| **Nucleic Acid Detection Tests** | | |
| Enterovirus RNA | Not detected | |
| Neisseria meningitidis DNA | Not detected | |
| Human parechovirus | Not detected | |

A retrospective review of the infant's medical notes from this admission demonstrated that:

- there was no documentation of maternal medicine use at any stage
- there was no mention of maternal Di-gesic® use in the infant's medical record
- potential drug exposure was not considered as a causative factor for the presenting symptoms of the infant
- vital observations on admission were unremarkable
- differential diagnosis on admission was sepsis/meningitis

## 3.3 Reported adverse reactions in breastfed infants attributed to opioids

Correct management of pain in the post-partum period is essential to minimise the risk of adverse outcomes to the mother and baby. Inadequate treatment of pain can lead to the development of anxiety and depression which can impact on a woman's physical and psychological well-being, as well as her ability to provide care for her baby (150). Opioids are known to cause adverse effects such as respiratory depression and death in breastfed infants (21, 151). Codeine has been associated with infant mortality and is no longer recommended for post caesarean analgesia due to the risk of accumulation and infant mortality particularly in CYP2D6 ultra-rapid metabolisers (152, 153). All other opioids carry the risk of causing infant adverse effects to some extent. Hence, many regulatory bodies such as the Royal Australian and New Zealand College of Obstetrics and Gynaecologists (RANZCOG) recommend the use of opioids be limited to the shortest duration possible. A recent study of calls to US poisons centres found that 88% of serious effects such as lethargy, cyanosis, respiratory depression and drowsiness in breastfeeding neonates was associated with maternal opioid use (38).

Due to the overwhelming evidence of maternal opioid use causing ADE in breastfed infants, it is recommended that postoperative pain management for caesarean section should be multimodal in approach with non-opioids such as paracetamol and non-steroidal anti-inflammatories to be the mainstay of treatment and opioids to be reserved for breakthrough pain only (154). Despite this, global evidence suggests that opioids are being prescribed postpartum often in quantities greater than needed and possibly without adequate maternal education (155, 156).

## 3.4 Reported adverse reactions in breastfed infants attributed to dextropropoxyphene

A case of a 10-day old infant with similar symptoms has been reported in the literature (157). In a case-control study of 12 breastfed term newborns with unexplained episodes of apnoea, bradycardia or cyanosis during the first week of life, maternal oral dextropropoxyphene was determined to be the probable cause in four cases (158). Breastfeeding associated adverse drug reactions reported to the French pharmacovigilance centre attributes 11 of the 174 ADRS reported between 1985 and 2011 to maternal dextropropoxyphene use (21).

Although the short-term use of opioids is considered safe during breastfeeding, drugs with long half-life or low clearance have the highest risk of causing adverse effects in breastfed infants (122, 151). Opioid use in breastfeeding mothers beyond 4 days has been associated with sedation and apnoea (151). Given the relatively long half-life of dextropropoxyphene and its active metabolite norpropoxyphene, coupled with low neonatal elimination capacity (159, 160), the possibility of accumulation in this case cannot be ruled out especially when other cause was not identified.

## 3.5 Pharmacokinetics of dextropropoxyphene

Dextropropoxyphene hydrochloride is a centrally acting, synthetic opioid analgesic structurally related to methadone. It binds to opioid receptors at many sites within the central nervous system. Paracetamol is a non-opioid analgesic and antipyretic. The combination of dextropropoxyphene with paracetamol produces greater analgesia than that produced by either drug administered alone. Dextropropoxyphene is readily absorbed from the gastrointestinal tract but is subject to considerable first-pass metabolism. Peak plasma concentrations of dextropropoxyphene are reached in 2 to 2½ hours. After a 65 mg oral dose of dextropropoxyphene hydrochloride, peak plasma levels of 0.05 to 0.1 microgram/mL are achieved (161).
Repeated doses of dextropropoxyphene at six hour intervals lead to increasing plasma concentrations, with a plateau after the ninth dose at 48 hours. Dextropropoxyphene is metabolised in the liver to yield norpropoxyphene. Dextropropoxyphene has a half-life of 6 to 12 hours, whereas that of

norpropoxyphene is 30 to 36 hours leading to an accumulation in the breastmilk (162, 163). Dextropropoxyphene has been shown to pass into breastmilk and should be used with caution during breastfeeding especially in the first two months postpartum due to the underdeveloped infant excretory and metabolic systems (164).

## 3.6 Dextropropoxyphene availability at the time of occurrence of this case

Due to safety concerns, dextropropoxyphene containing products have been withdrawn from various countries in Europe, the USA and New Zealand. Despite this, cases of serious harm due to this drug are still emerging (165). In Australia, any dextropropoxyphene containing medicines including Di-gesic® were supposed to be withdrawn from 1 March 2012, but the drug manufacturer sought a review in the Administrative Appeals Tribunal which ruled in 2013 that the drugs could be sold under strict conditions (166). One condition was that doctors were required to complete a prescriber confirmation form when prescribing this medicine to a patient, with copies of this form provided to the dispensing pharmacist and the patient. The completion of this form was meant to serve as an endorsement that the prescriber had considered alternatives and confirmed that there were none suitable, that they had considered recent changes to the patient's clinical presentation and biochemical markers, and that they had discussed with their patient the appropriate use of the dextropropoxyphene product including the risks of overdose (166). At the time of this incident in November 2016, dextropropoxyphene was available strictly under these conditions.

The mother stated that on the morning of her discharge from the hospital, the obstetrician in charge informed her that she would receive analgesics for pain but further details such as drug names or regimens were not discussed. The mother stated that she could not recall being visited by a pharmacist and consequently did not get to speak to one at all during the course of her admission and on discharge from the maternity hospital. The limited medicine counselling the mother received was part of the discharge facilitation process from the nurse on duty who reiterated the dosage instructions written on the labels of the dispensed medicines which included Di-

gesic® and diclofenac. However, no additional education or written medicines information were offered.

## 3.7 Discussion

Appropriate management of post-partum pain is essential for physical and psychological health and well-being of both mother and the baby (150). Although opioids have a place in therapy, their use in lactating women has been associated with adverse effects such as respiratory depression and death in breastfed infants (21, 151). Dextropropoxyphene hydrochloride, prescribed to the mother of this infant is a centrally acting, synthetic opioid analgesic structurally related to methadone (157). The half-life of dextropropoxyphene is 6 to 12 hours but the half-life of its metabolite, norpropoxyphene is 30 to 36 hours (167). Due to its long half-life, norpropoxyphene is known to accumulate in breastmilk and cause adverse effects especially in the first two months of an infant's life due to the underdeveloped infant excretory and metabolic systems (162, 163). Breastfeeding associated infant adverse drug reactions reported to the French pharmacovigilance centre attributes 11 of the 174 adverse drug reactions reported between 1985 and 2011 to maternal dextropropoxyphene use (21). Furthermore, several other cases of full term breastfed infants with unexplained episodes of apnoea, bradycardia or cyanosis during the first week of life have been attributed to maternal oral dextropropoxyphene (157, 158).

At the time of this incident there was adequate evidence linking dextropropoxyphene to adverse effects in breastfed infants to cause concern and warrant investigation of symptoms of toxicity in this infant. Despite this, frontline staff unreservedly dismissed maternal medicine use as a potential causative factor and assumed its safety because it was prescribed by an obstetrician. Although no opioid is considered absolutely safe in breastfeeding particularly with extended use, Di-gesic® seems to be a very questionable choice. Furthermore, the mother was ill-informed about the possible side effects of her medicines as she was not provided with adequate medicines education (written or oral) upon discharge from the hospital as required by legislation at the time.

All opioids have been shown to carry the risk of causing infant adverse effects and as such many regulatory bodies such as the Royal Australian and New Zealand College

of Obstetrics and Gynaecologists (RANZCOG) recommend the use of opioids be limited to the shortest duration possible (122, 168). In recent times, substantial evidence has emerged linking codeine, a commonly used weak opioid, with infant mortality. As a result, codeine is no longer recommended in lactation due to the risk of accumulation and infant mortality particularly in CYP2D6 ultra-rapid metabolisers (152, 153). Since the recommendations against codeine use in breastfeeding, tramadol and oxycodone are increasingly being utilised as the opioids of choice. While tramadol is considered safer in breastfeeding, it is a weak analgesic and may not provide adequate analgesia necessitating the use of more potent agents such as oxycodone (169). Evidence suggests that oxycodone is not much safer than codeine in causing infant adverse drug reactions, with some studies showing higher CNS depressant adverse effects compared to codeine (38, 122, 170, 171).

It is acknowledged that establishing a causal relationship between a clinical presentation in an infant and their exposure to medicines via breastmilk is inherently difficult, particularly when the signs and symptoms are not obvious or definitive. Most available breastfeeding related safety data is based upon small observational studies with short term follow up of less than a year. Long term studies or randomised controlled trials are generally not conducted in breastfeeding babies due to ethical considerations. This lack of large scale and quality breastfeeding related safety data may possibly lead to a concatenation of events whereby busy healthcare professionals are reluctant to commit resources to investigate and report such events to regulatory bodies which in turn prevents the generation of more data. This is apparent from research conducted by our team (unpublished data) which shows that breastfeeding related adverse events reported to the Therapeutics Goods Administration (TGA) is infrequent and incomplete. This case report is a perfect example of this phenomenon, where maternal concerns regarding transfer of dextropropoxyphene to the infant via breastmilk was not taken seriously and dismissed without appropriate investigation. Based on the reported interactions of the mother with doctors, it appears that unfamiliarity and a lack of knowledge about dextropropoxyphene were contributing factors as demonstrated by the doctor's remarks "*if the obstetrician prescribed it, it is safe*". Perhaps consideration should be given to the use of urine toxicology as part of the investigation of breastfed infants who present with non-specific symptoms where a history of maternal medicine use is

established. Liquid chromatography techniques are commonly used to reliably detect and quantify the presence of opioids including norpropoxyphene in urine samples (172, 173).

It is recognised that most doctors lack specialised breastfeeding related knowledge (102, 103, 174-176). The need for breastfeeding education to become an integral part of the medical school programmes has also been recognised by many researchers for a number of years (102, 103, 174-176). Patients associate dismissive behaviours and a lack of patient centered approach by doctors with patient dissatisfaction (177-179). In this case, the parents of this infant in retrospection expressed disappointment for not being provided any information about the mother's prescribed medicines especially the well-known side-effects of Di-gesic®. They reported having grave concerns for the health and safety of their newborn daughter when they witnessed her *"floppy body"* and her *"unresponsiveness to the pinching of her toes"*. The parents described spending three days in hospital and seeing their baby undergo a lumbar puncture as *"heart breaking and excruciatingly traumatic"* for what they believed could have *"possibly been a preventable cause"* but one that they *"will never find out for sure as breastmilk testing did not occur"*. The emotional pain undergone by the parents was described as *"far worse than the physical pain of the caesarean section"* by the mother who thereafter refused to take analgesics other than occasional paracetamol for the pain due to the fear of passing it to her baby through breastmilk. While they reported being grateful for the care that their daughter received at the hospital, they expressed displeasure at the dismissal of their concerns by the doctors and what they perceived as *"not being heard"* during a time that they described as *"the most traumatic experience for a new parent".*

## 3.8 Conclusion

The risk of infections and sepsis in a new born infant is not only high, but of great concern due to the associated morbidity and mortality, and should be thoroughly considered upon presentation of any signs and symptoms. However, other factors such as maternal medicine use (especially when high risk drugs with known adverse effects such as opioids are involved) in an exclusively breastfed infant should also be considered as an alternative diagnosis. It is therefore imperative that health professionals such as doctors, pharmacists and nurses are aware of the impact of

maternal medicine use and its potential effect in breastfed infants, particularly in the first two months of life, and ensure that it is not only managed but also investigated appropriately. Failure to give adequate consideration to maternal drug use may result in unnecessary investigative tests, inconclusive or inaccurate diagnosis and parental distress.

# Chapter 4 The role of efflux transporters in the transfer of medicines from maternal plasma to breastfed infant: A Longitudinal Study

## 4.1 Background

Most drugs enter breastmilk via passive diffusion (13, 68). Drug properties and pharmacokinetics play a key role in the determination of milk to plasma ratio (M: P), a measurement of the concentration of a drug in the breastmilk compared to its levels in the plasma at the same point in time. M:P is calculated using pharmacokinetic parameters such as drug lipophilicity, protein binding and pKa (180) . There are a number of drugs/toxicants such as lead, amisulpride, nitrofurantoin, acyclovir and cimetidine where the observed M:P has been significantly higher than their predicted M:P (65, 73, 75-79). This has been attributed to active transport mechanisms whereby transport proteins such as ABC transporters on the membrane of mammary epithelial cells actively pump these drugs into milk (76). The expression of these transporters has been found to vary between the lactating and the resting states of the human mammary gland, and animal studies suggest that their expression is lactation stage-dependent and vary as lactation duration increases. However, no studies investigating the lactation stage-dependent nature of active transporters have been undertaken in humans and little is known about their expression pattern in the lactating human mammary gland. A greater understanding of the expression pattern of these transporters can provide a useful insight into periods where the substrates of these transporters would have the greatest risk of being excreted into milk inadvertently exposing a breastfed infant and putting it at risk of toxicity.

Active cellular transport is mediated by integral cell membrane proteins like the ATP-binding cassette (ABC) superfamily. Active transporters are localised in the basolateral and apical membranes of the epithelial cells of various organs and their primary function is to transport molecules across the cell membrane using energy stored in adenosine triphosphate (ATP). The two main transporter superfamilies are ATP-binding cassette (ABC) transporters that primarily function as efflux transporters and the solute carrier (SLC) transporters. ABC transporters are one of the oldest gene family and are present in both prokaryotic and eukaryotic cells (181). Active transporters can be divided into two distinct classes a) importers (uptake) and b) exporters (efflux) as demonstrated in Figure 4.1. As uptake transporter or importer, these proteins mediate the uptake of essential nutrients into the cell. As an exporter, they are found in "vulnerable" sites such as the blood-brain barrier, blood placental barrier and blood-testes barrier, where they protect their vulnerable targets i.e. brain, developing foetuses and testis, respectively, from toxicity and harm by limiting access to these sites (182, 183). Efflux transporters (exporters) have been linked with multi-drug resistance in various disease states such as cancer where they contributed to treatment resistance by actively pumping the chemotherapeutic drug out of the cancer cells (184, 185).

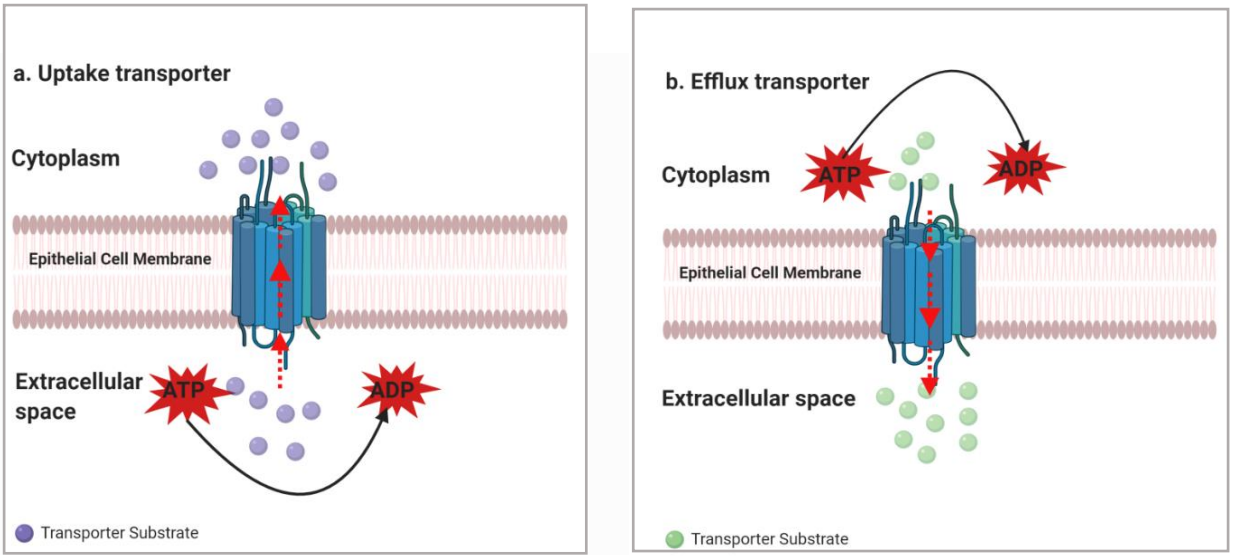



**Figure 4.1 Schematic of a primary active transporters such as ABC transporters utilising ATP for the transmembrane transport of a substrate against its concentration gradient as (a) an uptake transporter pushing the substrate into the cell and (b) as an efflux transporter transporting the substrate out of the cell into the extracellular space. (Figure created with Biorender.com)**

The role of efflux transporters in resistance to chemotherapy in breast cancer has been widely studied yet relatively little is known about their role in the lactating mammary gland (83, 186-188). In the lactating breast, the active transporters contribute to the nutrient content of breastmilk. For example, active transporters are essential in transport of vitamins such as riboflavin into the milk (85, 189). At the same time, they have also been shown to be involved in concentrating drugs and toxins in breastmilk, exposing breastfed infants to risk of drug-induced toxicity (76, 186).

The expression of active transporters in breastmilk derived mammary epithelial cells (MEC) has been shown to differentially express compared to resting MEC obtained through reduction mammoplasties (29). Transporters such as those belonging to the ATPase Binding Cassette (ABC) and the Solute Carrier (SLC) superfamilies are part of this large group with selectively varied expression (29). Although the role of these transporters in the mammary gland is still unclear, there is evidence of their differential expression between the lactating and non-lactating states suggesting that they have different functions in the differentiated gland compared to the undifferentiated gland. The composition of human breastmilk is known to be

strongly influenced by many maternal and environmental factors (190-193). Additionally, longitudinal animal studies indicate a lactation stage dependant fluctuation of the expression of active transporters in the lactating mammary gland (26, 30). However, longitudinal human studies looking at expression of active transport proteins is yet to be undertaken. If the expression of efflux transporters is lactation stage-dependent in humans and/or impacted by environmental factors, the composition of breastmilk will vary significantly between individuals making it rather difficult to predict the milk to plasma ratio of actively transported drugs. Variations in environmental and transporter expression profile would make it rather difficult to confidently and consistently determine a generalised drug safety profile during lactation and intra lactation-stage in the same woman at the different stages of breastfeeding. Hence, a personalised approach to the determination of drug safety is necessary, further highlighting the inadequacy of the currently used lactation compatibility categorisation of drugs.

## 4.2 Active transporters

In humans, there are two major families of active transporters, namely the ABC and the SLC. The SLC superfamily of transporters is the second largest family of membrane proteins after G protein-coupled receptors, but with relatively fewer known therapeutic drug substrates (194). The members of the SLC family of transporters mainly include organic anion-transporting polypeptides (OATP), organic anion transporters (OAT), organic cation transporters (OCT), organic cation/carnitine transporters (OCTN), peptide transporters (PEPT), and multidrug/toxin extrusions (MATE) (195). The ABC transporters are a large superfamily of proteins comprising of 49 members which are divided into seven subfamilies based on sequence similarities (196). BCRP, an ABC transporter is strongly induced during pregnancy and lactation and is known to contribute to the composition of breastmilk (75, 76, 197). In addition to BCRP, this thesis will focus on three other ABC transporters described in Table 4.1, as these have been implicated in multidrug resistance, drug disposition, drug-drug interactions and are also known to play a crucial role in defence against xenobiotics (198).

**Table 4.1 Key ABC transporters involved in drug absorption and disposition**

| **Key ABC transporters of interest** | | |
|---|---|---|
| **Protein name and synonyms** | **Tissue distribution** | **Polarized cell localization** |
| **ABCB1/P-gp/MDR1** | Blood-Brain Barrier, Liver, Intestines, Kidney, Placenta, Stem Cells | Apical |
| **ABCC2/MRP2** | Blood-Brain Barrier, Liver, Intestines, Kidney, Placenta, Lung | Apical |
| **ABCC1/MRP1** | Lung, Testes, Peripheral Blood Mononuclear Cells, Skeletal and Cardiac Muscles, Kidney, Placenta, Stem Cells | Basolateral |
| **BCRP/ABCG2** | Blood-Brain Barrier, Liver, Intestines, Kidney, Placenta, Breast | Apical |

Active efflux transporters are often expressed in tissues related to drug disposition, such as the small intestine, liver, and kidney, affecting intestinal absorption of drugs, uptake of drugs into hepatocytes, and renal/bile excretion of drugs. In the intestines, these proteins act as outward transporters thus reducing the bioavailability of their substrates (199). Efflux transporters are also expressed on the basolateral membrane of the intestinal polarized epithelium. These basolateral transporters are important in transporting drug metabolites (e.g. glucuronide conjugates) from enterocytes for those drugs undergoing first-pass metabolism (200). Consequently, the blood levels and the organ load of the drug are decreased, thereby reducing the efficacy of drug. Where the substrate is a toxicant, the action of these transporters work to remove them from the cell and reduce the risk of toxicity. In the context of drug disposition and drug interactions, P-glycoprotein (P-gp) also known as MDR1/ABCB1 is one of the most significant and widely studied active transporters. P-gp is predominantly found in the apical membranes of a number of epithelial cell types in the body and has a crucial protective role in the blood-brain barrier. P-gp is an efflux transporter that actively back-transports a large variety of hydrophobic amphipathic drugs out of the cell and is responsible for the poor penetration of many relatively large (>400 Da) hydrophobic drugs in the brain (201). The role of P-gp has been proven in numerous experiments with *in vitro* and *in vivo* models and with knockout mice where the absence of functional P-gp in the blood-brain barrier leads to highly

increased brain penetration of a number of important drugs (202). Another ABC transporter, the Breast Cancer Resistance Protein (BCRP) gets its name from its discovery in chemotherapeutic drug resistance in breast cancer even though it has subsequently been found in normal tissue through-out the body. BCRP has been shown to not only reduce the effectiveness of the chemotherapy drugs by actively pumping the drugs out of the cancerous cells but can also increase the risk of drug-drug interactions (203). The MRPs including MRP1 and MRP2 are also implicated in drug resistance in many disease states such as intractable epilepsy, breast and lung cancer (204-206). Drug-drug interactions occur when two competing drugs, either two substrates or a substrate and an inhibitor of the transporter, are co-administered. As shown in Figure 4.2 (a), co-administration of two substrates results in competition for the active transporter (an uptake transporter in this case), reducing the amount of the therapeutic drug reaching its active site and causing a suboptimal clinical response. Figure 4.2 (b) illustrates the case of a substrate being co-administered with an inhibitor, resulting in treatment failure because no drug (or sub therapeutic amounts) reach the target site.

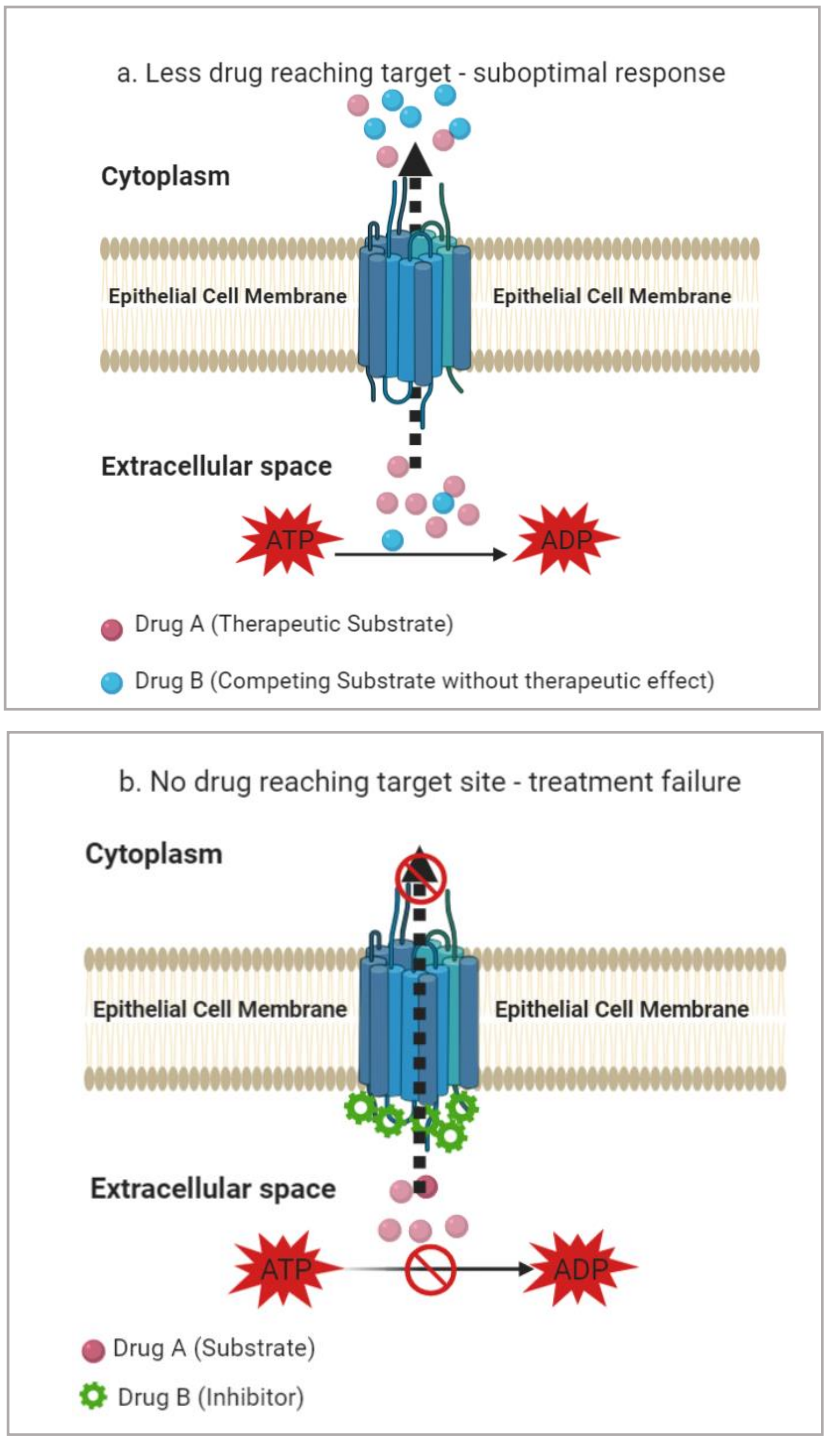



**Figure 4.2 Illustration depicting the function of ABC transporters in the presence of two competing substrates and inhibitors. a)When two substrates with differing therapeutic effects are competing to be transported across a cellular membrane by an active transporter, the competition leads to a reduced amount of the drugs reaching its target site, potentially leading to a reduced therapeutic outcome. b) When an active transporter substrate is competing with an inhibitor of the transporter, the inhibitor has the potential to block the transport capacity of the protein resulting in a lack of therapeutic response, resistance to treatment and treatment failure. (Figure created with Biorender.com)**

Only a few efflux transporters have been explored in context of drug kinetics and fewer yet have a well-defined role in the transport of clinically relevant drugs. ABC transporters such as P-gp, Breast Cancer Resistance Protein and Multi Resistant Associated Proteins (MRPs, mainly MRP1 and MRP2) are amongst those known to affect drug disposition. These transporters work together with metabolic enzymes such as CYP450 and often share common substrates (37, 198). It is important to note that due to the great degree of overlap between substrates and common tissue distribution of the various ABC transporters, together they have the capability to transport a wide range of molecules including cationic, anionic, neutrally charged molecules, conjugated organic anions such as those found in the environment, carcinogens, pesticides, metals and lipid peroxidation products (77, 207). Therefore, it is imperative to identify drug substrates of these transporters so that the risk of toxicity and interactions can be considered and mitigated.

## 4.3 Active transporters in the mammary gland

Due to the inherent difficulties in obtaining tissue samples of lactating mammary gland, breastmilk has been used as a non-invasive and reliable source of mammary epithelial cells (MEC). Alveolar and ductal as well as luminal-epithelial and myoepithelial cells are known to compose up to 99% of the cellular composition of human breastmilk and therefore is a reliable source, providing an insight into the molecular changes occurring in the gland (208).

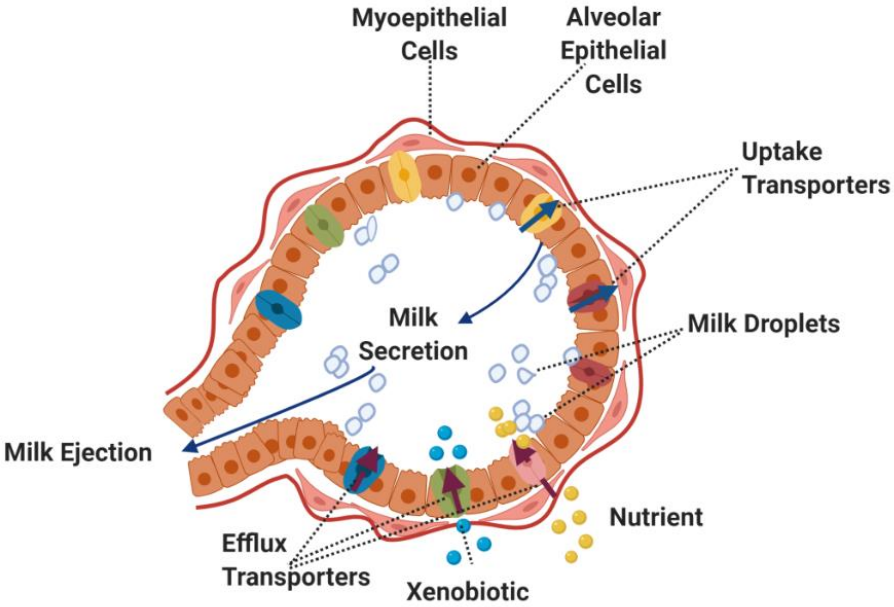



**Figure 4.3 Schematic representation of active efflux transporters in the lactating mammary gland. In the lactating mammary gland active transporters are located in the basolateral and apical membranes of alveolar epithelial cells. These transporters influence the composition of breastmilk by concentrating vitamins, nutrients, xenobiotics, drugs and pesticides into milk by pushing these substances against their concentration gradient. (Figure created with Biorender.com)**

In mammals, the mammary gland is an exocrine gland with a primarily secretory role that is responsible for the production of milk. As shown in Figure 4.3, the alveoli are lined with milk secreting epithelial cells and form the basic component of the mature mammary gland. Active transporters located in the basolateral and apical membranes of alveolar epithelial cells play a crucial role in increasing the nutrient content of breastmilk by concentrating certain vitamins and nutrients into breastmilk (37, 73, 74, 85, 189). Concomitantly, active transporters can also facilitate the secretion of toxic substances such as drugs, xenobiotics and pesticides into breastmilk which may be harmful to the nursing infant. The active efflux transporters usually assist in preventing accumulation of drugs into the tissues as they work against a concentration gradient and push drugs from the tissues back into the blood (29, 30, 77). However, the extent to which they affect drug transfer in the mammary gland is not fully understood (209). In the mammary gland, it is important to take into account the localization of these transporters. As demonstrated in Figure 1.6 (Chapter 1), their presence on the apical surface (MDR1, MRP2 and BCRP) may pump drugs

into milk and further place the suckling infant at risk of xenobiotic exposure (78). Conversely, when these transporters are located in the basolateral membrane of the cell (MRP1), then the substrate will be pumped out of the milk and into the mother's blood, thereby reducing infant exposure.

In the dairy industry, efflux transporters have been widely studied, due to their effect on the concentration of veterinary drugs in milk from animals where that milk is a human food source. There is potential for a negative health impact on the consumers of dairy if veterinary drugs accumulate in milk (78, 210, 211). By understanding the transfer mechanisms of drugs into milk, the use of potentially toxic substances can be avoided in these animals and risk of toxicity for the consumers of dairy is reduced. For example, ABCG2 has been identified as a major contributor to the accumulation of veterinary drugs in the milk of dairy cows (212). Animal studies have also shown and confirmed that there is a distinct variation in the expression of these transporters between the lactating and non-lactating mammary gland of animals, and that they are influenced by lactation stage (26, 30, 213).

In humans, the clinical impact of efflux transporters and their role in the transport of drugs from maternal plasma to the breastfed infant via breastmilk remains largely unknown. However, studies have shown a change in transporter RNA expression between lactating and non-lactating MEC with some transporters exhibiting multiple fold increases in gene expression (29). The expression of MRP1 (ABCC1), MRP2 (ABCC2) and MDR1 (ABCB1) are significantly lower in the lactating HMEC as compared to non-lactating HMEC whereas that of BCRP is significantly higher (76) . Longitudinal human studies have not been conducted to elucidate whether the expression of efflux transporters is lactation-stage dependent. However, a rat study highlighted the lactation-stage dependent expression of mammary epithelial cell transporters post-partum. Ling and co-workers discovered that the M:P ratio of the actively transported cephalosporin antibiotic drug, cefepime gradually reduced over time (86). This leads us to believe that as lactation progresses from stages of mammogenesis to lactogenesis to galactopoiesis, changes in the expression of efflux transporters along with changing hormones may influence the transfer of endogenous and exogenous substances from mother to baby via breastmilk. This means that the excretion of their various substrates (which includes many commonly used medicines as well as other xenobiotics) will vary between the different phases of

breastfeeding and consequently can expose a breastfed infant to unpredictable amounts of a xenobiotic, placing the infant at risk of exposure and toxicity.

## 4.4 Structure and function of active transporters

For this study four ABC active transporters (ABCB1, ABCG2, MRP1 and MRP2) with an established role in drug disposition were selected. These transporters are expressed in tissues important for absorption (e.g., lung and gut) and metabolism and elimination (liver and kidney) and therefore they have the ability to affect the pharmacokinetics (Absorption, Distribution, Metabolism and Elimination (ADME)) of substances that can be secreted into breastmilk (207).

### 4.4.1 P-glycoprotein (MDR1/ABCB1)

P-glycoprotein was first discovered in 1976 by Rudy Juliano and Victor Ling (214), who observed resistance to an anticancer chemotherapeutic agent in Chinese hamster ovary cells due to reduced permeability of the drug. Several years later the same transporter was found in the human BBB and in the apical membrane of most secretory tissues (215). P-gp is widely expressed in many other human tissues including the liver, kidneys, testis, placenta and the mammary epithelial cells (29). The main focus of research has so far been on the role of P-gp in cancer chemotherapy, including the bioavailability of drugs (including in the brain) and pharmacoresistance (184, 216). P-gp has been increasingly implicated in resistance to therapeutics in various tumours as it is readily induced in cancerous cells (184). Resistance is a result of the ability of these transporters to pump the anticancer drugs from the cell, lowering its concentration at the site of action and mediating drug excretion (217).

The important role of P-gp in the BBB, where it inhibits a wide range of substrates from entering the brain has garnered significant research interest (77, 218). P-gp also plays a crucial protective role during embryogenesis where it is expressed in the placenta providing foetal protection against naturally occurring toxins (219). Data regarding the role of P-gp in the lactating mammary gland is inconclusive. While some animal and *in vitro* studies suggest that P-gp may play a role in drug transfer in breastmilk, others suggest that P-gp is considerably downregulated during lactation, and thus its role in the transfer of medicines is relatively insignificant (29, 220).

Down regulation of P-gp in the lactating mammary epithelial cells theoretically can have a protective effect on the breast fed infant as this down regulation would mean less extrusion of its substrates into breastmilk. For example, very little nelfinavir, a P-gp substrate and HIV protease inhibitor, was excreted in murine milk, further supporting this theory (220). Some clinically relevant substrates of P-gp that may have implications in lactation include a wide range of antibiotics, domperidone, antihistamines such as fexofenadine, acid lowering drugs such as ranitidine and antihypertensives such as nifedipine (77, 221). Furthermore, the relevance and applicability of animal studies to humans should be closely considered. Most animal species used in the laboratory such as rats and mice have two genes (Mdr1a and Mdr1b) that code for P-gp in contrast to the one human gene (MDR1). This further complicates issues regarding induction, expression and drug-drug interactions (222). Therefore, in vitro models and animal studies may not always be conclusive and in some cases could be misleading due to the differences in experimental design and use of non-human systems and transporters (222-224).

One of the most striking features of P-gp is its diverse range of substrates which includes mostly hydrophobic, uncharged, weakly basic, acidic organic compounds of between 200 to 1900 Daltons containing both aromatic and non-aromatic (circular or linear) molecules (77). The only commonality between P-gp substrates seem to be their amphipathic nature. Table 4.2 lists common known substrates of P-gp (215, 221, 225, 226). Considering the role and function of P-gp in other organ systems, co-administration of medicines that are substrates or inhibitors of this transporter in a lactating mother could have potential safety consequences for the nursing infant.

**Table 4.2 List of known P-gp substrates**

| |
|---|
| Analgesics: asimadoline, fentanyl, morphine, pentazocine |
| Antiarrhythmics: amiodarone, digoxin, lidocaine, propafenone, quinidine, verapamil |
| Antibiotics: cefoperazone, ceftriaxone, clarithromycin, doxycycline, erythromycin, gramicidin A, gramicidin D, grepafloxacin, itraconazole, ketoconazole, levofloxacin, rifampicin, sparfloxacin, tetracycline, valinomycin |
| Anticancer drugs: 5-fluorouracil, actinomycin D, bisantrene, chlorambucil, colchicine, cisplatin, cytarabine, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, gefitinib, hydroxyurea, irinotecan (CPT-11), methotrexate, mitomycin C, mitoxantrone, paclitaxel, tamoxifen, teniposide, topotecan, vinblastine, vincristine |
| Antihistamines and acid lowering drugs: cimetidine, fexofenadine, ranitidine, terfenadine |
| Antilipidemic: lovastatin, simvastatin |
| Calcium channel blockers: azidopine, bepridil, diltiazem, felodipine, nifedipine, nisoldipine, nitrendipine, tiapamil, verapamil |
| Fluorescent dyes: calcein AM (calcein acetoxymethylester), Hoechst 33342, rhodamine 123 |
| HIV-protease inhibitors: amprenavir, indinavir, lopinavir, nelfinavir, saquinavir, ritonavir |
| Immunosuppressive agents: cyclosporin A, cyclosporin H, FK506, sirolimus, tacrolimus, valspodar (PSC-833) |
| Natural products: curcuminoids, flavonoids |
| Neuroleptics: chlorpromazine, phenothiazine |
| Others: BCECF-AM, bepridil, calcein-AM, endosulfan, leupeptin, methyl parathion, paraquat, pepstatin A, trifluoperazine, trans-flupentixol |

### 4.4.2 Breast Cancer Resistance Protein (BCRP)

The human breast cancer resistance protein is an ATP Binding Cassette transporter protein which was first cloned from a multidrug-resistant breast cancer cell line where it was found to confer resistance to chemotherapeutic agents such as mitoxantrone and topotecan (186). BCRP is known to have many physiological roles in mammalian tissues including the mammary gland, blood–brain, blood–testes, and maternal–foetal barriers. Similar to P-gp, BCRP has been characterized as an important element in self-defence systems, where apically expressed BCRP is

protective by eliminating substances from the maternal circulation, bile ducts, or intestinal lumen, via placental syncytiotrophoblasts, hepatocytes or intestinal mucosal cells respectively (227-229). BCRP is co-expressed in the majority of tissues, and shares many of its substrates with MDR1 as summarised in Table 4.6 (230). BCRP substrate rosuvastatin has been implicated in drug-drug interactions (DDI) especially with drugs that also inhibit OATP (organic anion transporters) such as cyclosporine. It has been postulated that the synergistic action of BCRP, MDR1, and the drug-metabolizing enzyme CYP3A4, particularly in the gastrointestinal tract can impact on the pharmacokinetics of many drug substrates (231). Despite the widespread expression of this protein in various tissues and its substrate poly-specificity, BCRP is not a viable therapeutic target. However, it is included in the list of important drug transporters that both the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) consider necessary to investigate for New Chemical Entities. Drugs whose ADME, and bioavailability in particular, is influenced by BCRP may require clinical investigation to reveal a potential DDI with potent clinical BCRP substrates and inhibitors.

It is known that BCRP is strongly induced and expressed during pregnancy and lactation (76). The expression of BCRP has been shown to be significantly higher in the mammary glands of several species including sheep, goat and cow (up to 10 fold) during pregnancy and lactation (31, 232). Both P-gp and BCRP are located in the apical membrane of alveolar epithelial cells of the mammary gland and actively transport their substrates into breastmilk in animal studies (76, 78). Due to its upregulation, BCRP has a significant role in accumulation of drugs and xenotoxins in breastmilk which could be either beneficial or detrimental to the breastfed infant's health depending on the drug administered (78). A BCRP substrate that is toxic can accumulate in milk and result in adverse effects in the infant whereas the accumulation of a drug such as aciclovir could be beneficial in reducing transmission of milk borne viruses from mother to baby. As shown in Table 4.2, one of the naturally occurring substrates of BCRP includes PhIP (2-amino-1-methyl-6-phenylimidazole [4, 5-b] pyridine), a known carcinogen, and major constituent of cigarette smoke and over-cooked meat (76). This compound has been found to be concentrated in milk, long term consumption of which can potentially cause carcinogenesis (77). Van Herwaarden. found that other substrates of this transporter such as heterocyclic amines and aflatoxin are also transferred into milk by BCRP, thereby posing a health risk to breast-fed infants and dairy consumers (197).

Table 4.3 contains a list of known BCRP substrates, some of which may cause infant ADRs if transferred into breastmilk (221, 233).

**Table 4.3 List of select known substrates of BCRP**

| |
|---|
| Antibiotics: ciprofloxacin, norfloxacin, ofloxacin, |
| Anticancer drugs: daunorubicin, doxorubicin, epirubicin, etoposide, gefitinib, imatinib, irinotecan, mitoxantrone, methotrexate, SN-38, teniposide, topotecan, diflomotecan |
| Antivirals: delavirdine, lopinavir, lamivudine, nelfinavir, zidovudine, dolutegravir |
| Antihypertensives: reserpine |
| Calcium channel blockers: nicardipine |
| Lipid lowering drugs: cerivastatin, pravastatin, rosuvastatin |
| Others: azidothymidine, chrysin, cyclosporin A, lamivudine, ortataxel, quercetin, sulfasalazine, coumestrol |

### 4.4.3 Multidrug Associated Resistance Protein (MRP1)

The multidrug resistance-associated protein (MRP1/ABCC1) is the first of the nine ABCC transporters to be originally identified in a lung cancer cell line which is chemoresistant to doxorubicin and other chemotherapeutic drugs. Over the last 20 years, the clinical importance of MRP1 has been highlighted further as it has been shown to affect drug response and prognosis in a number of malignancies (234). Additionally, MRP1 has many other physiological functions: it is involved in acute and chronic inflammation, cell metabolism, differentiation, proliferation, survival, and cell-cell communication. These processes influence a host of human diseases including cancer. Hence, MRP1 is thought to be more than a drug transporter or efflux pump and it has a much broader impact on human health and diseases which is yet to be fully understood (235). Studies in ABCC1 knockout mice have shown that Mrp1/MRP1 is an important determinant of drug disposition due to its presence in blood-organ barriers or pharmacological "sanctuary" sites (235). Substrates of MRP1 as shown in Table 4.4 include hydrophobic natural products, antineoplastic agents (*e.g.* vincristine, doxorubicin), various antibiotics, opiates, antiviral agents, citalopram, and statins (221, 235-238)

**Table 4.4 List of known substrates of MRP1**

| |
|---|
| Antibiotics: ciprofloxacin, norfloxacin, ofloxacin, grepafloxacin |
| Anticancer drugs: daunorubicin, doxorubicin, epirubicin, etoposide, gefitinib, imatinib, irinotecan, mitoxantrone, methotrexate, SN-38, teniposide, topotecan |
| Antivirals: delavirdine, lopinavir, lamivudine, nelfinavir, zidovudine, saquinavir |
| Antihypertensives: reserpine |
| Calcium channel blockers: nicardipine |
| Lipid lowering drugs: cerivastatin, pravastatin, rosuvastatin |
| Others: azidothymidine, chrysin, cyclosporin A, lamivudine, ortataxel, quercetin |

The expression of MRP1 has been shown to be altered in lactating MEC compared to resting/non-lactating MEC such that it decreases gradually throughout lactation (29, 30, 76). The role and function of MRP1 in the transfer of nutrients in the lactating mammary gland is not well understood but given its broad substrate base, it is possibly involved in mechanisms for the transport of breastmilk constituents. Murine model studies suggest that the expression of this transporter is decreased during gestation and lactation (239). Additionally, recent studies indicate that its expression

is influenced by mastitis resulting in significant upregulation during infection (239, 240).

### 4.4.4 Multidrug Resistance Associated Protein 2 (MRP2)

The multidrug resistance associated protein 2 (MRP2/ABCC2) is an ATP-binding cassette transporter which was first cloned from rat liver and was known as cMOAT (canalicular multi-specific organic anion transporter). MRP2 is an organic anionic transporter which transports a number of compounds, mainly conjugates of lipophilic substances with glutathione, glucuronate and sulphate, which are products of phase II biotransformation (228). Hence, MRP2 plays an important role in detoxification and chemoprotection. Additionally, MRP2 can also transport uncharged compounds in cotransport with glutathione, and thus can modulate the pharmacokinetics of many drugs. MRP2 is specifically expressed on the apical membrane domain of polarised cells such as hepatocytes, renal proximal tubular cells, enterocytes and syncytiotrophoblasts of the placenta, often together with MDR1 (77, 199). Genetic mutations resulting in the absence of MRP2 have been associated with diseases such as human Dubin-Johnson syndrome which causes conjugated hyperbilirubinaemia (199). Table 4.5 lists some of the known substrates of MRP2 (199, 221, 238).

**Table 4.5 List of substrates of MRP2**

| |
|---|
| Anticancer drugs: cisplatin, doxorubicin, epirubicin, etoposide, irinotecan, mitoxantrone, tenitoposide, daunorubicin, idarubicin, vincristine and vinblastine |
| HIV drugs: adefovir, cidofovir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir |
| Others: arsenite, cysteinyl $LTC_4$, conjugated estrogen, $E_2$17βG, reduced and oxidized glutathione (GSH and GSSG), fexofenadine, Ezetimib |

In the mammary gland, MRP2 expression is low compared to other tissues (241). Despite the low levels of expression of this transporter, a small decrease (-1.2-fold) is seen in the lactating MEC compared to the resting tissue suggesting downregulation. However, the significance of this is not well known. Herbal drugs such as St John's Wort have been implicated in increasing the expression of liver MRP2 in mothers consuming this product during pregnancy (241). However, its effect is not known on mammary MRP2. Table 4.4 shows a list of known common substrates of MDR1, BCRP and MRP2 (242).

**Table 4.6 Comparison of substrate specificity between different active efflux transporters**

| Drug name | MDR1 | BCRP | MRP2 |
|---|---|---|---|
| Vinblastine | Yes | No | Yes |
| Vincristine | Yes | No | Yes |
| Doxorubucin | Yes | Yes | Yes |
| Mitoxantrone | Yes | Yes | No |
| Topotecan | Yes | Yes | No |
| Quinidine | Yes | No | No |
| Indinavir | Yes | No | Yes |
| Ritonavir | Yes | No | Yes |
| Nelfinavir | Yes | No | Yes |
| Ciprofloxacin | Yes | No | Yes |
| Levofloxacin | Yes | No | Yes |
| Calcium AM | Yes | No | Yes |
| Rhodamine 123 | Yes | Yes | Yes |

## 4.5 Aim

The aim of this study was to investigate the expression profile of four efflux transporters namely MDR1, MRP2, BCRP and MRP1 in the human mammary gland using epithelial cells that are sloughed into breastmilk during lactation. This longitudinal study aimed to investigate the changes in the expression of these transporters as lactation duration increased, and to ascertain if these could impact the transfer of xenobiotics, drugs and toxins that are substrates of these transporters into breastmilk. These transporters were chosen as they have been implicated in drug disposition.

## 4.6 Materials and Methods

### 4.6.1 Ethics approval and participant recruitment

This study was approved by the Human Research Ethics Committee of Curtin University (HR110/2012). Breastfeeding women who intended to breastfeed for a minimum of six months were recruited during pregnancy or early postpartum through word of mouth, and advertising through lactation consultants at two maternity hospitals (St John of God Hospital Subiaco and St John of God Hospital Murdoch). These hospitals did not have a requirement for additional ethics approval

and considered the university approved ethics application sufficient. Meetings were held with senior nurses and midwives, who agreed to display a poster (Appendix A) in their maternity suites and in antenatal classes. The inclusion criteria for this study included healthy pregnant women not on any prescribed medications (other than prenatal vitamins and supplements) with an intention to breastfeed for 12 months. Withdrawal from the study was explained to be at the discretion of the participant. Participants were excluded from the study if they or their newborn had developed medical conditions requiring long-term treatment or if they had inadequate milk supply. Through this recruitment process, 27 healthy pregnant women intending to breastfeed for 12 months or more were enrolled in the study. All participants were provided with an information sheet detailing the requirements of the study and all participants provided written informed consent. Over the duration of this study five participants withdrew at various stages due to inadequate milk supply, cessation of breastfeeding and post-partum medical conditions. Participants with less than three donated samples were also excluded from the study.

### 4.6.2 Sample collection

Breastmilk samples were collected at 5 time points representing increasing months post-partum; namely 1 (T1), 3 (T2), 5 (T3), 9 (T4) and 12 (T5) months post-partum. When participants were recruited, they indicated an intention to breastfeed for 12 months. However, many participants did not breastfeed for the entire year. Therefore, all five timepoints are available for only 10 participants. In this study we controlled for factors that are known to influence gene expression and/or breastmilk composition including fore vs hind milk, maternal drug/alcohol use, maternal and infant infection, differences in pumps used (or manual expression), maternal diet, and maternal general health (243). All participants were healthy and were not taking any prescribed medications during the study period. At the time of sample collection, the breastfeeding dyads were required to be healthy with no signs of local or systemic infections as infections are known to affect the cellular composition of breastmilk (208, 244). Participants were provided with a Pigeon electric breast pump and they were asked to use the pump when expressing breastmilk for our study. Mothers were instructed to express milk after feeding the baby and at around the same time of the day for each collection. Mothers were asked to inform the investigator if they or their baby were unwell with any minor illnesses. Collection dates were postponed until the dyad had fully recovered.

#### 4.6.2.1 Quantitative Real-Time Polymerase Chain reaction (qRT-PCR)

An established RT-PCR protocol as per Hassiotou's laboratory was used for this assay and is described below (245).

#### 4.6.2.2 Breastmilk sample collection

Participants expressed breastmilk samples using an electric breast pump under aseptic conditions. Samples were protected from light and transported to the laboratory at room temperature immediately after being expressed. Milk samples were processed and breastmilk derived cells were measured for viability and RNA was extracted for RT-PCR analysis.

#### 4.6.2.3 Breastmilk cell isolation

Breastmilk was diluted with equal amounts of sterile phosphate buffered saline (PBS pH 7.4, Gibco, Grand Island NY) and centrifuged at 800 *g* for 20 minutes at 20°C. After the removal of the skim milk and the fat layer, the cell pellet was washed with PBS twice, centrifuged at 400 *g* for 5 minutes and resuspended in PBS. Cell numbers and viability was determined using a Neubauer haemocytometer by Trypan Blue (0.4%) exclusion. The cell pellet was stored at -80°C until RNA extraction. Blood derived cells were not isolated from lactocytes and the myoepithelial cells, primarily because the immunological cells were expected to be largely washed off during the isolation and washing process as they are lighter than epithelial cells. Therefore, the blood derived cells were not expected to have a significant impact on dilution of the samples (244-246).

#### 4.6.2.4 RNA extraction

Total RNA was extracted with the mini-RNeasy extraction kit (Cat No. 74104; Qiagen, Valencia, CA) following manufacturer's directions. Cell pellets were incubated in 600 µL of RLT buffer for 10 minutes and transferred to a separate microfuge tube where they were triturated through a 21G needle syringe 10 times to ensure cell rupture was complete. The cell lysates were then mixed with an equal volume of 70% ethanol before centrifuging it through the supplied spin column at maximum speed of 8000 *g* for 30 seconds. This was followed by the addition of 700 µL of RW1 solution to the spin column and centrifuging at 8000 × *g* for 30 seconds. Then 500 µL of RPE buffer was added and the spin column was spun at 8000 × *g* for 30 seconds. This was repeated after the flow through was discarded, and the column was centrifuged for 2 minutes at 8000 *g*. The spin column was placed in a sterile new tube and 30 µL of RNAse free water was added to the centre of the column. After a 10-minute incubation period on ice, the column was spun at 8000 ×*g* for one minute and the flow through containing the RNA was collected and measured using a Nanodrop® 1000 spectrophotometer. RNA quality was determined to be acceptable only if 260/280 absorbance ratio was 1.9 to 2.1.

#### 4.6.2.5 cDNA generation

Total RNA was reverse transcribed using the high-capacity cDNA kit (Applied Biosystems, Carlsbad, CA) following manufacturer's directions. The cDNA contains RT Buffer (10×), RT Random Primers (10×), 100mM dNTP Mix (×25) and MultiScribe Reverse Transcriptase (50 U/µL). The prescribed volumes of each component contained within the kit to make up the cDNA master mix. A 50 µL reaction was created by adding the master mix to 25 µL of the RNA diluted in ultrapure RNAse free water (Gibco®). Samples were incubated in a Bio-Rad® C1000 96 well gradient block thermo cycler and held at 25° C for 10 minutes, 37° C for 120 minutes, 85° C for 5 minutes and held at 4°C. The cDNA was stored at -20° C until required for quantitative real-time polymerase chain reaction (*q*RT-PCR).

#### 4.6.2.6 Quantitative Real-Time Polymerase Chain Reaction (*q*RT-PCR)

Gene transcription was quantified by PCR using hydrolytic probes (Table AppE1) (Taqman®; Applied Biosystems) with the 7500 FAST RT-PCR system (Applied Biosystems). Each sample was measured in triplicate or in a few cases in duplicate when the extracted RNA was inadequate. Genes were standardised to MCF10A (a normal human mammary epithelial cell line) and each sample was controlled with housekeeping gene, Glyceraldehyde 3-phospahe dehydrogenase (GAPDH). GAPDH expression was monitored for stability during the different timepoints. Fold change in gene expression for each sample and experimental condition was calculated as $2^{Ct(control)-Ct(sample)} \pm$ SD and relative quantitation was determined for each replicate. Repeated measures of the samples were averaged, and the standard deviations were calculated. Standard deviations were used for quality control of the data and means were used for statistical analysis.

### 4.6.3 Protein quantitation by iTRAQ (Isobaric tags for relative and absolute quantitation)

iTRAQ allows four, six, or eight samples to be multiplexed in a single run and is a quantitation method that is increasingly being used in biological sciences. As the iTRAQ tag has a balancer group to equalize all states of a labelled peptide to the same mass, the differentially labelled iTRAQ peptides are mixed before chromatography and elute as a single combined peak in mass spectrometry.

Breastmilk cells were isolated as described above and were stored at -80 °C until processing. Samples from eight donors, each of whom had provided milk samples for all five timepoints (1, 3, 5, 9 and 12 months post-partum), were pooled per timepoint for this assay. As this assay was being carried out with 4-plex reagents, we were restricted to the use of only four of the five available timepoints. An additional assay to include the remaining timepoint could not be undertaken due to the significant cost implications. A decision to exclude timepoint2 (T2) ($3^{rd}$ month post-partum) was made as this timepoint fell close to two other timepoints (1-month post-partum and 5 months post-partum). Additionally, RT-PCR showed a peak in the expression of BCRP at 5 month (T3) post-partum. Therefore, in this assay T1 (1-month post-partum), T3 (5 months post-partum), T4 (9 months post-partum) and T5 (12 months postpartum) were used.

iTRAQ analysis was undertaken as described by Casey (247). Breastmilk cells were isolated as described above. The sample with the least amount of protein (19.5 µg) was used as the standard mass for the 8 samples at each timepoint. The eight samples were combined for each of the 4 timepoints and the resultant 4 protein samples were analysed for protein concentration using the Direct Detect infrared method [Merck Millipore]. 100 µg of each of the four samples were desalted, reduced, alkylated and trypsin digested according to the iTRAQ protocol (Sciex). The four samples were then labelled using the iTRAQ reagents. All labelled samples were combined to make a pooled sample. Peptides were desalted on a Strata-X 33 µm polymeric reversed phase column (Phenomenex) and dissolved in a buffer containing 2% acetonitrile and 0.1% formic acid. The sample was analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence Nano HPLC system [Shimadzu] coupled to a 5600 TripleTOF mass spectrometer (Sciex). Peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm column (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). 4 µg of the pooled sample was loaded on the mass spectrometer. Spectral data was analysed against *Homo sapiens* peptide database using the SwissProt database, facilitated by ProteinPilot™ 5.0 software (Casey, 2016, Proteomics International results report, Appendix F)

### 4.6.4 Immunostaining

Immunostaining is not a recommended quantitative method for determining protein abundance and quantification. However, with the low total protein in these breastmilk samples, a more accurate assay such as Western Blot could not be performed. Therefore, immunostaining was chosen to be used as a semi-quantitative method given the limited supply of protein.

#### 4.6.4.1 Breastmilk cell isolation

The cells in the collected breastmilk were isolated as described in section 4.6.1.3.

#### 4.6.4.2 Processing of breastmilk derived cells for immunostaining

Freshly isolated cells were fixed (formaldehyde 4%; sucrose 2% in sterile PBS) for 15 minutes at room temperature. Fixed cells were placed on glass slides followed by centrifugation in supplied cradles in the MPW223c centrifuge® (HD Scientific Supplies) at 1000 RPM for 3 minutes to dry the pellet. Cells were then permeabilised on the slide using 0.2% Triton-X100 in PBS (PBST), followed by blocking with 4% foetal bovine serum in PBST for 30 min. The cells were then washed with PBS and incubated for 4 hours or overnight with mouse primary antibody. Optimised antibody concentrations were used as per Table App E2. After washing the cells three times, they were incubated with AF594 labelled secondary goat anti-mouse antibody for one hour. For each sample, a secondary only negative control was used. Nuclear staining was achieved by using a DAPI mountant (Slowfade Gold Antifade Mountant® - Life Technologies). The cells were then visualised and imaged using UltraView confocal microscope (PerkinElmer).

#### 4.6.4.3 Image analysis

Slides were analysed on an Ultra VIEW VoX confocal imaging system with Volocity 6.0.1 software (PerkinElmer, Massachusetts, USA). HMEC that adhered to the slides varied between samples. In order to have a random selection of cells and get an appropriate representation of the cell population, ten random images were recorded from each slide at two different wavelengths (480 nm for DAPI and 594 nm for Alexa Fluor 594). The images were then overlaid, and a 50% margin (mask) was created around the nuclei. Protein abundance was calculated as a proportion of the total mask area that emitted red light (Alexa Fluor 594) indicating that antibody binding had occurred. The student t-test and one-way analysis of variance (ANOVA) were used to determine differences between the different timepoints in GraphPad PRISM® 8.0. Differences were considered significant if $p<0.05$.

## 4.7 Results

### 4.7.1 Sample details

A total of 88 breastmilk samples from 22 participants were used for this study. The demographic characteristics of the study participants (N=22) and the breastmilk sample characteristics (n=88) are shown in Table 4.7.

**Table 4.7 Demographic and breastmilk sample characteristics of the study participants (n=22).**

| | **Median** | **Range** |
|---|---|---|
| **Maternal Characteristics** | | |
| Age (years) | 31 | 21 - 36 |
| Parity | 1 | 1 - 2 |
| **Breastmilk samples** | | |
| Cell viability (%) | 99 | 93 - 100 |
| Volume of breastmilk provided (mL) | 64 | 10 - 190 |
| Total breastmilk cell count ($\times 10^6$) | 17.7 | 0.028 - 585 |
| Breastmilk cell content (cell/mL milk, $\times 10^5$) | 3.49 | 0.06 - 35.5 |

### 4.7.2 *q*RT-PCR

Gene expression was determined by relative quantitation (RQ) compared to the control MCF10A. PCR showed that the gene expression for BCRP was higher compared to the other three transporters as shown in Figure 4.4. While the RQ for BCRP was in the thousands, MDR1 expression was in the tens, peaking at 30, and MRP1 and MRP2 were in the hundreds. The data for MRP1 and MRP2 showed a great degree of variation and consequently comprised a significant number of outliers.

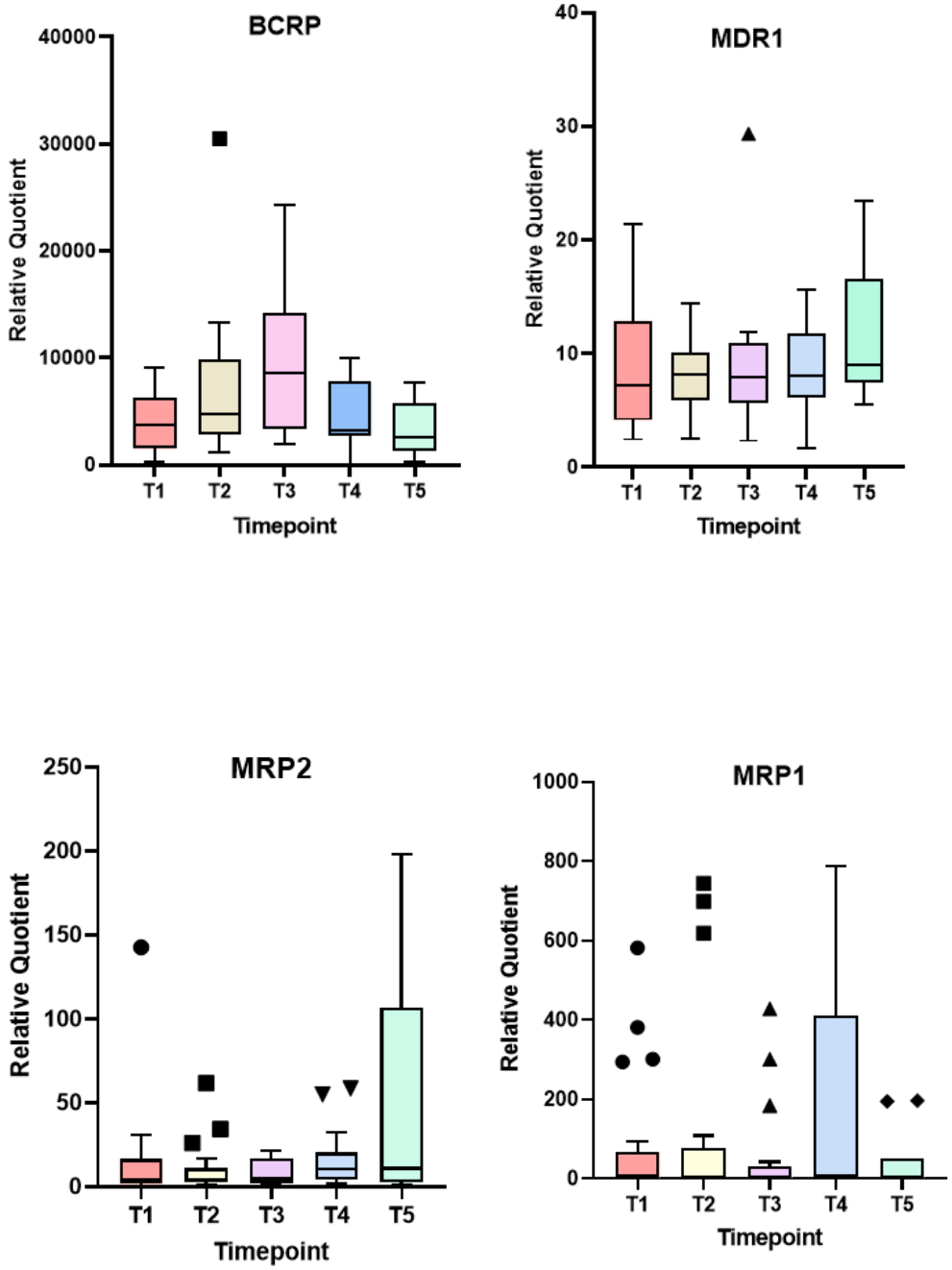



**Figure 4.4 Distribution of gene expression (RQ) of BCRP, MDR1, MRP2 and MRP1 by human breastmilk cells in 88 milk samples from 22 participants. Box plots represent gene expression distribution where tails show the minimum and maximum values (excluding outliers) and upper and lower interquartile ranges; middle line represents the median. (● = T1 outlier, ■ = T2 outlier, ▲= T3 outlier, ▼= T4 outlier, ♦ = T1 outlier). Individual PCR reactions were normalised against internal control (GAPDH) and relative to the expression level of MCF10A. Bars represent the mean±SEM.**

#### 4.7.2.1 Statistical analyses

All data were tested for normality. While data for BCRP and MDR1 largely passed normality tests using Shapiro-Wilk and Kolmogorov-Smirnov tests, MRP1 and MRP2 did not pass normality tests due to the significant sample variation. Due to the magnitude of difference between the mRNA expression of BCRP and the other three transporters (MRP1, MRP2 and MDR1), their clinical relevance in drug disposition is uncertain. However, further studies will need to be done to confirm this. This analysis will focus largely on the expression of BCRP, the most abundantly expressed and therefore predicted to have the largest significance in transfer of xenobiotics from maternal plasma to breastmilk.

Data were analysed by fitting a mixed model rather than by repeated measures ANOVA due to missing data for some timepoints. The mixed effects analysis showed that the expression of BCRP was statistically significant between the different timepoints ($p = 0.0063$). Post hoc analysis was performed using Tukey's multiple comparison test which confirmed a significant difference in the expression of BCRP over the 5 timepoints as stated in Table 4.8 and Figure 4.5. Inter-individual differences and changes in the expression of BCRP in each woman over time was also significant as demonstrated in Figure 4.6 and further analysed in Table App E3. In 18 of the 22 participants, variations in BCRP expression over time were found to be statistically significant ($p<0.05$) with peak levels most often occurring at T3.

**Table 4.8 Mixed effects model using multiple comparisons test for BCRP**

| Tukey's multiple comparisons test | Significant | Adjusted *P* Value |
|---|---|---|
| T1 vs. T2 | No | 0.1908 |
| **T1 vs. T3** | **Yes*** | **0.0176** |
| T1 vs. T4 | No | 0.748 |
| T1 vs. T5 | No | 0.9857 |
| T2 vs. T3 | No | 0.2855 |
| T2 vs. T4 | No | 0.9975 |
| **T2 vs. T5** | **Yes*** | **0.0406** |
| T3 vs. T4 | No | 0.9988 |
| **T3 vs. T5** | **No** | **0.0509** |
| **T4 vs. T5** | **Yes*** | **0.0176** |

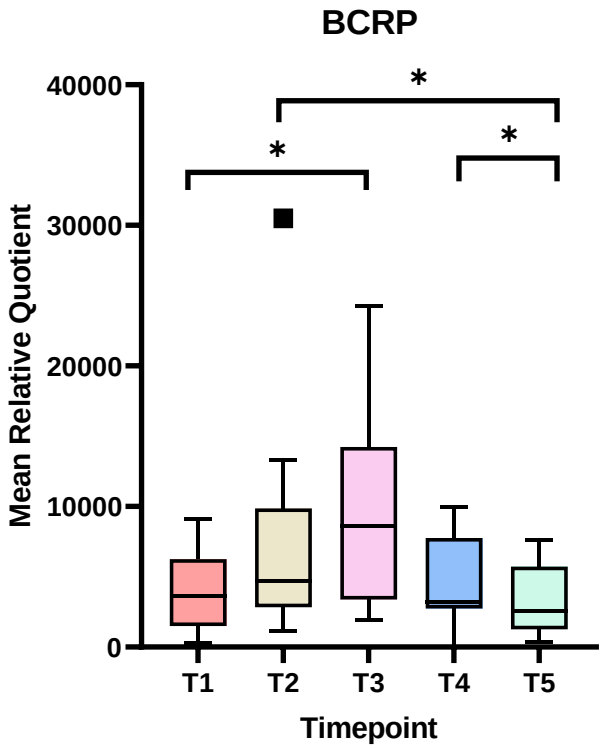



**Figure 4.5 Longitudinal expression of BCRP over 12 months (Timepoints 1 to 5) of lactation in 22 participants. (■ = T2 outlier)**

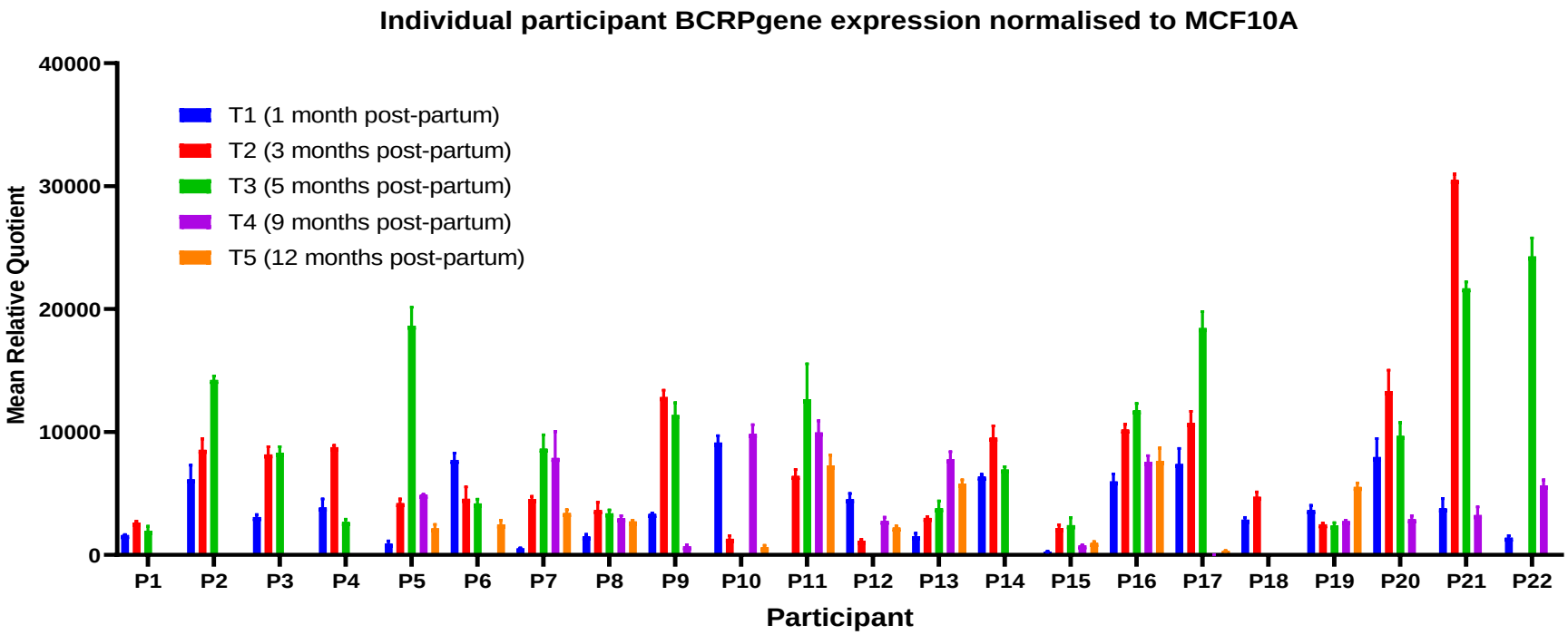



**Figure 4.6 Quantitative real-time polymerase chain reaction (qRT-PCR) assay for longitudinal expression of BCRP by human breastmilk cell samples from 22 participants (P1 – P22) over five time points (T1-T5). Individual PCR reactions were normalised against internal controls (GAPDH) and plotted relative to the expression level of the MCF10A. Bars represent the mean ± SEM. Supporting information in Table App E3.**

### 4.7.3 Isobaric tags for relative and absolute quantitation (iTRAQ)

The iTRAQ proteomics analysis did not detect any transporter proteins. A limitation of the proteomics analysis is that proteins that are present in large quantities can overshadow and block the detection of those expressed in smaller quantities. As expected, the proteins of interest were not detected by this isobaric labelling method. However, this technology allowed the evaluation of the changing properties of breastmilk at a cellular level longitudinally over a lactation period of 12 months post-partum. As shown in Table 4.9 and Table 4.10, a number of breastmilk proteins (n=17) were found to be differentially expressed, with 10 being downregulated and 7 being upregulated over time.

**Table 4.9 iTRAQ Summary results showing a total of 143 proteins with 2 or more peptides (95%CI) detected in the breastmilk cell protein samples with 17 of these proteins being differentially expressed.**

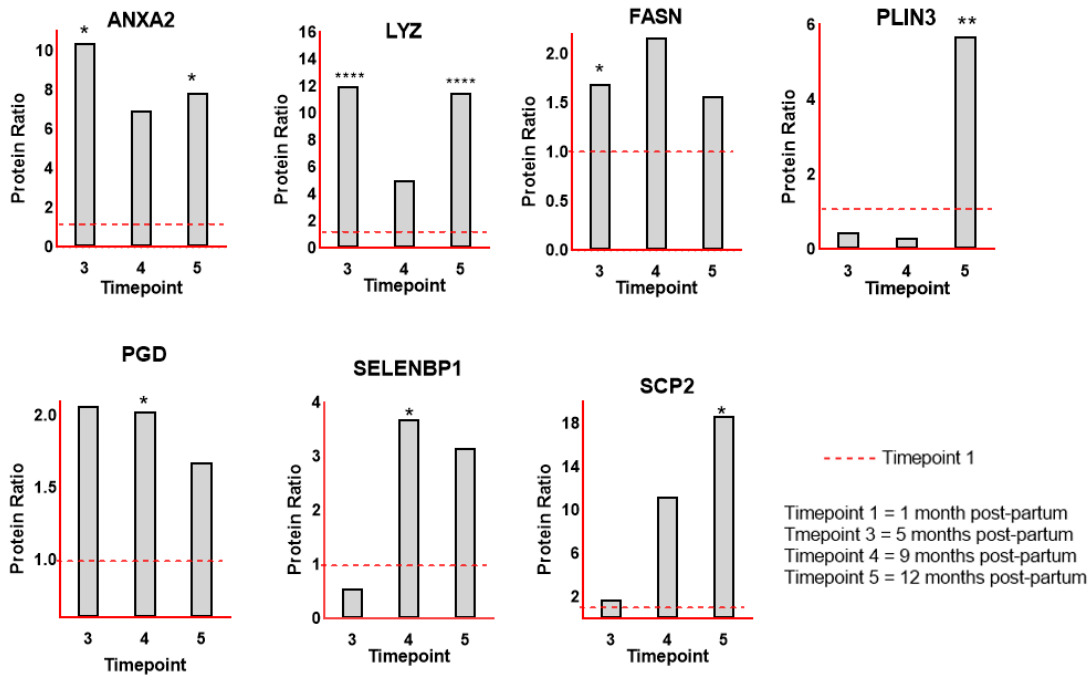
| Features | | Value |
|---|---|---|
| No. of proteins detected (≥1 peptide with >95% confidence) | | 203 |
| No. of proteins detected (≥2 peptides with >95% confidence) | | 143 |
| No. of distinct peptides with >95% confidence | | 1172 |
| Normalization | B *vs.* A | 0.6 |
| | C *vs.* A | 0.8 |
| | D *vs.* A | 0.8 |
| Global False Discovery Rate | | <0.1% |
| Local False Discovery Rate | | <0.1% |
| Confidence level of protein detection | | >95% |
| Unused ProtScore cut off | | >1.3 |
| No. of differentially expressed proteins | | |
| B *vs.* A | | 7 |
| C *vs.* A | | 7 |
| D *vs.* A | | 11 |

**Table 4.10 List of differentially expressed proteins in breastmilk.**

| **Downregulated proteins** | | **Upregulated proteins** | |
|---|---|---|---|
| **Gene** | **Function** | **Gene** | **Function** |
| XDH | Enzyme | ANXA2 | Immune protein |
| UGP2 | Enzyme | LYZ | Immune protein |
| ALB | Transport | FASN | Enzyme |
| CNDP2 | Enzyme | SELENBP1 | Transport |
| ENO1 | Enzyme | PGD | Enzyme (glycotic) |
| HBB | Transport | PLIN3 | Transport |
| GSN | Other | SCP2 | Transport |
| MDH1 | Enzyme (glycotic) | | |
| LDHB | Enzyme (glycotic) | | |
| ANXA5 | Membrane Protein | | |

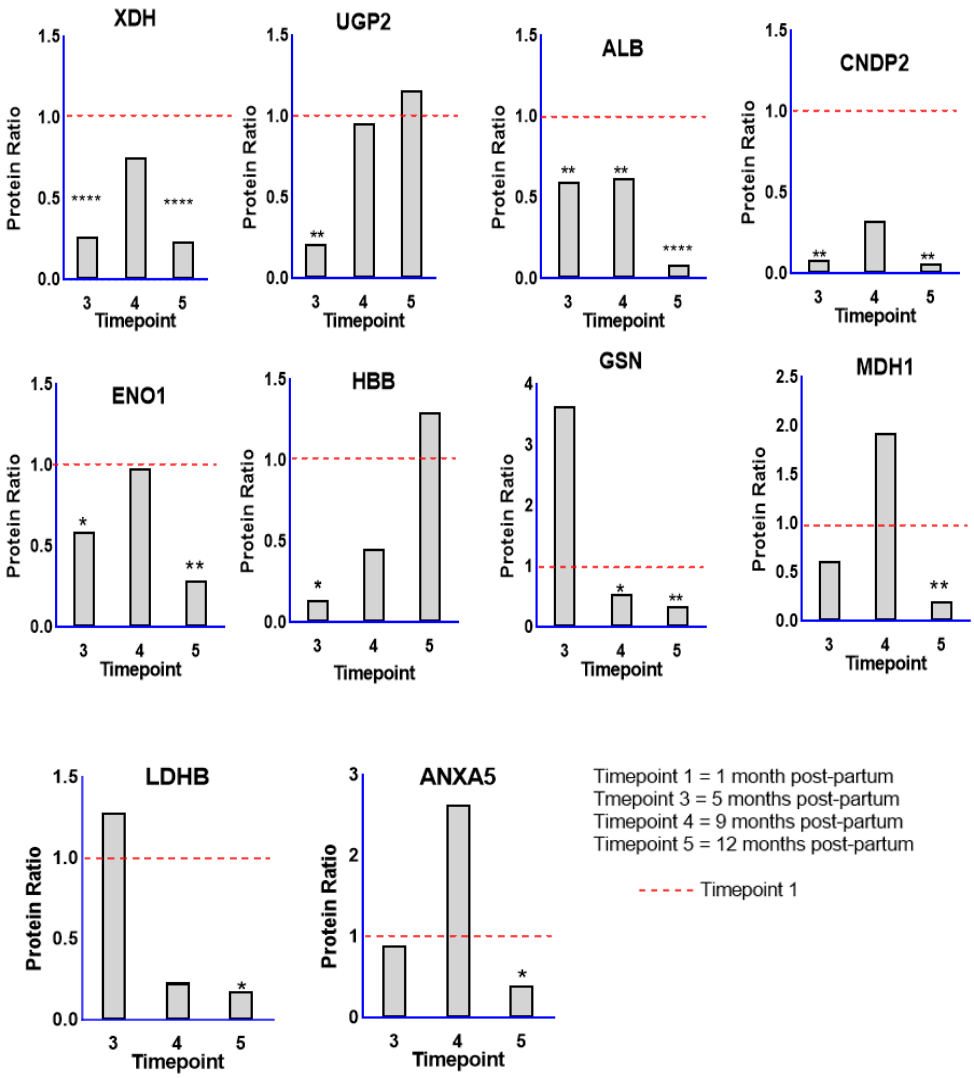
(XDH = Xanthine dehydrogenase/oxidase; UGP2 = UTP--glucose-1-phosphate uridylyl transferase; ALB = Serum Albumin; CNDP2 = Cytosolic non-specific dipeptidase; ENO1 = Alpha-enolase; HBB = Haemoglobin subunit beta; GSN = Gelsolin; MDH1 = Malate dehydrogenase; LDHB = L-lactate dehydrogenase B chain; ANXA5 = Annexin A5; ANXA2 = Annexin A2; LYZ = Lysozyme; FASN = Fatty Acid Synthase; SELENBP1 = Selenium-binding protein 1; PGD = 6-phosphogluconate dehydrogenase; PLIN3 = Perilipin; SCP2 = Non-specific lipid-transfer protein)

The proteins that were differentially expressed vary in function with majority being enzymes and transport proteins. A brief description of the proteins and their functions is provided in Table 4.11 while Figure 4.7 and Figure 4.8 illustrate their expression over time in comparison to Timepoint 1 (one month post-partum).




***p* value <0.05; ****p* value <0.01; *****p* value <0.001; ******p* value <0.0001

**Figure 4.7 Upregulated proteins in lactation normalised to Timepoint 1 (---) as shown by iTRAQ analysis.**




***p* value <0.05; ****p* value <0.01; *****p* value <0.001; ******p* value <0.0001**

**Figure 4.8 Downregulated proteins in lactation normalised to Timepoint 1 (---) as shown by iTRAQ analysis.**

**Table 4.11 Brief description of differentially expressed proteins in breastmilk and their functions.**

| Name | Gene | Brief description and function (248) |
|---|---|---|
| **Annexin A2** | ANXA2 | • Members of this calcium-dependent phospholipid-binding protein family play a role in the regulation of cellular growth and in signal transduction pathways.<br>• This protein functions as an autocrine factor which heightens osteoclast formation and bone resorption. |
| **Lysozyme** | LYZ | • Lysozymes have primarily a bacteriolytic function; those in tissues and body fluids are associated with the monocyte-macrophage system and enhance the activity of immunoagents. |
| **Fatty Acid Synthase** | FASN | • Fatty acid synthase (FASN) is a multienzyme that catalyzes the conversion of acetyl-CoA and malonyl-CoA to the 16-carbon fatty acid palmitate. |
| **Perilipin** | PLIN3 | • Required for the transport of mannose 6-phosphate receptors (MPR) from endosomes to the trans-Golgi network. |
| **6-phosphogluconate dehydrogenase** | PGD | • Catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and $CO_2$, with concomitant reduction of NADP to NADPH. |
| **Selenium-binding protein 1** | SELENBP1 | • Selenium is an essential nutrient that exhibits potent anticarcinogenic properties, and deficiency of selenium may cause certain neurologic diseases.<br>• The effects of selenium in preventing cancer and neurologic diseases may be mediated by selenium-binding proteins.<br>• Decreased expression of this protein may be associated with several types of cancer and may play a selenium-dependent role in ubiquitination/deubiquitination-mediated protein degradation. |

| Name | Gene | Brief description and function |
|---|---|---|
| **Non-specific lipid-transfer protein** | SCP2 | • Mediates in vitro the transfer of all common phospholipids, cholesterol and gangliosides between membranes. May play a role in regulating steroidogenesis. |
| **Xanthine dehydrogenase/oxidase** | XDH | • Key enzyme in purine degradation<br>• Catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid.<br>• Contributes to the generation of reactive oxygen species. |
| **Alpha-enolase** | ENO1 | • Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses.<br>• May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons.<br>• Stimulates immunoglobulin production. |
| **Haemoglobin subunit beta** | HBB | • Involved in oxygen transport from the lung to the various peripheral tissues. |
| **Serum albumin** | ALB | • Most abundant protein in human blood.<br>• Regulates blood plasma colloid osmotic pressure and acts as a carrier protein for a wide range of endogenous molecules including hormones, fatty acids, and metabolites, as well as exogenous drugs.<br>• Exhibits an esterase-like activity with broad substrate specificity.<br>• A peptide derived from this protein, EPI-X4, is an endogenous inhibitor of the CXCR4 chemokine receptor. |

| Name | Gene | Brief description and function |
|---|---|---|
| **UTP--glucose-1-phosphate uridylyl transferase** | UGP2 | • An important intermediary in mammalian carbohydrate interconversions. It transfers a glucose moiety from glucose-1-phosphate to form UDP-glucose.<br>• In lactating mammary gland UDP-glucose is converted to UDP-galactose which is then converted to lactose.<br>• In liver and muscle tissue, UDP-glucose forms glycogen. |
| **Cytosolic non-specific dipeptidase** | CNDP2 | • CNDP2, also known as tissue carnosinase and peptidase A (EC 3.4.13.18), is a nonspecific dipeptidase rather than a selective carnosinase |
| **Gelsolin** | GSN | • Calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping). It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed. Plays a role in ciliogenesis. |
| **Malate dehydrogenase** | MDH1 | • Plays a key role in the malate-aspartate shuttle that allows malate to pass through the mitochondrial membrane to be transformed into oxaloacetate for further cellular processes. |
| **L-lactate dehydrogenase B chain** | LDHB | • Catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+ in a post-glycolysis process. |
| **Annexin A5** | ANXA5 | • Annexin-5 is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and a potential role in cellular signal transduction, inflammation, growth and differentiation.<br>• Annexin 5 has also been described as placental anticoagulant protein I, vascular anticoagulant-alpha, endonexin II, lipocortin V, placental protein 4 and anchorin CII. |

### 4.7.4 Immunostaining and protein abundance

Immunostaining is usually not used as a quantitative measure of protein expression or abundance as more definitive and precise methods such as Western Blot or ELISA are preferred. Due to inadequate protein content in the milk derived cell samples, immunostaining was carried out to provide semi quantitative data to support gene studies. Due to variability in the samples, the results were not statistically significant as shown in Figure 4.9. However, the presence of BCRP can be seen in immunostained images as demonstrated in Figure 4.10 Examples of immunostained images (x20 magnification). Breastmilk derived cells were stained using DAPI (nuclei, blue) and Alexa Fluor 594 (BCRP, red) then visualised at 480nm and 594nm. Area of co-localisation highlighted by arrows.

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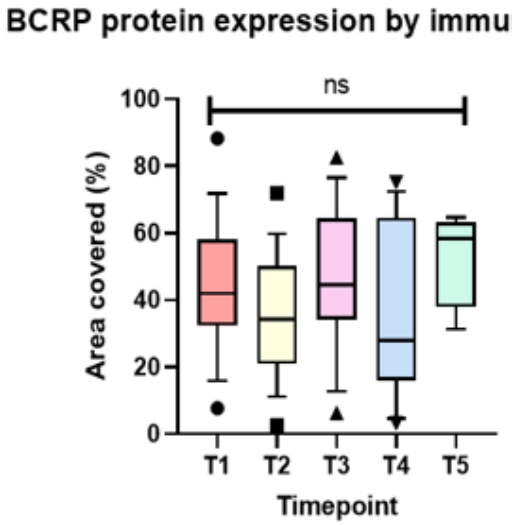


| Tukey's multiple comparisons test | Adjusted *P* Value |
|---|---|
| T1 vs. T2 | 0.5692 |
| T1 vs. T3 | 0.9986 |
| T1 vs. T4 | 0.6541 |
| T1 vs. T5 | 0.9440 |
| T2 vs. T3 | 0.4085 |
| T2 vs. T4 | >0.9999 |
| T2 vs. T5 | 0.4713 |
| T3 vs. T4 | 0.4980 |
| T3 vs. T5 | 0.9784 |
| T4 vs. T5 | 0.5117 |

**Figure 4.9 BCRP protein expression by human breastmilk cells in milk samples from 22 participants quantified by immunohistochemistry using mouse primary antibodies and AF594 labelled secondary goat anti-mouse antibody. Box plots represent gene expression distribution where tails show the minimum and maximum values (excluding outliers) and upper and lower interquartile ranges; middle line represents the median. (● = T1 outlier, ■ = T2 outlier, ▲= T3 outlier, ▼= T4 outlier, ♦ = T1 outlier).**

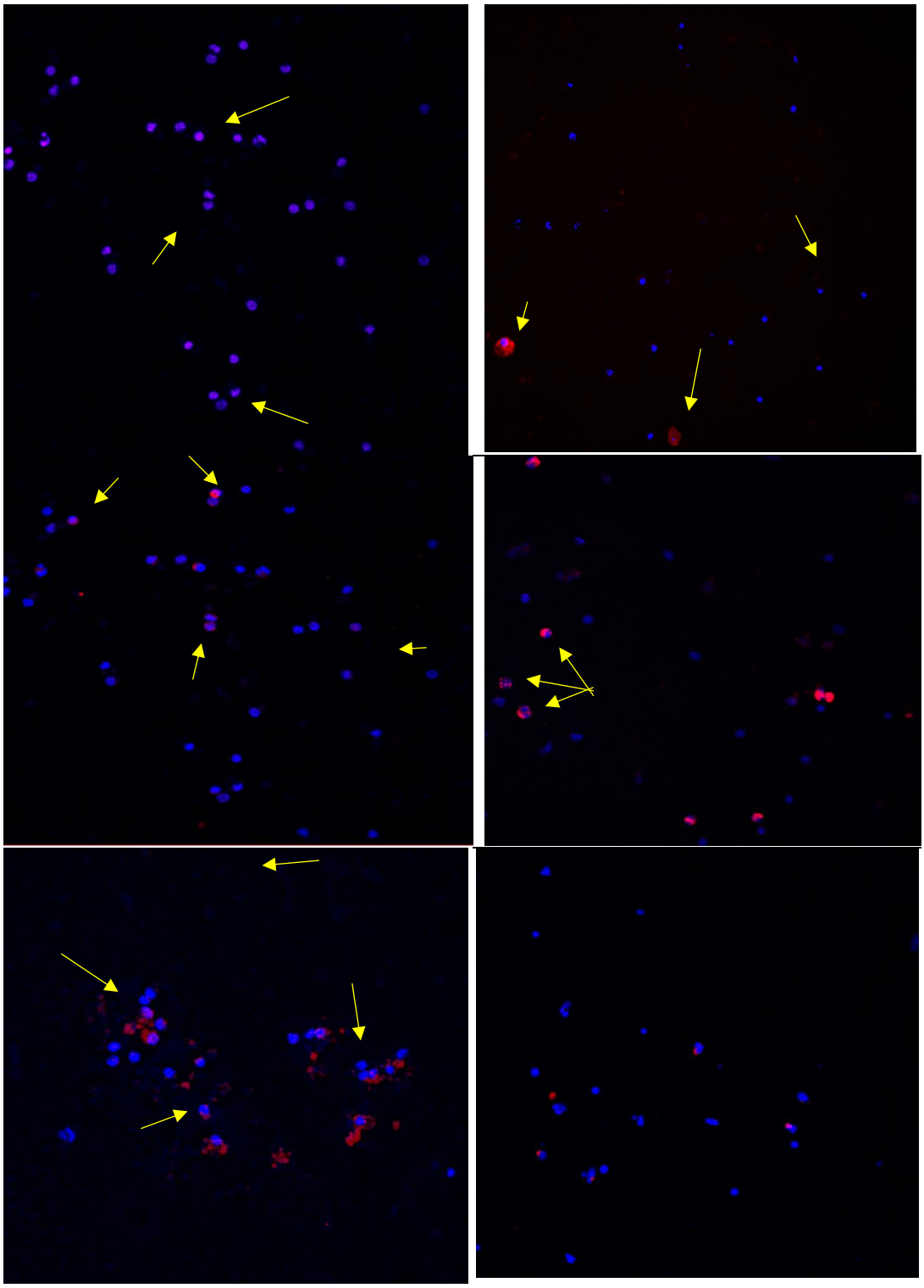

**Figure 4.10 Examples of immunostained images (x20 magnification). Breastmilk derived cells were stained using DAPI (nuclei, blue) and Alexa Fluor 594 (BCRP, red) then visualised at 480nm and 594nm. Area of co-localisation highlighted by arrows.**

## 4.8 Discussion

Active efflux transporters such as those belonging to the ABC family which are located in the apical membrane of the human mammary epithelial cells influence the composition of breastmilk by actively pumping substrate drugs and substances into breastmilk (76). BCRP is known to be strongly expressed during lactation, suggesting that substrates of BCRP are likely to be excreted into milk inadvertently exposing a breastfed infant to a xenobiotic and putting it at risk of toxicity (76, 85). Breastmilk is a readily available source of mammary epithelial cells that are sloughed during lactation and can provide insight into the lactating mammary gland at a molecular level (208).

Studies have shown that the RNA extracted from milk cells are representative of gene expression in the mammary gland and provide an insight at a molecular level (249). Using PCR, the presence of BCRP was confirmed. It was also shown that that BCRP is strongly induced during lactation and its mRNA expression peaks at around 5 months post-partum (T3). While the presence of other efflux transporters were also confirmed, their expression remained at much lower levels compared to BCRP and there was great inter-individual variability affecting their statistical significance. The role of BCRP in drug disposition in breastmilk was anticipated to be more prominent compared to the other three transporters due to the relative overexpression of BCRP mRNA in the lactating mammary gland. Furthermore, many studies have shown BCRP to be involved in regulating the composition of breastmilk (76, 186, 197).

Although relative mRNA abundance provides useful information regarding the cellular processes that drive protein synthesis, gene expression studies alone without protein expression/abundance studies may not provide clinically relevant conclusions. Due to the inadequacy of protein content in the samples, Western Blot, a definitive and proven assay to determine protein expression was not able to be conducted and immunostaining was used as a third-line semi-quantitative assay (250, 251). While statistically significant results were unable to be shown by immunostaining, the presence of active efflux transporters was confirmed. Studies with a larger sample size should be conducted to ascertain transporter protein abundance in breastmilk samples to support the gene expression data.

Isobaric tags for relative and absolute quantitation (iTRAQ) has been previously used to study biological samples and the milk proteome across species. While iTRAQ has been successfully used in identification of many biological markers, the method has some limitations and has also been shown to produce less reliable quantification in complex biological samples such as breastmilk (252). For instance, iTRAQ labelling has been linked to a reduction in the number of identifiable proteins due to the introduction of undesirable charge enhancements (253). iTRAQ analysis of these breastmilk samples did not detect any proteins of interest. MDR1, MRP1 and MRP2 were expected to be present at low levels, certainly compared to BCRP. However, not even BCRP was able to be detected. This could possibly be due to the previously mentioned limitations of this technique. Although the efflux transporter proteins were not detected by the isobaric labelling method, this technology allowed us to obtain a picture of the changing composition of the breastmilk at a molecular level.

In this study strict identification criteria with a false discovery rate (FDR) of 0.1% were used whereas other studies have used FDR of up to 5% resulting in the identification of a larger number of proteins albeit with a lower precision level (254). For this study, due to resource limitations, only four time points could be added to a 4-plex assay. A total of 32 samples from eight participants were pooled for the 4 timepoint for iTRAQ analysis. As also identified with the PCR assay, there was a great degree of inter-individual variability between samples. This variability was possibly due to maternal and environmental factors previously discussed that influence milk composition (255). iTRAQ analysis of our breastmilk samples showed a total of 17 proteins to be differentially expressed at the four timepoints spread from 1 to 12 months post-partum ($p<0.005$). While 10 proteins were upregulated, 7 were downregulated over time. Figure 4.11 adapted from Zhang *et al*. shows some of these proteins that are involved in lipid transport and metabolism in the lactating mammary gland (255).

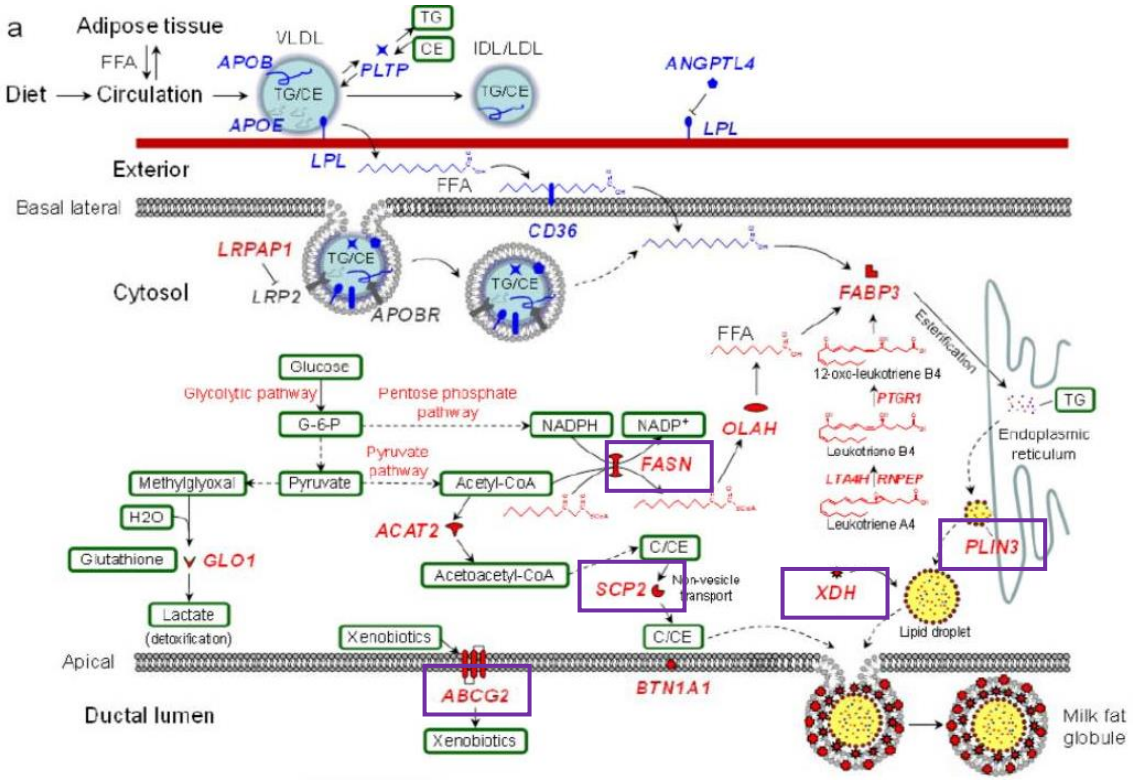


**Figure 4.11 Schematic representation of extracellular and intracellular pathways in lipid transport and metabolism.**

Serum albumin, a major component of human breastmilk was found to be increasingly downregulated over time. Most milk proteins are synthesised within the mammary gland but a few such as serum albumin may be transferred from maternal blood (256). The overall protein content of human milk is known to gradually reduce over time as the weight gain slows after the initial months reducing the need for protein. These findings were in alignment with currently available literature which show a linear decline in the albumin content of milk as the consumption of other foods is increased by the breastfed infant over the first year of lactation (257-259). However, one study showed an increase in the serum albumin content over a six-month period which is in contradiction to these findings (255).

Similarly, xanthine oxidase/dehydrogenase (XDH), an enzyme in breastmilk attributed to a reduced risk of gastroenteritis caused by *Escherichia coli* and *Salmonella enteritides* was also downregulated over time. Xanthine dehydrogenase generates radical nitric oxide which inhibits the growth of these bacteria. Breastfed infants have a lower risk of gastroenteritis due to the antibiotic effects of the naturally occurring Xanthine oxidase in breastmilk (260, 261). The gradual reduction

seen in the longitudinal study is in alignment with available literature showing a higher level of XDH in the first month of lactation. Although the results for the third timepoint (9 months post-partum) show a non-significant relative increase, it is thought to be due to a variation in the sample at that timepoint as the level in T5 is very similar to the level at T3 (5 months post-partum). Gao and colleagues also showed that XDH is downregulated in lactation (262). We have now demonstrated that the overall downregulation in XDH manifests as a gradual decrease as lactation progresses.

This study shows that lysozyme is progressively upregulated as lactation progresses. This is in alignment with previous research showing consistent upregulation of lysozyme over the duration of lactation of up to 26 months (263, 264). Lysozyme have a bacteriolytic function and enhance the activity of immunoagents in body tissues and fluids. The progressive upregulation can be explained as a protective mechanism for a growing infant with increased mobility, who may be increasingly exposed to pathogens. Another enzyme that is also upregulated is Fatty acid synthase (FASN), a crucial enzyme in cellular de novo fatty acid synthesis in the mammary gland which is the main source of short and medium-chain fatty acids of breastmilk. Animal studies have shown that FASN is upregulated during lactation (265). Our study extends these observations into humans.

This study is the first longitudinal human study of the expression of efflux transporters in the mammary gland. As can be seen from the results, this study confirms that there is a great degree of inter-individual variability in the expression of efflux transporters in the studied population. Infections, including mastitis, due to the body's response to infection have been associated with acute and transient regulatory mechanisms that are capable of inducing a change in the expression of efflux transporters as the body's response to infection influences expression of these transporters (240, 266). We ensured that all our breastfeeding dyads were healthy and free from infection at the time of sample collection. However, the possibility of subclinical infection impacting the results cannot be discounted. Other factors with potential to introduce variability were strictly controlled. These included breastmilk collection techniques, transfer of samples and cell storage. Personal electric breast pumps were provided by the researcher to each participant for sample collection. Consideration was given to the uniformity of sample collection process including the

apparatus, processing times and transfer to the laboratory ensuring these were kept uniform between samples. These factors were closely monitored and verified at each sample collection. All cell pellets were stored under the same conditions in -80 °C prior to RNA extraction. Mothers were instructed to notify the candidate if they felt unwell or their baby was unwell with any symptoms of infection including mild illnesses such as colds. Sample collection was delayed until both mother and baby had fully recovered. Due to the rigorous control of external factors, the sample variability shown in this study is thought to be due to known intersubjective and intra-subjective factors.

Lactogenic hormones such as prolactin, insulin and hydrocortisone have an important role in modulating expression of transporters (267). Prolactin, being the key hormone affecting the induction and maintenance of lactation, has been shown to enhance the expression of PEPT2 transporter through signalling pathways that involve the activation of JAK2/STAT5 transcription factors (266, 267). Although little is known about the factors influencing expression of the efflux transporters in the mammary gland, data are emerging that associates this variability to epigenetic factors (266, 268). Epigenetic mechanisms biochemically alter the DNA such that the DNA sequence is unaltered, but gene expression is affected via changes in their accessibility to replicating mechanisms in response to various environmental factors (243, 269). Some common and best-known epigenetic mechanisms in humans include DNA methylation, post-translational modifications of histone proteins, and modulation of gene expression by noncoding RNAs (270). Genetic polymorphism related to the ABCG2 gene is attributed to the differences in response to chemotherapy in breast cancer (271). These changes can alter tissue-specific expression of genes in various cell types including transporter proteins. Although currently there is no evidence of epigenetic mechanisms in expression of efflux transporters during lactation, it is interesting to note that many malignancies exhibit drug resistance primarily due to the presence of active efflux transporter proteins (227, 272-275), suggesting a possible link between epigenetics and the expression of efflux transporter proteins, which may also be applicable to the lactating mammary gland. This is an emerging field that requires further investigation.

## 4.9 Conclusion

This study was the first longitudinal study of efflux transporters in humans. It has demonstrated that from its mRNA levels it is likely that BCRP is a relatively highly expressed efflux transporter in the lactating mammary gland that could potentially be involved in the disposition of drugs and facilitating their excretion in breastmilk. Other active transporters such as P-gp (MDR1), MRP2 and MRP1 are also expressed to a relatively lower level. Given the magnitude of expression of BCRP in the lactating mammary gland and available data that shows its contribution to the composition of breastmilk, substrates of BCRP can potentially be transferred into breastmilk. It is likely that a nursing infant may be at risk of toxicity from BCRP substrates, particularly around the 5-6 month post-partum period owing to the upregulation of BCRP mRNA at this time. However, the lack of relevant conclusive protein expression data and intra-individual variability prevents the potential role of BCRP in breastfed infant ADRs from being categorically confirmed.

Although the focus of this chapter was mainly the longitudinal expression of active transporters in the lactating mammary gland, iTRAQ proteomic analysis of breastmilk samples revealed that a number of breastmilk proteins were differentially expressed over time. Interestingly, immune proteins such as Lysozyme and Annexin 2 were upregulated over time, while enzymes such as xanthine dehydrogenase and UTP-glucose-1- phosphate uridylyltransferase were down regulated. However, iTRAQ analysis was not able to detect transporter proteins in pooled milk samples, potentially due to their relatively low expression compared to other milk proteins. While this study confirmed that efflux transporters such as MDR1, MRP1, MRP2 and BCRP were expressed during lactation, further research is required to elucidate their role and impact in drug transport into breastmilk.

# Chapter 5 Development of a model using breastmilk derived cells

## 5.1 Background

The influence of maternal and environmental factors makes human breastmilk a "live tissue" that is constantly adapting (9, 243, 276). Breastmilk has the potential to give insight into the molecular changes that occur in the lactating breast. In Chapter 4, we showed that the expression of BCRP, an active transporter with an established role in the lactating mammary gland varied between participants and was lactation stage dependent (75, 76). Hence, in order to predict the transferability of an actively transported drugs into breastmilk, it is important to take a personalised approach where each subject's breastmilk composition and their lactation stage needs to be considered in order to determine the safety of a particular drug. Being a rich source of epithelial cells, breastmilk provides an ideal tool to investigate the impact of its dynamic composition on the expression of efflux transporters and potentially provide greater insight into the safety of actively transported drugs into breastmilk. In this study, we aimed to use epithelial cells derived from breastmilk to establish a monolayer culture *in vitro* which could then be used to conduct drug transport studies with the aim of providing a personalised drug safety profile.

Primary cells are the preferred *in vitro* models for pre-clinical and investigative biological research as primary cells generally retain normal morphology, cellular function, growth characteristics, signalling and genetic integrity when propagated in culture (277). While the greatest advantage of primary cells is their high biological relevance, as they retain the *in vivo* tissue genetic make-up, a major disadvantage is their finite lifespan, slow growth and their tendency to lose expression of drug transporters and metabolizing enzymes rather quickly. In contrast to primary cells, immortalised cell lines grow well in culture, but they have been found to exhibit different characteristics *in vitro* compared to *in vivo* (278, 279). For example, most breast cancer cell lines *in vitro* closely resemble the phenotype of the normal mature luminal cell (*in vivo)* but when cultured, normal HMEC with this phenotype show

the least proliferative potential (280). Breastmilk is rich in epithelial cells as well as pluripotent stem cells and therefore has the potential to be used as a tool to study mammary gland diseases (40, 281). The use of primary human mammary epithelial cells (HMEC) derived from breastmilk containing no mutations and chromosomal abnormalities can serve as a good indicator of progression of early-stage disease. Breastmilk derived epithelial cells also have great potential in exploring drug safety of actively transported drugs during lactation as active transporters such as BCRP are localised on the membrane of the secretory epithelial cells. Cregan's group showed that epithelial cell cultures derived from expressed breastmilk contained a heterogeneous population of cells similar to those seen in established mammary tissue (282). This further highlighted that if breastmilk derived epithelial cells could be consistently grown *in vitro*, their application as a tool to study drug safety in lactation as well as the study of development/progression of diseases such as breast cancer would be vast.

The establishment of a primary cell culture with breastmilk sourced cells has been somewhat challenging. Establishing a primary culture of milk derived epithelial cells can take anywhere from a week to almost two months (282). This is attributed to the differences in milk characteristics. In our laboratory we had previously used "conventional" milk cell medium adapted from Cregan (282) which resulted in 70-80% confluence after an average of 35 days (unpublished data; Sim, TF and Tee, L). Generally, culturing primary HMEC have been shown to have many limitations including a slow and variable rate of growth, finite life span and failure to reach senescence (282-284).

The role of different media to support breastmilk derived cell growth in culture while maintaining the lineage heterogeneity has been well established (245, 277). Stem cells found in breastmilk have been successfully grown *in vitro* into various cell lineages such as chondrogenic, osteogenic and neuronal lineages using specific cell growth medium (40, 285, 286). However, many studies report that the rate at which breastmilk derived epithelial cells divide depends on the growth conditions, and the characteristics of the milk sample which is heavily influenced by many maternal and environmental factors (287). Tang *et al*. (288) showed that the maturity of breastmilk i.e. whether it is colostrum, transitional or mature milk as well as the culture media used heavily influenced the growth of breastmilk derived cells.

Human breastmilk can be used to provide an individualised, non-invasive tool to obtain real-time information about the changes that occur in the lactating mammary gland. HMECs are known to grow in colonies and do not form tight junctions (208, 289). This makes them less ideal for traditional bidirectional transport studies. However, accumulation and efflux assays can still be carried out with cells growing on the bottom of a well or a plate (290, 291). If breastmilk derived epithelial cells are being propagated in culture with the ultimate aim of determining the safety of a drug at a specific stage of lactation, it is crucial that the time it takes for the cells to be useful *in vitro* is short so that recommendations are as close as possible to real-time. Furthermore, the cells should exhibit normal morphology, cellular function, growth characteristics and genetic integrity. This will allow for a model that is personalised and has the potential to be recreated at different stages of lactation to accommodate the evolving nature of breastmilk. In this study we aimed to optimise the culture conditions of breastmilk derived HMEC *in vitro* and test their *in vivo* relevance with respect to the expression of active transporters including BCRP, MDR1, MRP1 and MRP2.

## 5.2 Aim

The objectives of this study were:

- To optimise growth conditions for human breastmilk derived human mammary epithelial cells (HMEC).
- To determine whether breastmilk derived cells retain their ABC transporter expression characteristics in culture medium in order for them to be utilised as a non-invasive, reliable, personalised, quick and cost-effective model to predict the transferability of actively transported drugs into breastmilk.

## 5.3 Materials and Methods

### 5.3.1 Sample collection

Donors were selected from healthy volunteers ranging from 17 to 97 weeks postpartum. This study was approved by the Curtin University Human Research

Ethics Committee (HR2012/110) and all volunteers gave written consent for providing the milk sample. After the collection of breastmilk samples using electric breast pumps, the milk samples were aseptically transferred into 50 mL conical tubes and transferred to the laboratory at room temperature as soon as possible with no more than 2 hours between expression and processing of the sample.

### 5.3.2 Total cell isolation and purification

Working within a sterile environment, inside a laminar flow hood, the milk was diluted with equal parts of RPMI medium and was centrifuged at 805 g for 20 min in a swinging bucket (Beckman Coulter Allegra X-12R) centrifuge at 20°C. The milk fat layer and the supernatant were aspirated, and the cell pellet was washed with RPMI medium and transferred into a 15 mL conical tube. The cell suspension was placed back in the centrifuge for five min at 394 g at room temperature. This step was repeated to facilitate complete removal of fat and milk. This washing step also allow for enrichment of the epithelial cells by removing the relatively smaller and lighter immune cells. The cells were counted, checked for cell viability using Trypan Blue exclusion test and were resuspended in the required amount of milk cell medium prepared as per the formula in Section 5.3.3.

### 5.3.3 Preparation of Conventional Milk Cell Medium for primary HMEC culture

Milk cell medium was prepared according to the formula shown in Table 5.1 adapted from Cregan's laboratory (282).

**Table 5.1 Conventional milk cell medium ideal for the growth and proliferation of human mammary epithelial cells.**

| Constituents | Storage | Stock Concentration | Working Concentration | Final Volume in 100 mL | Company/ Source |
|---|---|---|---|---|---|
| **RPMI-1640 + L-Glut** | 4°C | 1 x | 1 x | 79 mL | Gibco |
| **Foetal Bovine Serum (FBS)** | -20°C | 100% | 20% | 20 mL | Gibco |
| **Penicillin/Streptomycin** | -20°C | 100 x | 1 | 1.0 mL | Gibco |
| **Amphotericin B** | 4°C | 2 mg/mL | 2 μg/mL | 100 μL | Thermo Fisher Scientific |
| **Insulin** | 4°C | 4 mg/mL | 4 μg/mL | 100 μL | Sigma |
| **Epidermal growth factor (EGF)** | -20°C | 100 μg/mL | 20 ng/mL | 20 μL | Invitrogen |
| **Hydrocortisone** | -20°C | 50 μg/mL | 0.5 μg/mL | 1.0 mL | Sigma |
| **Cholera Toxin** | 4°C | 1 mg/mL | 60 ng/mL | 6.0 μL | Sapphire Biosciences |

### 5.3.4 Participant details

Breastmilk isolates were obtained from a total of seven participants (n=7). As stated in Table 5.2, samples were collected from mothers who were at various stages of breastfeeding ranging from one week postpartum to 97 weeks. All milk samples were mature milk and contained both fore and hind milk.

**Table 5.2 Milk sample details: Pre-optimisation**

| Participant | Weeks post-partum | Amount of breastmilk collected (mL) | Viable cell count /mL of breastmilk |
|---|---|---|---|
| 1 | 35 | 70 | $72 \times 10^3$ |
| 2 | 1 | 125 | $188 \times 10^3$ |
| 3 | 15 | 85 | $144 \times 10^3$ |
| 4 | 56 | 42.5 | $647 \times 10^3$ |
| 5 | 97 | 47.5 | $212 \times 10^3$ |
| 6 | 91 | 42.5 | $275 \times 10^3$ |
| 7 | 68 | 73 | $111 \times 10^3$ |

### 5.3.5 Growth and differentiation of primary HMEC

The breastmilk cells were seeded at a density of 500,000 and 200,000 cells per well in 6 and 24 well plates, respectively. The cell cultures were incubated at 37°C in a humidified incubator with 5% $CO_2$. The culture medium was first replaced 3 days after seeding and non-adherent cells were removed by washing the cultures with sterile RPMI. Cell cultures were considered to be confluent when approximately 70-90% of the surface of the well was covered by the epithelial cells. Alternatively, a culture was considered confluent when clonal expansion ceased, and the cells started to become senescent.

Only one of seven samples seeded in the 24 well plates demonstrated adherence to the plate and an extension of the cytoplasmic membrane by day 5, as seen in Figure 5.1 B. Culture medium was changed every 3 to 5 days. Occasionally, adherent cells were observed in some wells (Figure 5.1 B) but there was an absence of clonal expansion. The majority of other samples showed no such attachment to the plate and had begun to float by day 5. These wells were observed but no medium change was done (as changing the medium would have resulted in disposal of the cells). By day 15, no changes were observed in these wells, after which they were discarded.

In the single well that demonstrated adherence, the attached cells showed very slow growth of "neuron or fibroblast" like cells and, as demonstrated in Figure 5.1C, these cells were sparse. By day 30, these cells had failed to differentiate into typical epithelial cells (Figure 5.1E and F) as their long structures resembled fibroblasts more than epithelial cells. These cultured cells failed to reach the defined confluence

parameters with coverage of approximately 40% to 50% of the surface of the 24 well plate. Cells were discarded on day 35 when no further growth was observed, and senescence was noted.

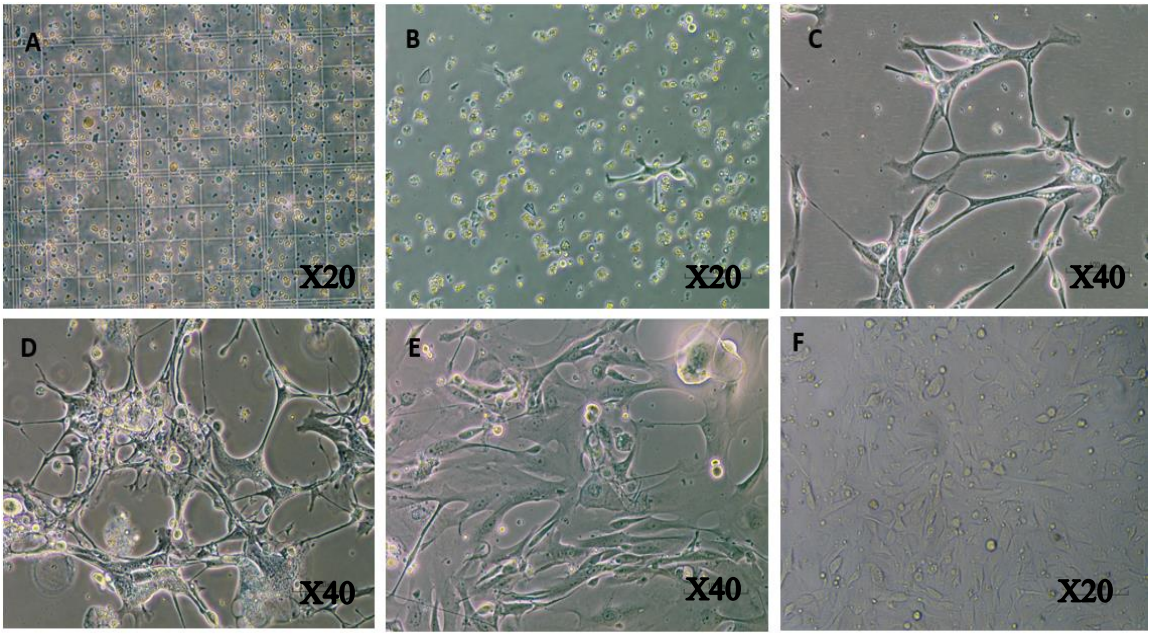


**Figure 5.1 Culture of breastmilk derived HMEC in the traditional milk cell medium at days 0, 5, 15, 20, 30 and 35. [A = Day 0; B = Day 5; C = Day 15; D = Day 20; E = Day 30; F= Day 35]**

### 5.3.6 Optimisation of milk cell medium and culture environment

The abovementioned results reflected the high failure rate of prior experiments conducted in our laboratory using the traditional milk cell medium formula. To optimise this medium, several variations were made to the formula. These included:

a) Varying insulin concentration between 2 µg/mL and 10 µ g/mL
b) Reducing amphotericin concentration to 1 µ g/mL
c) Increasing EGF to 30 ng/mL
d) Increasing hydrocortisone to 1 µ g/mL
e) Use of 24 well plates, 6 well plates and 25 $cm^2$ flask ± collagen. (The plates were collagen coated by placing collagen solution, and incubated for 2 hours under UV light in a laminar airflow hood. The collagen was removed from the plate/flask by direct aspiration and the plates were left overnight under UV light for decontamination.)

f) Using 5 different seeding density of the cells between 1 $\times 10^5$ and 1 $\times 10^6$ cells per well ($1\times10^5$, $2.5\times10^5$, $5\times10^5$, $7.5\times10^5$, $1\times10^6$)

All these variations were made individually on breastmilk samples collected from one mother between weeks 50 and 54 post-partum. Cells were incubated at 37°C in 5% $CO_2$. The cells were assessed for adherence and gross morphology at three day intervals and imaged. Mammary epithelial cells grow in colonies and often the culture dish exhibited areas with no growth and areas where the cells were adherent and numerous as shown in Figure 5.2 C (280, 284).

As demonstrated in Figure 5.2, none of the modifications made to the conventional milk cell medium enhanced the growth of the breastmilk derived HMEC. While there was no improvement, halving the concentration of amphotericin B from 2 µg/mL to 1 µg/mL resulted in fungal contamination of the cultured cells (Figure 5.2 H). Changes to the concentration of insulin, EGF and hydrocortisone resulted in no growth as cells were afloat with no adherence at 10 days after seeding. Coating of the plates with collagen had no obvious impact on growth or adherence. The 24 well plates were found to have a slightly higher proportion of attached cells and relatively more visible colonies of attached cells compared to the 25$cm^2$ flask or the 6 well plates (Figure 5.2 C). The recommended seeding density for HMEC is 2500 cells/$cm^2$ (292). However, seeding at a higher density than the recommended 2500 cells/$cm^2$ has been shown to improve adherence (284, 288).

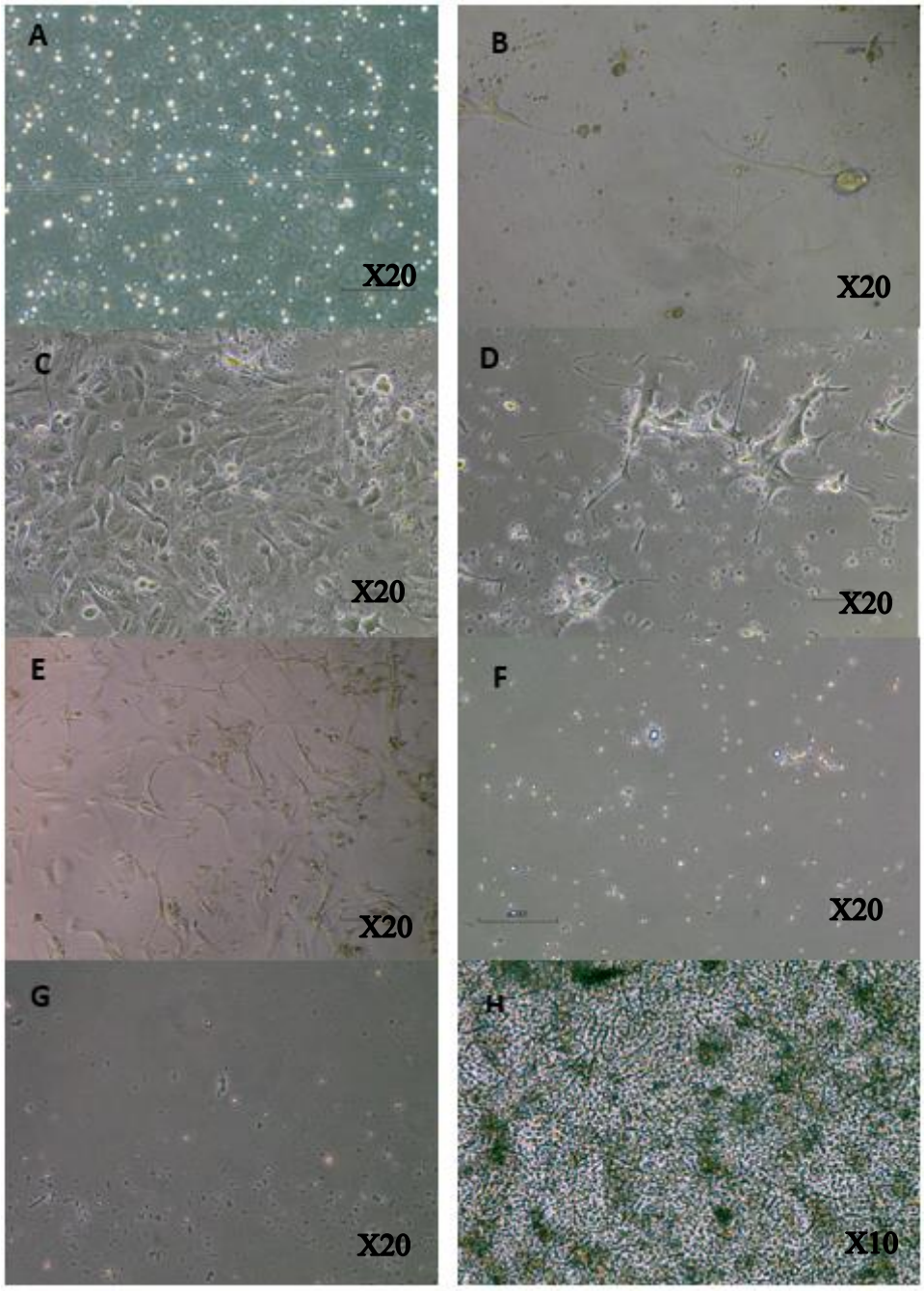


**Figure 5.2 Culture of breastmilk derived HMEC in modified milk cell medium. A = Day 0; B and C = Traditional milk cell media at days 5 and 35 (24 well plate) respectively; D= collagen coated plate at day 20; E= Increased EGF concentration 30 ng/mL day 10; F, G = increased insulin concentration (2 µg/mL and 5 µg/mL respectively) day 10; H = reduced amphotericin concentration day 10.**

### 5.3.7 A new approach: Specialised HuMEC Ready Medium® for mammary epithelial cells

HuMEC® Ready Medium (ThermoFisher Scientific) is marketed by the manufacturing company as an optimised medium specifically for the growth of human mammary epithelial cell lines. At the time of our experiments, we believe we were the first group to use this medium to grow primary epithelial cells derived from BREASTMILK.

The HuMEC® Ready Medium consists of HuMEC basal serum free medium, a supplement kit and bovine pituitary extract. The supplement kit includes 5 ml of supplement mix containing epidermal growth factor, hydrocortisone, isoproterenol, transferrin, and insulin. The HuMEC® Ready Medium was modified by the addition of heat inactivated foetal bovine serum (FBS) and antibiotic/antimycotic as shown in. Although breastmilk is a sterile fluid while in the mammary gland, when expressed it is easily contaminated and culturing requires the addition of an antibiotic and antifungal to prevent contamination as seen in Figure 5.2H.

**Table 5.2 HuMEC Ready Medium® modified to grow human mammary epithelial cells from freshly expressed breastmilk.**

| Ingredient | Volume added | Final Concentration |
|---|---|---|
| HuMEC® basal serum free medium | 500 ml | - |
| Foetal Bovine Serum | 75 mL | 15% |
| Pituitary extract | 2.5 ml (supplied) | - |
| Supplement | 5 mL (supplied) | - |
| Amphotericin (2 mg/mL) | 0.583 mL | 2% |
| Penicillin/streptomycin (5,000 units/5,000 µg per mL) | 5.825 mL | 1% |

#### 5.3.7.1 Growth of breastmilk derived primary epithelial cells in the HuMEC Ready Medium®

Breastmilk samples were processed and epithelial cells were isolated as described in Section 5.3.2. Cell cultures established in the HuMEC medium improved culture characteristics like cell adhesion and growth. Cells were seeded in 24 well plates, 6 well plates and 25 $cm^2$ flasks at densities ranging from $1\times10^5$ to $1\times10^6$cells in each of the 6 and 24 well plates, and between $1\times10^6$ to $3\times10^6$ cells for the 25$cm^2$ flasks.

All samples exhibited growth regardless of the size of the vessel. However, best growth was seen in 25cm$^2$ flasks (all seeding densities) followed by 24 well plates at $5\times10^5$ cells per well. Hence, a further five samples (n=5) were collected from five participants as described in Table 5.3.

### 5.3.7.2 Breastmilk sample characteristics

**Table 5.3 Milk sample details for primary culture in HuMEC Ready Medium®.**

| Participant | Weeks post-partum | Approx. number of days taken to reach confluence |
|---|---|---|
| 1 | 22 | 23 |
| 2 | 43 | 14 |
| 3 | 78 | 26 |
| 4 | 26 | 21 |
| 5 | 16 | 21 |

Cells were isolated and purified as described in Section 5.3.2. Cells were seeded at a concentration of $5\times10^5$ cells per well in 24 well plates, and $3\times10^6$ cells in 25cm$^2$ flasks, using culture conditions as described above.

These cultured primary human mammary epithelial cells showed extensive growth. Confluence (70-80%) was reached at an average of 21$\pm$4.4 days. All the samples followed a similar growth pattern as shown in the images in Figure 5.3.

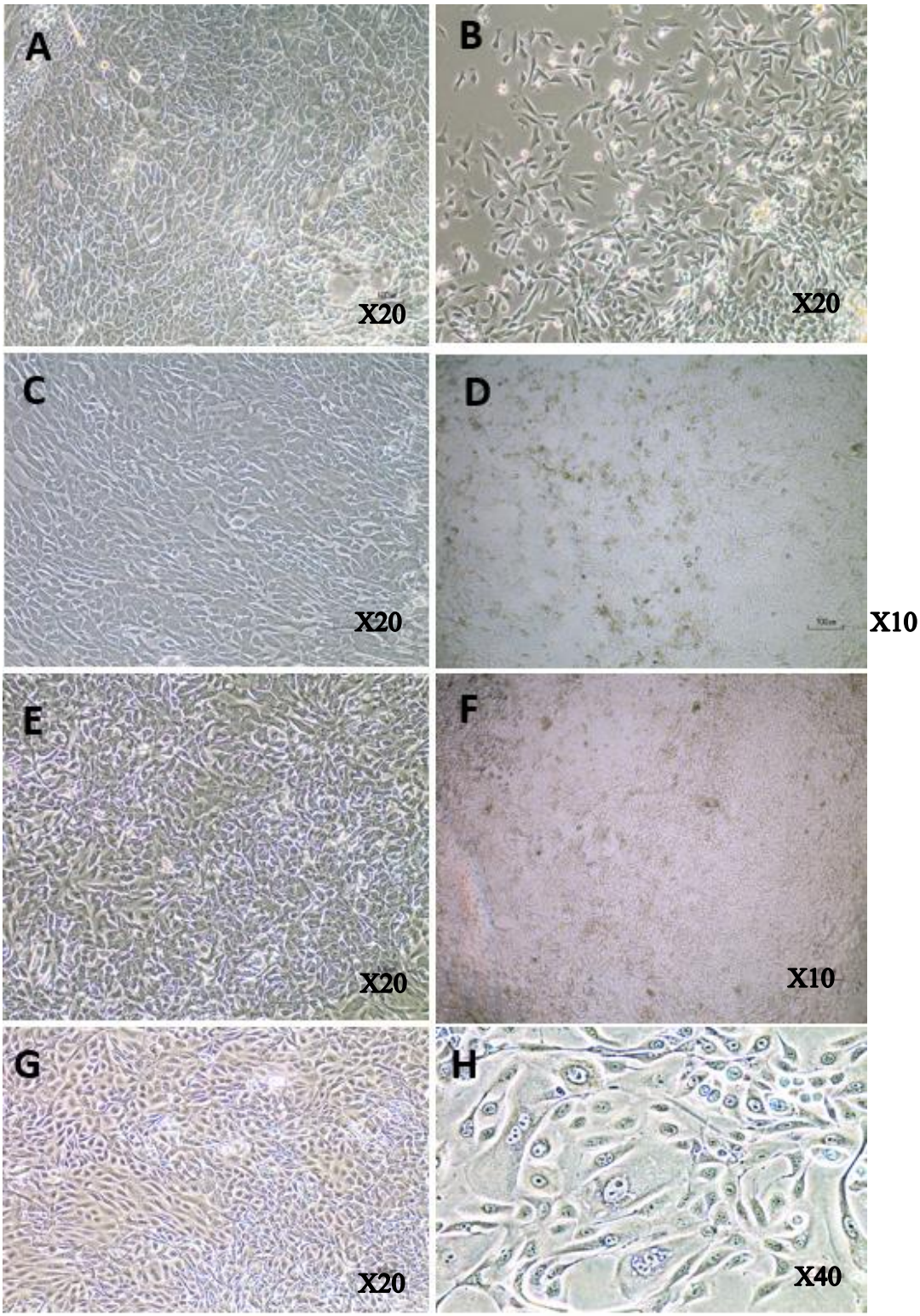


**Figure 5.3 Primary Human mammary epithelial cells derived from expressed breastmilk in HuMEC Ready Medium® at various incubation periods and magnifications. (A & B = Participant 2 at day 14; C & D = Participant 3 at day 26; E & F = Participant 4 at day 21; G and H = Participant 5 at day 21).**

## 5.4 Suitability of primary culture of breastmilk derived HMEC for active drug transport studies

After the optimisation of growth conditions for breastmilk derived HMEC and achieving a 100% success rate regardless of the stage of lactation, these cells were harvested to assess their suitability for use in determining drug safety during lactation. To achieve this, their RNA expression was compared to that of cells that were obtained from freshly expressed breastmilk from the same donor at the same time.

### 5.4.1 Cell harvest

The cells were prepared for harvest when the well appeared confluent. The medium was aspirated. Cells were detached using TrypLE express®, an animal-origin free recombinant enzyme (Life Technologies® Cat. No. 12604013). The cells were visually checked every five minutes until they became rounded and lifted off the plate surface. Equal amounts of HuMEC Ready Medium® were added to deactivate the TrypLE express®. The solution was centrifuged at 394 x *g* for five minutes in a swinging bucket (Beckman Coulter Allegra X-12R) centrifuge at room temperature. The cells were washed again with PBS, counted and stored at -80°C for RT-PCR analysis.

#### 5.4.1.1 RNA extraction and integrity

RNAeasy minikit for RNA extraction (Qiagen) was used to extract RNA from the primary culture breastmilk samples in accordance with the manufacturer's instructions as described in Section 4.6.1.4 The RNA content was measured using a Nanodrop® 1000 spectrophotometer (ThermoFisher Scientific). RNA samples with a concentration of no less than 150 µg/mL and an OD 260/280 ratio of 1.9 to 2.1 were considered for further analysis. Only two of the five successful cultures had an adequate amount of RNA. Therefore, a further 5 samples were collected from 5 participants (lactation stage 8-52 weeks postpartum). Table 5.3 summarises the characteristics of the samples. These samples were cultured using the modified HuMEC Ready Medium®. Confluence was achieved at 22.8 days (range 21-26

days) after incubation. The RNA quality and content of all samples were high as summarised in the Table 5.4.

**Table 5.4 RNA content (µg/mL) of cultured cells derived from expressed breastmilk in HuMEC Ready Medium®.**

| Sample | No. of days to reach confluence | No. of cells harvested (x10^6) | RNA concentration (µg/mL) | OD 260/280 |
|---|---|---|---|---|
| 1 | 22 | 7 | 777.7 | 2.08 |
| 2 | 21 | 22.2 | 513.0 | 2.04 |
| 3 | 22 | 7 | 362.8 | 2.05 |
| 4 | 23 | 18 | 171.6 | 2.04 |
| 5 | 26 | 14.3 | 254.3 | 2.05 |

### 5.4.1.2 cDNA generation

A high-capacity cDNA reverse transcription kit (Applied Biosystems) was used to prepare the cDNA as per the manufacturer's instructions as described in section 4.6.1.5. cDNA was prepared in aliquots containing either 1.5 µg of RNA or 5 µ g of RNA. The cDNA was stored at -20°C until needed.

### 5.4.1.3 Quantitative Real -Time Polymerase Chain Reaction (qRT-PCR)

The cultured cells and the matched fresh cells were analysed for the expression of mRNA for the four proteins of interest namely BCRP, MDR1, MRP1 and MRP2. Gene transcription was measured by qRT-PCR using hydrolytic probes (Taqman, Applied Biosystems) with the 7500 Fast RT-PCR system (Applied Biosystems). [Taqman probes used include: Hs00324085_m1 (MDR1); Hs00910358_s1 (MRP1); Hs01385685_m1 (MRP2); HS03929097_g1 (GAPDH).] Each sample was measured in triplicate except for a few cases with inadequate RNA for three distinct assays. GAPDH was used as the housekeeping gene and the samples were normalised against MCF10A. MCF10A was chosen as it is a non-tumorigenic cell line derived from healthy breast epithelial cells and was also used in the longitudinal study (Chapter 4).

## 5.5 Results

Repeated measures of the samples were averaged, and the standard deviations were calculated. Standard deviations were used for quality control of the data and means were used for statistical analysis. As shown in Table 5.5, the gene expression of BCRP, MDR1, MRP1 and MRP2 varied greatly between the fresh and cultured cells with the cultured cells expressing relatively small amount of transporter mRNA. This shows that the HMEC when cultured *in vitro* lose their characteristics and genetic integrity. As shown in Figure 5.4 A, the RQ data were logged due to magnitude changes that existed between samples. Using a mixed model analysis, only BCRP and MDR1 were found to have statistically significant differences ($p<0.05$) between the fresh and cultured cells as depicted in Figure 5.4 B.

**Table 5.5 The relative mRNA expression of transporters in human mammary epithelial cells derived from freshly expressed breastmilk cells and primary culture of breastmilk cells.**

| Transporter Gene | BCRP (RQ) | | MDR1 (RQ) | | MRP1 (RQ) | | MRP2 (RQ) | |
|---|---|---|---|---|---|---|---|---|
| Participant | Fresh | Cultured | Fresh | Cultured | Fresh | Cultured | Fresh | Cultured |
| **1** | 8233.78 | 4.58 | 7.5 | 0.9 | 2.1 | 0.5 | 4.6 | 1.8 |
| **2** | 12266.12 | 12.14 | 5.1 | 0.77 | 1.0 | 0.9 | 9.7 | 3.6 |
| **3** | 7560.17 | 10.03 | 14.4 | 1.6 | 601.2 | 1.6 | - | 8.4 |
| **4** | 3484.10 | 12.14 | 6.7 | 1.2 | 415.0 | 0.51 | 160.4 | 3.2 |
| **5** | 1986.08 | 7.41 | 18.2 | 1.4 | 341.6 | 0.3 | 677.3 | 2.2 |
| **6** | 14211.84 | 3.12 | 18.3 | 1.9 | 1.95 | 1.1 | 6.4 | 1.8 |
| **7** | 19620.16 | 12.57 | 13.5 | 0.87 | 46.3 | 0.90 | 9.9 | 0.8 |

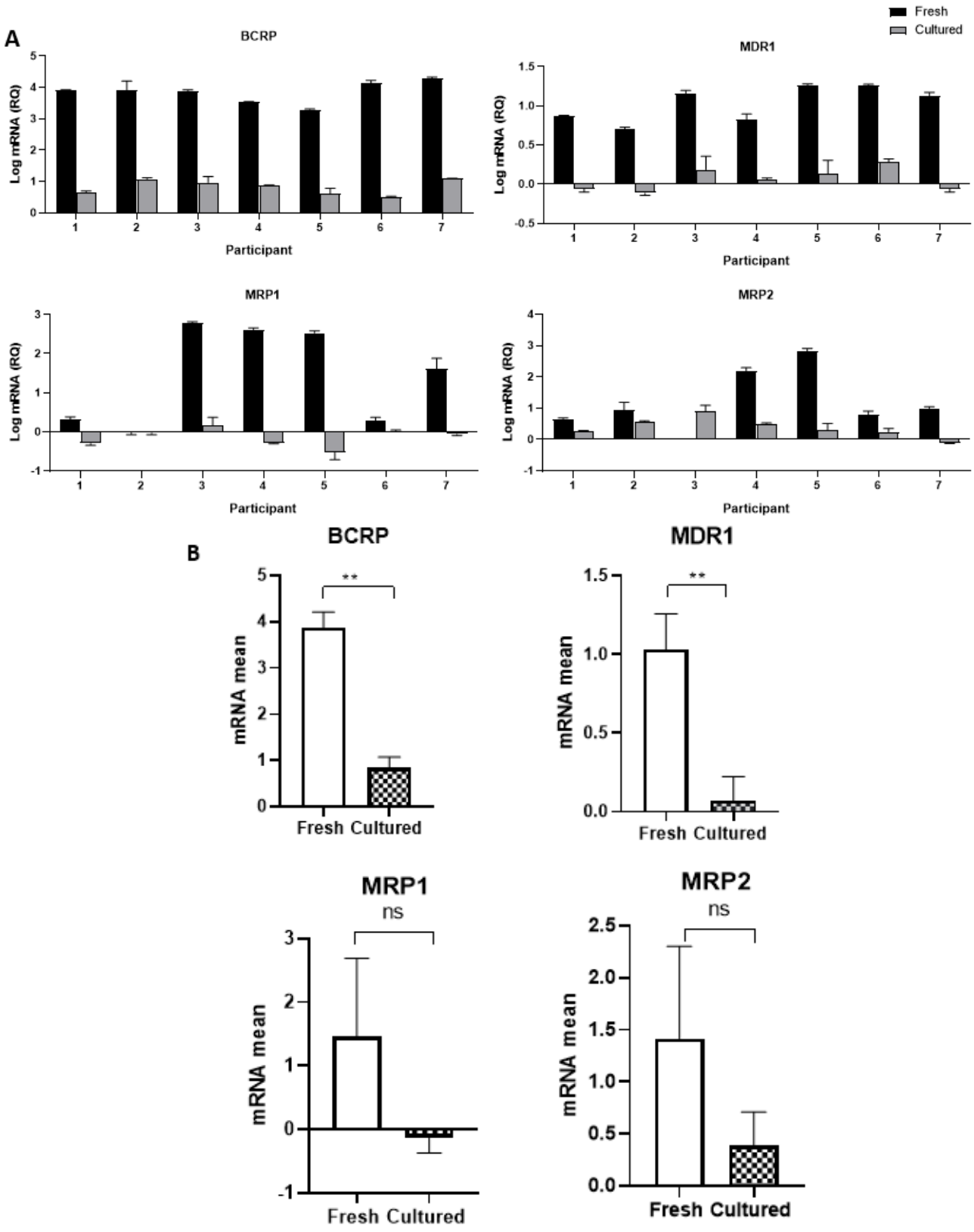


**Figure 5.4 (A) Log mRNA (RQ =Relative Quotient) expression of BCRP, MDR1, MRP1 and MRP2 relative to MCF10A normalised to GAPDH in seven breastmilk derived mammary epithelial cell samples. (B) Comparison of means of mRNA expression of BCRP ($p$ = 0.0017), MDR1 ($p$ = 0.0016), MRP1 ($p$ = 0.0537) and MRP2 ($p$ = 0.1094). F=breastmilk derived cells; C=breastmilk derived cells grown in culture medium.**

## 5.6 Discussion

Breastmilk can be used as a non-invasive tool to better understand the functioning of the mammary gland and associated pathologies. However, successful culturing of breastmilk derived cells in vitro has been hampered by slow growth and variable growth, and a finite life span. The variability in the *in vitro* growth of breastmilk derived HMEC has been attributed to the composition of breastmilk which in turn is influenced by many maternal and environmental factors such as BMI, parity, mode of foetal delivery and breastfeeding practices amongst others (35). The stage of lactation and the degree of emptiness/fullness of the breast, as well as hind vs fore milk are also known to affect *in vitro* growth of breastmilk cells (193, 293).

Human breastmilk is rich in epithelial cells that are exfoliated from the lining of the ducts and alveoli as milk is ejected from the breast during suckling or when vacuum is applied by breast pumps. Being a rich source of epithelial cells, breastmilk has been used to study the expression of active transporters such as BCRP (29, 186). Therefore, it is a great tool to study the potential impact of active transport proteins that are found on the surface of secretory epithelial cells. Due to inter-individualt variability of breastmilk, the expression of active transporters significantly differs between individuals as described in the longitudinal study (Chapter 4) necessitating a personalised approach to the determination of safety of actively transported drugs.

Difficulties in successfully and reliably growing primary cultures of breastmilk derived cells have hampered efforts in its widespread use. This study aimed to optimise cell culture conditions for breastmilk derived epithelial cells with a view to develop a personalised non-invasive, reliable model to study active drug transport mechanisms in the lactating mammary gland. In this study, we report the first use of HuMEC Ready Medium® (Thermofisher Scientific Catalogue No. 12752010) which was modified for culturing primary human mammary epithelial cells derived from breastmilk. The HuMEC Ready Medium® is marketed as a serum free medium for mammary epithelial cell lines sourced from reduction mammoplasties. The various milk cell media that have been reported in the literature show variable growth of HMEC resulting in long incubation periods of up to 50 days and significant variation in the results, achieved attributed to the inter-individual variation of breastmilk (282, 294). However, with the HuMEC Ready Medium®, it was found that regardless of

the stage of lactation, milk derived primary HMEC were cultured successfully, reaching confluence at around day 22 (median 22.8 days: range 21-26 days). Modification of this medium to suit primary culture of HMEC by adding heat inactivated foetal bovine serum (15%) and antimicrobials (penicillin/streptomycin and amphotericin B) to prevent microbial contamination. This was not only much quicker than the 35 days that traditional milk cell medium previously achieved in our laboratory, but also more uniform between samples and compared to literature reports of between 7 and 50 days (282). Additionally, the success rate of culturing was 100% (n=10) compared to 28.5% (2/7) with the traditional milk cell medium. The addition of the penicillin-streptomycin and amphotericin (final concentration of 1% and 2% respectively) helped in preventing cross contamination of the cultures with normal commensal bacteria which is expected in breastmilk samples. Although the HuMEC Ready Medium® is marketed for human mammary epithelial cell lines, we have shown that by including foetal bovine serum and antimicrobials, it can be successfully and reliably used for primary culture of breastmilk derived HMEC.

Unlike traditional culture studies where cells are allowed to undergo growth and proliferation and become immortalised, (as a loss of senescence checkpoints and immortalisation is thought to be involved in tumorigenesis) for this study we wanted to maintain the *in vivo* properties of these cells under *in vitro* culture conditions (295). One aim was to not only improve the growth rate of milk derived primary epithelial cells but also to establish that the cellular characteristics of these cells are unaffected by the culture environment and the cultured cells maintain the same level of transporter expression as the fresh cells. Quantitative Real-Time Polymerase Chain Reaction showed differences in the expression of the studied efflux transporters from the isolated cells when used fresh compared to those that were cultured. This difference was noted in all seven samples and all four ABC transporters studied with the differences in BCRP and MDR1 being statistically significant ($p$ = 0.0017and 0.0016 respectively) using a mixed model analysis. As shown in Figure 5.4, MRP1 and MRP2 did not show statistically significant results overall when the mean expression of these transporters was analysed in fresh cells and compared with expression in cultured primary cells ($p$ = 0.0537 and 0.1094 respectively). This could be attributed to the small sample size (n=7). As MRP1, MDR1 and MRP2 are expressed in relatively much smaller amounts compared to

BCRP in the lactating breast (as demonstrated in Chapter 4), a larger sample size in future studies is recommended.

Although the growth and success rate of breastmilk derived HMEC in culture medium has been significantly improved compared to our previous investigations and published literature, it was found that the use of breastmilk derived cells as a model to predict drug transfer from mother's plasma to breastmilk needs further culture condition optimisation (282, 284, 289). The magnitude of the difference between fresh cells and cultured cells ranged from 10 to 1000-fold in some cases, which highlights that culturing the primary cells to enable drug transport studies would not represent the patient's clinical situation with vastly lower expression of the main transporters present in the breast tissue. In our laboratory we have shown that Caco-2 cells, a human adenocarcinoma cell line that is widely used to examine bidirectional drug transport due to its ability to form tight junctions, has inadequate expression of MDR1 in early passage cell lines, with significantly increased expression with increasing number of passages, thereby linking differentiation with increasing number of passages (296). Other studies have also shown that the expression of other transporters including BCRP, MRP1 and MDR1 increases with increased differentiation (297). This may explain the significant differences in the expression of the efflux transporters between the fresh and cultured cells. To overcome this problem, once these cells reached confluence, they were passaged and grown in the modified HuMEC Ready Medium® using the same conditions as described in section 5.3.7.1. However, the cells failed to survive and differentiate as before, and the passaged cells senesced five days after seeding. Due to a lack of expression of these transporters in the primary culture of the breastmilk derived HMEC and the finite life span of these cells within this study time period, this proposed model utilising milk derived epithelial cells to grow primary culture of HMEC is deemed unsuitable for drug transport and accumulation/efflux studies unless single cell efflux analysis becomes achievable. Furthermore, our study serves to warn that *in vitro* studies are not a true representation of *in vivo* processes and therefore, application of *in vitro* data should be done with great caution.

It is important to acknowledge that the interaction of a cell with its environment may hold the key to the differences seen in *in vitro* cell culture models compared to cells within their *in vivo* settings. Bissell and colleagues believe that "petri dish" based *in*

*vitro* cell cultures do not represent essential cellular functions of living tissues and may limit their potential to predict the *in vivo* cellular responses (298). It is suggested that three-dimensional (3D) *in vitro* cultures are a better cellular model that mimic the functions of living tissues and is closer to the *in vivo* environment (299). However, establishing 3D cultures as a mainstream approach requires the development of standard protocols and quantitative analysis methods, which include well-suited three-dimensional imaging techniques. A 3D cell culture would allow the interaction of various cells with each other, the extracellular matrix and through biochemical and mechanical signalling. It is also believed that the 3D cell cultures allow re-establishment of the necessary interactions between the cells and their extra cellular matrix and can better maintain the specificity of the tissue (298). The use of 3D cell cultures is beyond the scope of this thesis but may be explored in future studies.

## 5.7 Study limitations

A limitation of this study was the small sample size. Although a statistically significant difference was only shown in the expression of two of the four proteins of interest in the fresh versus cultured cells, a larger sample size could assist in obtaining more definitive results. While a specific medium designed for mammary epithelial cells was used on a sample that is known to contain 99% epithelial cells (208), the identity of the cultured cells as exclusively epithelial cells, which could be done through cytokeratin staining was not undertaken (208, 282). Cytokeratin staining was initially planned but was not conducted due to the large variation in the gene expression of the cells between the fresh and cultured cells indicating that pursuing this avenue was not in our interest beyond what we had already discovered. Alveolar and ductal epithelial cells stain positively to CK18 and CK 19 respectively, and myoepithelial cells to CK14. In future studies it is intended to use these markers to purify epithelial cells from different tissue origins and lactation.

## 5.8 Conclusion

Despite the limitations of this study, culture conditions were optimised which allowed the uniform growth of milk derived HMEC independent of the stage of

lactation and maternal factors. Additionally, the time taken for primary breastmilk derived HMEC was reduced significantly from 35 days to 24 days. A commercially available cell culture medium, the HuMEC Ready Medium® marketed for immortalised cell lines was modified and successfully used to grow primary cultures of breastmilk derived HMEC. This study showed that breastmilk derived cells have significantly different characteristics related to active efflux proteins when these cells were grown in culture medium compared to newly isolated cells *in vivo*. Their mRNA expression related to these efflux proteins is much lower once allowed to divide on culture plates. Therefore *in vitro* data should be used with great caution especially with respect to breastmilk derived epithelial cells. At this point it cannot be concluded that cultured breastmilk derived cells are a viable model to study or predict drug transfer for actively transported substances from mother's plasma to breastmilk.

# Chapter 6 General discussion and conclusion

The ultimate objective of this thesis was to add to the body of available literature on medicine safety during lactation so that women who require medicines while breastfeeding can have better safety assurances for the wellbeing of their breastfed infant. Infant ADRs attributed to the transfer of drugs via breastmilk are considered uncommon (21, 96). It was hypothesised that the reported infant ADRs is an under representation of their actual occurrence as barriers such as inability to recognise infant ADRs and difficulty to establish causal relationship between the ADR and maternal medication intake may avert reporting. Furthermore, there is heavy reliance on small studies for lactation related medication safety data as ethical challenges hamper large scale clinical studies in this population. However, due to the great degree of inter-individual variability of breastmilk composition which can affect drug pharmacokinetics, small studies are unlikely to provide definitive information. This can be seen with the current classification of lactation drug safety where a binary system of safety identifying a drug as compatible or otherwise is drawn from these studies. Additionally, new mothers in the early stages of post-partum are likely to be establishing a sense of normality in their developing infant and as a consequence may fail to recognise subtle signs of an ADR which may be taken as expected behaviour. Therefore, they may not be recognising and reporting the ADR.

In order to ascertain whether the occurrence of infant ADRs due to breastfeeding was uncommon as reported in the literature, an online survey of breastfeeding mothers was conducted. This survey aimed to obtain the mother's account or perception of whether they experienced an ADR in their breastfed infant that they attributed to a medicine that they had consumed. This study also aimed to evaluate the impact of a perceived ADR on the continuation of breastfeeding and maternal treatment. The study was completed by 339 women (95% CI) who were breastfeeding or had breastfed in the last 12 months. Over 80 percent of the survey participants were from Western Australia. In Western Australia, an average of between 32,000 to 35,000 births are reported annually (135). Based on this, the study was completed by approximately 1% of women who had given birth in that year. In the study

population, medicine use during breastfeeding was commonly reported with 42% taking one or more medicines while breastfeeding. Infant ADRs were self-reported in a small number (n=23; 6.7%), of which 16 (4.7%) were found to have a positive Naranjo score associating the maternal medications use with the infant ADR with possible or probable association. While ten participants reported the perceived ADR to their healthcare professional, none of these ADRs which typically range from gastrointestinal behavioural or skin reactions, are thought to have been further reported to the TGA by the healthcare professional. The most common medicine group attributed to these ADRs was antibiotics. While side-effects such as gastrointestinal upset are expected, most antibiotics are deemed compatible with breastfeeding. However, evidence of detrimental long-term effects of antibiotic exposure in early life on neurobehavioural development has recently emerged. This further highlights the need for better reporting and pharmacovigilance of medication use in lactation owing to the vulnerability of the infant to adverse effects even if the drug is deemed pharmacokinetically compatible with lactation (89, 138, 139). In this study, one of the reasons attributed to the lack of ADR reporting is thought to be the nature and seriousness of the ADR. As the ADR experienced by the survey participants was an expected side-effect of this class of drugs (i.e. antibiotics causing gastrointestinal upset), the healthcare professionals may have chosen not report them as adverse reactions. An evaluation of the TGA ADR reports from 2003 to 2016 found only 60 cases of poor-quality infant ADRs nationally (approximately 7 cases per year). This study confirmed that infant ADRs due to maternal medication use occurs more commonly than reported and drugs deemed compatible with breastfeeding such as antibiotics not only commonly cause infant ADRs but also have long-term consequences which may go unnoticed due to underreporting.

While conducting this survey, a more detailed case study on one participant's experience of a perceived ADR in their 6-day old infant was initiated. This case-study highlighted the inappropriate prescribing of dextropropoxyphene, an opioid analgesic for post caesarean pain which resulted in a perceived ADR in the breastfed infant. This case study also highlighted a lack of healthcare professional knowledge on medication safety in lactation and their inadequate response to a suspected ADR. Despite there being ample evidence linking dextropropoxyphene to adverse effects in breastfed infants, maternal medicine use was dismissed as a potential causative factor

because it was prescribed by an obstetrician and was seemingly considered safe. Furthermore, the mother was ill-informed about the possible side effects of her medicines as she was not provided with medicines information (written or oral) upon discharge from the hospital. This case study highlighted the lack of clinician knowledge of breastfeeding related medication information and their suboptimal response to the management of a potential lactation related infant ADR. This case also highlighted that failure to give adequate consideration to maternal medication use may result in unnecessary investigative tests, inconclusive or inaccurate diagnosis and parental distress.

In order to add to the available lactation related medication safety data, this thesis investigated the potential role of efflux transporters as a mechanism in the transfer of drugs from mother to baby via breastmilk. In a longitudinal study using breastmilk as a readily available source of human mammary epithelial cells, this study investigated the expression of four active transporters namely MDR1, MRP1, MRP2 and BCRP in the lactating mammary gland. Analytical tools such as quantitative Real-Time Polymerase Chain Reaction, iTRAQ quantitation and immunostaining experiments were used to conduct this study. This study showed that the BCRP gene was strongly overexpressed in the lactating mammary gland. Compared to BCRP, the other three transporters (MRP1, MRP2 and MDR1) were relatively less expressed. BCRP was found to be at the highest level of expression five months post-partum. As BCRP has been shown to contribute to the composition of breastmilk by pumping nutrients and xenobiotics into milk, this high-level expression of BCRP at five months post-partum could potentially mean a time of high risk of exposure and toxicity for a breastfed infant to BCRP substrates. Additionally, this study highlighted the significant inter-individual variability that exists between women and variation in the expression of this transporter in the same woman over time. Protein studies (iTRAQ and immunostaining) were not able to confirm statistically significant results to support RNA expression studies. This was attributed to various factors including a potential lack of sensitivity and a small sample size. Future studies investigating protein levels through validated assays such as Western Blot or ELISA using more sensitive antibodies than currently available (given the low protein yield of breastmilk samples) would be useful to quantify and ascertain the role of BCRP in the transfer of its substrates in the lactating mammary gland at various stages of lactation.

The inter-individual variation in the expression of BCRP uncovered in the longitudinal study further highlighted the need for a personalised approach to the determination of medication safety in breastfeeding. The current pharmacokinetic based "one size fits all" methodology is deemed to be imprecise and may result in inadvertent infant exposure, and toxicity. Therefore, the final study of this thesis was planned as a follow-up to optimise the growth of breastmilk derived epithelial cells *in vitro* to assess their suitability as a personalised drug transport model. The potential for breastmilk derived epithelial cells to be used for the development of a non-invasive, reliable, personalised, quick and cost-effective model to predict the transferability of actively transported drugs into breastmilk was evaluated. The advantages of primary cells in providing a reflective picture of *in vivo* process is well established and recognised but the difficulties associated with culturing of primary HMEC have hampered their widespread use and greater application (208, 246, 282, 294, 295). Breastmilk derived HMEC have been shown to have a variable and slow growth rate especially with the commonly utilised "conventional milk cell media" (282). Before investigating the viability and feasibility of cultured mammary epithelial cells for use as a model for drug transport studies, the growth of breastmilk derived mammary epithelial cells in culture needed to be optimised. Many culture media combinations were explored without success until HuMEC Ready Medium® (Life Technologies) designed for already immortalised mammary epithelial cell lines was investigated. With appropriate modification of this media the breastmilk derived primary mammary epithelial cells began to respond positively with a reduced time to reach confluence from 35 days to 22 days. The growth was reproducible indicating that the use of an appropriate culture medium can have a tremendous impact on the growth of these cells. Once growth conditions were improved, qRT-PCR was conducted on the cell sample propagated in culture comparing their expression to cells derived from the same donated milk sample. The cultured cells showed a significant reduction for BCRP and MDR1, where the expression of these transporters in the fresh milk cells were magnitudes higher compared to the cultured cells. Due to the differences in the *in vitro* and *in vivo* characteristics of milk derived cells, the use of breastmilk as a tool to conduct drug transport studies is not currently possible. This study also highlighted that despite cultured primary cells being regarded as a better alternative to cell lines, the extrapolation of cultured primary cell data to *in vivo* processes should be conducted with great caution.

In summary this work shows that available lactation related medication safety information is inadequate. At the molecular level, little is known about controlling the transfer of maternal medication into breastmilk. At the clinical level, identification and reporting of infant ADRs is decidedly inadequate. Further research to investigate the role of the overexpressed BCRP in the lactating mammary gland is warranted. This study also highlighted that breastfeeding related infant ADRs are under-reported and potentially does not reflect the true occurrence of ADRs in breastfed infants. Larger studies to identify whether this is a nationwide/worldwide problem are warranted. Due to inadequacies in lactation related medicine safety data and inadequate reporting of infant ADRs, there is an increased need for long term follow up of breastfed children exposed to medicines. This can be undertaken in the form of a national registry. This is particularly important as clinical trials in this subpopulation will continue to be difficult to conduct. Additionally, breastfeeding mothers should be educated on the possibility and risks of infant toxicity due to maternal medication use during their hospital admission. Future work should be directed towards increasing awareness and reporting of infant ADRs. This can be achieved through a systemic supportive infrastructure focussed on a) educating new mothers to recognise and report any untoward infant ADRs to their healthcare professional and the TGA; b) better engagement and education of frontline healthcare professionals to identify, investigate and report breastfeeding related infant ADRs; c) adoption of a personalised approach to the determination of drug safety in lactation where possible.

# References


1. National Health and Medical Research Council. Eat for health; Infant feeding Guidelines Summary Australia2012 [cited 2019 28th February]. Available from: https://www.nhmrc.gov.au/about-us/publications/infant-feeding-guidelines-information-health-workers#block-views-block-file-attachments-content-block-1.
2. World Health Organisation. Planning Guide for national implementation of the Global Strategy for Infant and Young Child Feeding 2012 [Available from: https://apps.who.int/iris/bitstream/handle/10665/43619/9789241595193_eng.pdf;jsessionid=7D47B8073EB464AB11FCE057F1C01736?sequence=1.
3. Victora CG, Bahl R, Barros AJ, França GV, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. Lancet. 2016;387(10017):475-90.
4. Dieterich CM, Felice JP, O'Sullivan E, Rasmussen KM. Breastfeeding and health outcomes for the mother-infant dyad. Pediatr Clin North Am. 2013;60(1):31-48.
5. Horta BL, de Sousa BA, de Mola CL. Breastfeeding and neurodevelopmental outcomes. Curr Opin Clin Nutr Metab Care. 2018;21(3):174-8.
6. Victora CG, Horta BL, Loret de Mola C, Quevedo L, Pinheiro RT, Gigante DP, et al. Association between breastfeeding and intelligence, educational attainment, and income at 30 years of age: a prospective birth cohort study from Brazil. Lancet Glob Health. 2015;3(4):e199-205.
7. Li DP, Du C, Zhang ZM, Li GX, Yu ZF, Wang X, et al. Breastfeeding and ovarian cancer risk: a systematic review and meta-analysis of 40 epidemiological studies. Asian Pac J Cancer Prev. 2014;15(12):4829-37.
8. Victora CG, Bahl R, Barros AJ, Franca GV, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. Lancet. 2016;387(10017):475-90.
9. Piper KM, Berry CA, Cregan MD. The bioactive nature of human breastmilk. Breastfeed Rev. 2007;15(3):5-10.
10. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. Pediatrics. 1993;91(5):867-72.
11. Indumathi S, Dhanasekaran M, Rajkumar JS, Sudarsanam D. Exploring the stem cell and non-stem cell constituents of human breast milk. Cytotechnology. 2013;65(3):385-93.
12. Fleishaker JC, Desai N, McNamara PJ. Factors affecting the milk-to-plasma drug concentration ratio in lactating women: physical interactions with protein and fat. J Pharm Sci. 1987;76(3):189-93.
13. Hale TW, Rowe HE. Medications & Mothers' milk 17th ed. New York: Springer publishing company; 2017.
14. Walters DD, Phan LTH, Mathisen R. The cost of not breastfeeding: global results from a new tool. Health Policy Plan. 2019;34(6):407-17.
15. Chowdhury R, Sinha B, Sankar MJ, Taneja S, Bhandari N, Rollins N, et al. Breastfeeding and maternal health outcomes: a systematic review and meta-analysis. Acta Paediatr. 2015;104(467):96-113.
16. Bartick MC, Schwarz EB, Green BD, Jegier BJ, Reinhold AG, Colaizy TT, et al. Suboptimal breastfeeding in the United States: Maternal and pediatric health outcomes and costs. Matern Child Nutr. 2017;13(1).
17. Department of Health. Australian National Breastfeeding Strategy: 2019 and Beyond 2019 [31st October 2019]. Available from: http://www.coaghealthcouncil.gov.au/Portals/0/Australian%20National%20Breastfeeding%20Strategy%20-%20FINAL%20.pdf.
18. Ogbo FA, Eastwood J, Page A, Arora A, McKenzie A, Jalaludin B, et al. Prevalence and determinants of cessation of exclusive breastfeeding in the early postnatal period in Sydney, Australia. Int Breastfeed J. 2016;12:16.

19. Department of Health. Australian National Breastfeeding Strategy: 2019 and Beyond Canberra, ACT: The Coag Health Council 2019 [Available from: https://www1.health.gov.au/internet/main/publishing.nsf/Content/health-pubhlth-strateg-brfeed-index.htm.
20. Australian Institute of Health and Welfare. Australia's mothers and babies data visualisations: Australian Institute of Health and Welfare; 2019 [Available from: https://www.aihw.gov.au/reports/mothers-babies/australias-mothers-babies-data-visualisations/contents/demographics-of-mothers-and-babies/maternal-age.
21. Soussan C, Gouraud A, Portolan G, Jean-Pastor MJ, Pecriaux C, Montastruc JL, et al. Drug-induced adverse reactions via breastfeeding: a descriptive study in the French Pharmacovigilance Database. European journal of clinical pharmacology. 2014;70(11):1361-6.
22. Hegedus E, Oakes DJ, Hill M, Ritchie HE, Kennedy DS. Calls to a Major Teratogen Information Service Regarding Exposures During Breastfeeding. Breastfeed Med. 2019;14(9):674-9.
23. Alvarez AI, Perez M, Prieto JG, Molina AJ, Real R, Merino G. Fluoroquinolone efflux mediated by ABC transporters. J Pharm Sci. 2008;97(9):3483-93.
24. Smits A, Annaert P, Allegaert K. Drug disposition and clinical practice in neonates: cross talk between developmental physiology and pharmacology. Int J Pharm. 2013;452(1-2):8-13.
25. Anderson PO, Manoguerra AS, Valdes V. A Review of Adverse Reactions in Infants From Medications in Breastmilk. Clin Pediatr (Phila). 2016;55(3):236-44.
26. Ito N, Ito K, Ikebuchi Y, Kito T, Miyata H, Toyoda Y, et al. Organic cation transporter/solute carrier family 22a is involved in drug transfer into milk in mice. J Pharm Sci. 2014;103(10):3342-8.
27. Petzinger E, Geyer J. Drug transporters in pharmacokinetics. Naunyn Schmiedebergs Arch Pharmacol. 2006;372(6):465-75.
28. Zhang JT. Use of arrays to investigate the contribution of ATP-binding cassette transporters to drug resistance in cancer chemotherapy and prediction of chemosensitivity. Cell Res. 2007;17(4):311-23.
29. Alcorn J, Lu X, Moscow JA, McNamara PJ. Transporter gene expression in lactating and nonlactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. J Pharmacol Exp Ther. 2002;303(2):487-96.
30. Gilchrist SE, Alcorn J. Lactation stage-dependent expression of transporters in rat whole mammary gland and primary mammary epithelial organoids. Fundam Clin Pharmacol. 2010;24(2):205-14.
31. Lindner S, Halwachs S, Wassermann L, Honscha W. Expression and subcellular localization of efflux transporter ABCG2/BCRP in important tissue barriers of lactating dairy cows, sheep and goats. J Vet Pharmacol Ther. 2013;36(6):562-70.
32. Chung MY. Factors affecting human milk composition. Pediatr Neonatol. 2014;55(6):421-2.
33. Casavale KO, Ahuja JKC, Wu X, Li Y, Quam J, Olson R, et al. NIH workshop on human milk composition: summary and visions. Am J Clin Nutr. 2019;110(3):769-79.
34. Lee S, Kelleher SL. Biological underpinnings of breastfeeding challenges: the role of genetics, diet, and environment on lactation physiology. Am J Physiol Endocrinol Metab. 2016;311(2):E405-22.
35. Moossavi S, Sepehri S, Robertson B, Bode L, Goruk S, Field CJ, et al. Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. Cell Host Microbe. 2019;25(2):324-35.e4.
36. Shennan DB, Peaker M. Transport of milk constituents by the mammary gland. Physiol Rev. 2000;80(3):925-51.
37. Garcia-Lino AM, Alvarez-Fernandez I, Blanco-Paniagua E, Merino G, Alvarez AI. Transporters in the Mammary Gland-Contribution to Presence of Nutrients and Drugs into Milk. Nutrients. 2019;11(10).

38. Beauchamp GA, Hendrickson RG, Horowitz BZ, Spyker DA. Exposures Through Breast Milk: An Analysis of Exposure and Information Calls to U.S. Poison Centers, 2001-2017. Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine. 2019.
39. Klein LD, Breakey AA, Scelza B, Valeggia C, Jasienska G, Hinde K. Concentrations of trace elements in human milk: Comparisons among women in Argentina, Namibia, Poland, and the United States. PLoS One. 2017;12(8):e0183367.
40. Ninkina N, Kukharsky MS, Hewitt MV, Lysikova EA, Skuratovska LN, Deykin AV, et al. Stem cells in human breast milk. Hum Cell. 2019;32(3):223-30.
41. Kaingade PM, Somasundaram I, Nikam AB, Sarang SA, Patel JS. Assessment of Growth Factors Secreted by Human Breastmilk Mesenchymal Stem Cells. Breastfeed Med. 2016;11(1):26-31.
42. Sani M, Ebrahimi S, Aleahmad F, Salmannejad M, Hosseini SM, Mazarei G, et al. Differentiation Potential of Breast Milk-Derived Mesenchymal Stem Cells into Hepatocyte-Like Cells. Tissue Eng Regen Med. 2017;14(5):587-93.
43. Rawn DFK, Sadler AR, Casey VA, Breton F, Sun WF, Arbuckle TE, et al. Dioxins/furans and PCBs in Canadian human milk: 2008-2011. Sci Total Environ. 2017;595:269-78.
44. LaKind JS, Lehmann GM, Davis MH, Hines EP, Marchitti SA, Alcala C, et al. Infant Dietary Exposures to Environmental Chemicals and Infant/Child Health: A Critical Assessment of the Literature. Environ Health Perspect. 2018;126(9):96002.
45. Lehmann GM, LaKind JS, Davis MH, Hines EP, Marchitti SA, Alcala C, et al. Environmental Chemicals in Breast Milk and Formula: Exposure and Risk Assessment Implications. Environ Health Perspect. 2018;126(9):96001.
46. Neville MC, Keller R, Seacat J, Lutes V, Neifert M, Casey C, et al. Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation. Am J Clin Nutr. 1988;48(6):1375-86.
47. McNally S, Martin F. Molecular regulators of pubertal mammary gland development. Ann Med. 2011;43(3):212-34.
48. Hassiotou F, Geddes D. Anatomy of the human mammary gland: Current status of knowledge. Clin Anat. 2013;26(1):29-48.
49. Musumeci G, Castrogiovanni P, Szychlinska MA, Aiello FC, Vecchio GM, Salvatorelli L, et al. Mammary gland: From embryogenesis to adult life. Acta Histochem. 2015;117(4-5):379-85.
50. Tortora GJ, Grabowski SR. Principles of Anatomy and Physiology. 10th ed. Hoboken: John Wiley & Sons, Inc.; 2003.
51. Huebner RJ, Ewald AJ. Cellular foundations of mammary tubulogenesis. Semin Cell Dev Biol. 2014;31:124-31.
52. Neville MC, McFadden TB, Forsyth I. Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia. 2002;7(1):49-66.
53. Pang WW, Hartmann PE. Initiation of human lactation: secretory differentiation and secretory activation. J Mammary Gland Biol Neoplasia. 2007;12(4):211-21.
54. Grattan DR, Pi XJ, Andrews ZB, Augustine RA, Kokay IC, Summerfield MR, et al. Prolactin receptors in the brain during pregnancy and lactation: implications for behavior. Horm Behav. 2001;40(2):115-24.
55. Need EF, Atashgaran V, Ingman WV, Dasari P. Hormonal regulation of the immune microenvironment in the mammary gland. J Mammary Gland Biol Neoplasia. 2014;19(2):229-39.
56. Neville MC, Anderson SM, McManaman JL, Badger TM, Bunik M, Contractor N, et al. Lactation and neonatal nutrition: defining and refining the critical questions. J Mammary Gland Biol Neoplasia. 2012;17(2):167-88.
57. Neville MC, Morton J, Umemura S. Lactogenesis. The transition from pregnancy to lactation. Pediatr Clin North Am. 2001;48(1):35-52.
58. Voogt JL, Lee Y, Yang S, Arbogast L. Regulation of prolactin secretion during pregnancy and lactation. Prog Brain Res. 2001;133:173-85.

59. Tucker HA. Endocrinology of lactation. Semin Perinatol. 1979;3(3):199-223.
60. Ostrom KM. A review of the hormone prolactin during lactation. Prog Food Nutr Sci. 1990;14(1):1-43.
61. Milsom SR, Breier BH, Gallaher BW, Cox VA, Gunn AJ, Gluckman PD. Growth hormone stimulates galactopoiesis in healthy lactating women. Acta Endocrinol (Copenh). 1992;127(4):337-43.
62. Naylor MJ, Oakes SR, Gardiner-Garden M, Harris J, Blazek K, Ho TWC, et al. Transcriptional changes underlying the secretory activation phase of mammary gland development. Molecular endocrinology (Baltimore, Md). 2005;19(7):1868-83.
63. Buhimschi CS. Endocrinology of lactation. Obstet Gynecol Clin North Am. 2004;31(4):963-79, xii.
64. Uvnas-Moberg K. Neuroendocrinology of the mother-child interaction. Trends Endocrinol Metab. 1996;7(4):126-31.
65. Weaver SR, Hernandez LL. Autocrine-paracrine regulation of the mammary gland. J Dairy Sci. 2016;99(1):842-53.
66. Fok D, Aris IM, Ho JH, Chan YH, Rauff M, Lui JK, et al. Early initiation and regular breast milk expression reduces risk of lactogenesis II delay in at-risk Singaporean mothers in a randomised trial. Singapore Med J. 2019;60(2):80-8.
67. Peaker M, Wilde CJ. Feedback control of milk secretion from milk. J Mammary Gland Biol Neoplasia. 1996;1(3):307-15.
68. Anderson PO. Drugs in Lactation. Pharm Res. 2018;35(3):45.
69. Begg EJ, Atkinson HC. Modelling of the passage of drugs into milk. Pharmacol Ther. 1993;59(3):301-10.
70. Ito S, Lee A. Drug excretion into breast milk--overview. Advanced drug delivery reviews. 2003;55(5):617-27.
71. C Schaefer PP, R Miller. Drugs during pregnancy and lactation- Treatment options and risk assessment Second ed. London GB: Acacdemic Press; 2007.
72. Buhimschi CS, Weiner CP. Medications in pregnancy and lactation: part 1. Teratology. Obstet Gynecol. 2009;113(1):166-88.
73. Dostal LA, Weaver RP, Schwetz BA. Excretion of high concentrations of cimetidine and ranitidine into rat milk and their effects on milk composition and mammary gland nucleic acid content. Toxicol Appl Pharmacol. 1990;102(3):430-42.
74. Gerk PM, Kuhn RJ, Desai NS, McNamara PJ. Active transport of nitrofurantoin into human milk. Pharmacotherapy. 2001;21(6):669-75.
75. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Mol Pharmacol. 2005;67(5):1758-64.
76. Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, et al. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat Med. 2005;11(2):127-9.
77. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev. 2003;55(1):3-29.
78. van Herwaarden AE, Schinkel AH. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. Trends Pharmacol Sci. 2006;27(1):10-6.
79. Teoh S, Ilett KF, Hackett LP, Kohan R. Estimation of rac-amisulpride transfer into milk and of infant dose via milk during its use in a lactating woman with bipolar disorder and schizophrenia. Breastfeed Med. 2011;6(2):85-8.
80. Liu X. SLC Family Transporters. Adv Exp Med Biol. 2019;1141:101-202.
81. Tee L.B.G ST, Williams D, Crowe A, Illett K Drug transporters in human mammary epithelial cells. 16th World Congress of Basic and Clinical Pharmacology,; Denmark2010.
82. Obaidat A, Roth M, Hagenbuch B. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. Annu Rev Pharmacol Toxicol. 2012;52:135-51.

83. Choi YH, Yu AM. ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des. 2014;20(5):793-807.
84. Han LW, Gao C, Mao Q. An update on expression and function of P-gp/ABCB1 and BCRP/ABCG2 in the placenta and fetus. Expert Opin Drug Metab Toxicol. 2018;14(8):817-29.
85. van Herwaarden AE, Wagenaar E, Merino G, Jonker JW, Rosing H, Beijnen JH, et al. Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. Mol Cell Biol. 2007;27(4):1247-53.
86. Ling B, Alcorn J. Lactation stage influences drug milk-to-serum values and neonatal exposure risk. Int J Toxicol. 2010;29(4):411-7.
87. Brucker MC, King TL. The 2015 US Food and Drug Administration Pregnancy and Lactation Labeling Rule. J Midwifery Womens Health. 2017;62(3):308-16.
88. Slykerman RF, Thompson J, Waldie KE, Murphy R, Wall C, Mitchell EA. Antibiotics in the first year of life and subsequent neurocognitive outcomes. Acta Paediatr. 2017;106(1):87-94.
89. Lavebratt C, Yang LL, Giacobini M, Forsell Y, Schalling M, Partonen T, et al. Early exposure to antibiotic drugs and risk for psychiatric disorders: a population-based study. Transl Psychiatry. 2019;9(1):317.
90. Kronenfeld N, Berlin M, Shaniv D, Berkovitch M. Use of Psychotropic Medications in Breastfeeding Women. Birth Defects Res. 2017;109(12):957-97.
91. Schoretsanitis G, Augustin M, Saßmannshausen H, Franz C, Gründer G, Paulzen M. Antidepressants in breast milk; comparative analysis of excretion ratios. Arch Womens Ment Health. 2019;22(3):383-90.
92. Amir LH, Pirotta MV, Raval M. Breastfeeding--evidence based guidelines for the use of medicines. Aust Fam Physician. 2011;40(9):684-90.
93. Schirm E, Schwagermann MP, Tobi H, de Jong-van den Berg LT. Drug use during breastfeeding. A survey from the Netherlands. Eur J Clin Nutr. 2004;58(2):386-90.
94. Saha MR, Ryan K, Amir LH. Postpartum women's use of medicines and breastfeeding practices: a systematic review. Int Breastfeed J. 2015;10:28.
95. Stultz EE, Stokes JL, Shaffer ML, Paul IM, Berlin CM. Extent of medication use in breastfeeding women. Breastfeed Med. 2007;2(3):145-51.
96. Anderson PO, Pochop SL, Manoguerra AS. Adverse drug reactions in breastfed infants: less than imagined. Clin Pediatr (Phila). 2003;42(4):325-40.
97. Amir LH, Pirotta MV. Medicines for breastfeeding women: a postal survey of general practitioners in Victoria. Med J Aust. 2009;191(2):126.
98. Al-Sawalha NA, Sawalha A, Tahaineh L, Almomani B, Al-Keilani M. Healthcare providers' attitude and knowledge regarding medication use in breastfeeding women: a Jordanian national questionnaire study. J Obstet Gynaecol. 2018;38(2):217-21.
99. Szucs KA, Miracle DJ, Rosenman MB. Breastfeeding knowledge, attitudes, and practices among providers in a medical home. Breastfeed Med. 2009;4(1):31-42.
100. Feldman-Winter LB, Schanler RJ, O'Connor KG, Lawrence RA. Pediatricians and the promotion and support of breastfeeding. Arch Pediatr Adolesc Med. 2008;162(12):1142-9.
101. Pound CM, Williams K, Grenon R, Aglipay M, Plint AC. Breastfeeding Knowledge, Confidence, Beliefs, and Attitudes of Canadian Physicians. J Hum Lact. 2014;30(3):298-309.
102. Brodribb W, Fallon A, Jackson C, Hegney D. Breastfeeding and Australian GP registrars--their knowledge and attitudes. J Hum Lact. 2008;24(4):422-30.
103. Brodribb W, Fallon AB, Jackson C, Hegney D. Breastfeeding knowledge - the experiences of Australian general practice registrars. Aust Fam Physician. 2009;38(1-2):26-9.
104. Australian Institute of Health and Welfare. Australians mothers and babies data visualisations 2020 [updated 29 May 2020. Available from: https://www.aihw.gov.au/reports/mothers-babies/australias-mothers-babies-data-visualisations/contents/demographics-of-mothers-and-babies/maternal-age.

105. Hudzik B, Zubelewicz-Szkodzinska B. Antithyroid drugs during breastfeeding. Clin Endocrinol (Oxf). 2016;85(6):827-30.
106. Sultana J, Cutroneo P, Trifiro G. Clinical and economic burden of adverse drug reactions. J Pharmacol Pharmacother. 2013;4(Suppl 1):S73-7.
107. Ahmadzai H TL, Crowe A. Pharmacological role of efflux transporters: Clinical implications for medication use during breastfeeding. World J Pharmacol. 2014;3(4):153-61.
108. Seyberth HW, Kauffman RE. Basics and dynamics of neonatal and pediatric pharmacology. Handb Exp Pharmacol. 2011;205:3-49.
109. de Ponti M, Stewart K, Amir LH, Hussainy SY. Medicine use and safety while breastfeeding: investigating the perspectives of community pharmacists in Australia. Aust J Prim Health. 2015;21(1):46-57.
110. Bettiol A, Lombardi N, Marconi E, Crescioli G, Bonaiuti R, Maggini V, et al. The use of complementary and alternative medicines during breastfeeding: results from the Herbal supplements in Breastfeeding InvesTigation (HaBIT) study. Br J Clin Pharmacol. 2018;84(9):2040-7.
111. Ceftriaxone-AFT. 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=ceftriaxone&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=94110001_2.
112. Polaramine (Dexchlorpheniramine). 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=polaramine&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=13130004_2.
113. Ozin (Olanzapine). 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=olanzapine&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=92020001_2.
114. Zyrtec Nasal Spray 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=Zyrtec&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=13090001_2.
115. Eleva (Sertraline). 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=Sertraline&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=74010001_2.
116. Augmentin Duo Forte. 2020. In: MIMS Online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=Augmentin&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=38020001_2.
117. Cephalexin APO. 2020. In: MIMS online [Internet]. Crows Nest (NSW). Available from: https://www-mimsonline-com-au.dbgw.lis.curtin.edu.au/Search/AbbrPI.aspx?ModuleName=Product%20Info&searchKeyword=cephalexin&PreviousPage=~/Search/QuickSearch.aspx&SearchType=&ID=15710001_2.
118. Shields KE, Lyerly AD. Exclusion of pregnant women from industry-sponsored clinical trials. Obstet Gynecol. 2013;122(5):1077-81.
119. Amir LH, Grzeskowiak LE, Kam RL. Ethical Issues in Use of Medications During Lactation. J Hum Lact. 2020;36(1):34-9.
120. Sachs HC. The transfer of drugs and therapeutics into human breast milk: an update on selected topics. Pediatrics. 2013;132(3):e796-809.

121. eTG Complete. Melbourne(Vic): Therapeutic Guidelines Ltd; 2017 [cited 2018 25th Dec]. Available from: https://tgldcdp-tg-org-au.dbgw.lis.curtin.edu.au/etgAccess.
122. Ito S. Opioids in Breast Milk: Pharmacokinetic Principles and Clinical Implications. J Clin Pharmacol. 2018;58 Suppl 10:S151-s63.
123. The Therapeutics Goods Administration. Reporting Adverse Effects 2018 [30th November 2018]. Available from: https://www.tga.gov.au/overview-how-tga-manages-medicine-adverse-event-reports.
124. Australian Bureau of Statistics. Births, Australia, 2016 2016 [Available from: https://www.abs.gov.au/AUSSTATS/abs@.nsf/Lookup/3301.0Main+Features32016?OpenDocument#:~:text=There%20were%20311%2C104%20births%20registered%20in%20Australia%20in%202016.
125. Murayama H, Sakuma M, Takahashi Y, Morimoto T. Improving the assessment of adverse drug reactions using the Naranjo Algorithm in daily practice: The Japan Adverse Drug Events Study. Pharmacol Res Perspect. 2018;6(1).
126. Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther. 1981;30(2):239-45.
127. Belhekar MN, Taur SR, Munshi RP. A study of agreement between the Naranjo algorithm and WHO-UMC criteria for causality assessment of adverse drug reactions. Indian J Pharmacol. 2014;46(1):117-20.
128. Kattah AG, Garovic VD. The management of hypertension in pregnancy. Adv Chronic Kidney Dis. 2013;20(3):229-39.
129. Wilkerson RG, Ogunbodede AC. Hypertensive Disorders of Pregnancy. Emerg Med Clin North Am. 2019;37(2):301-16.
130. Ito S, Blajchman A, Stephenson M, Eliopoulos C, Koren G. Prospective follow-up of adverse reactions in breast-fed infants exposed to maternal medication. Am J Obstet Gynecol. 1993;168(5):1393-9.
131. Brandstetter S, Atzendorf J, Seelbach-Göbel B, Melter M, Kabesch M, Apfelbacher C. Sociodemographic factors associated with health literacy in a large sample of mothers of newborn children: cross-sectional findings from the KUNO-Kids birth cohort study. Eur J Pediatr. 2020;179(1):165-9.
132. Slomian J, Bruyère O, Reginster JY, Emonts P. The internet as a source of information used by women after childbirth to meet their need for information: A web-based survey. Midwifery. 2017;48:46-52.
133. Tan SS, Goonawardene N. Internet Health Information Seeking and the Patient-Physician Relationship: A Systematic Review. J Med Internet Res. 2017;19(1):e9.
134. Bianco A, Zucco R, Nobile CG, Pileggi C, Pavia M. Parents seeking health-related information on the Internet: cross-sectional study. J Med Internet Res. 2013;15(9):e204.
135. Department of Justice. Registry of Births, deaths and Marriages 2016 [26th March 2020]. Available from: https://bdm.justice.wa.gov.au/S/statistics_print.aspx.
136. Cliff-Eribo KO, Sammons H, Choonara I. Systematic review of paediatric studies of adverse drug reactions from pharmacovigilance databases. Expert Opin Drug Saf. 2016;15(10):1321-8.
137. Nahum GG, Uhl K, Kennedy DL. Antibiotic use in pregnancy and lactation: what is and is not known about teratogenic and toxic risks. Obstet Gynecol. 2006;107(5):1120-38.
138. Mathew JL. Effect of maternal antibiotics on breast feeding infants. Postgrad Med J. 2004;80(942):196-200.
139. Slykerman RF, Coomarasamy C, Wickens K, Thompson JMD, Stanley TV, Barthow C, et al. Exposure to antibiotics in the first 24 months of life and neurocognitive outcomes at 11 years of age. Psychopharmacology (Berl). 2019;236(5):1573-82.
140. Ito S, Koren G, Einarson TR. Maternal noncompliance with antibiotics during breastfeeding. Ann Pharmacother. 1993;27(1):40-2.
141. Olesen C, Søndergaard C, Thrane N, Nielsen GL, de Jong-van den Berg L, Olsen J. Do pregnant women report use of dispensed medications? Epidemiology. 2001;12(5):497-501.

142. Spiesser-Robelet L, Brunie V, de Andrade V, Gagnayre R. Knowledge, Representations, Attitudes, and Behaviors of Women Faced With Taking Medications While Breastfeeding. J Hum Lact. 2017;33(1):98-114.
143. Lupattelli A, Spigset O, Nordeng H. Adherence to medication for chronic disorders during pregnancy: results from a multinational study. Int J Clin Pharm. 2014;36(1):145-53.
144. Bennett IM, Marcus SC, Palmer SC, Coyne JC. Pregnancy-related discontinuation of antidepressants and depression care visits among Medicaid recipients. Psychiatr Serv. 2010;61(4):386-91.
145. Sim TF, Sherriff J, Hattingh HL, Parsons R, Tee LB. The use of herbal medicines during breastfeeding: a population-based survey in Western Australia. BMC Complement Altern Med. 2013;13:317.
146. Sim TF, Hattingh HL, Sherriff J, Tee LB. Perspectives and attitudes of breastfeeding women using herbal galactagogues during breastfeeding: a qualitative study. BMC Complement Altern Med. 2014;14:216.
147. Hussainy SY, Dermele N. Knowledge, attitudes and practices of health professionals and women towards medication use in breastfeeding: A review. Int Breastfeed J. 2011;6:11.
148. Lefever S, Dal M, Matthíasdóttir Á. Online data collection in academic research: advantages and limitations. British journal of educational technology. 2007;38.
149. Marcano Belisario JS, Jamsek J, Huckvale K, O'Donoghue J, Morrison CP, Car J. Comparison of self-administered survey questionnaire responses collected using mobile apps versus other methods. Cochrane Database Syst Rev. 2015(7):Mr000042.
150. Bisson DL, Newell SD, Laxton C. Antenatal and Postnatal Analgesia: Scientific Impact Paper No. 59. BJOG : an international journal of obstetrics and gynaecology. 2019;126(4):e114-e24.
151. Hendrickson RG, McKeown NJ. Is maternal opioid use hazardous to breast-fed infants? Clinical toxicology (Philadelphia, Pa). 2012;50(1):1-14.
152. Koren G, Cairns J, Chitayat D, Gaedigk A, Leeder SJ. Pharmacogenetics of morphine poisoning in a breastfed neonate of a codeine-prescribed mother. Lancet (London, England). 2006;368(9536):704.
153. Willmann S, Edginton AN, Coboeken K, Ahr G, Lippert J. Risk to the breast-fed neonate from codeine treatment to the mother: a quantitative mechanistic modeling study. Clinical pharmacology and therapeutics. 2009;86(6):634-43.
154. Sutton CD, Carvalho B. Optimal Pain Management After Cesarean Delivery. Anesthesiology clinics. 2017;35(1):107-24.
155. Badreldin N, Grobman WA, Chang KT, Yee LM. Opioid prescribing patterns among postpartum women. Am J Obstet Gynecol. 2018;219(1):103.e1-.e8.
156. Osmundson SS, Schornack LA, Grasch JL, Zuckerwise LC, Young JL, Richardson MG. Postdischarge Opioid Use After Cesarean Delivery. Obstet Gynecol. 2017;130(1):36-41.
157. Rigourd V, Amirouche A, Tasseau A, Kintz P, Serreau R. Retrospective diagnosis of an adverse drug reaction in a breastfed neonate: liquid chromatography-tandem mass spectrometry quantification of dextropropoxyphene and norpropoxyphene in newborn and maternal hair. J Anal Toxicol. 2008;32(9):787-9.
158. Naumburg EG, Meny RG. Breast milk opioids and neonatal apnea. Am J Dis Child. 1988;142(1):11-2.
159. Gow PJ, Ghabrial H, Smallwood RA, Morgan DJ, Ching MS. Neonatal hepatic drug elimination. Pharmacol Toxicol. 2001;88(1):3-15.
160. Allegaert K, van de Velde M, van den Anker J. Neonatal clinical pharmacology. Paediatr Anaesth. 2014;24(1):30-8.
161. Flanagan RJ, Johnston A, White AS, Crome P. Pharmacokinetics of dextropropoxyphene and nordextropropoxyphene in young and elderly volunteers after single and multiple dextropropoxyphene dosage. British journal of clinical pharmacology. 1989;28(4):463-9.
162. Koyyalagunta D. Opioid Analgesics. 2nd ed2007. 939-64 p.

163. Kunka RL, Venkataramanan R, Stern RM, Ladik CF. Excretion of propoxyphene and norpropoxyphene in breast milk. Clin Pharmacol Ther. 1984;35(5):675-80.
164. Propoxyphene. Drugs and Lactation Database (LactMed). Bethesda (MD): National Library of Medicine (US); 2006.
165. Delcher C, Chen G, Wang Y, Slavova S, Goldberger BA. Fatal poisonings involving propoxyphene before and after voluntary withdrawal from the United States' market: An analysis from the state of Florida. Forensic Sci Int. 2017;280:228-32.
166. Buckley NA, Faunce TA. Trials and tribulations in the removal of dextropropoxyphene from the Australian Register of Therapeutic Goods. Med J Aust. 2013;199(4):257-60.
167. Drugs and Lactation Database (LactMed). Propoxyphene: national Library of Medicine (US); 2006 [updated 28th Oct 2018. Available from: https://www.ncbi.nlm.nih.gov/books/NBK501250/.
168. van den Anker JN. Is it safe to use opioids for obstetric pain while breastfeeding? J Pediatr. 2012;160(1):4-6.
169. Palmer GM, Anderson BJ, Linscott DK, Paech MJ, Allegaert K. Tramadol, breast feeding and safety in the newborn. Arch Dis Child. 2018;103(12):1110-3.
170. Lam J, Kelly L, Ciszkowski C, Landsmeer ML, Nauta M, Carleton BC, et al. Central nervous system depression of neonates breastfed by mothers receiving oxycodone for postpartum analgesia. J Pediatr. 2012;160(1):33-7.e2.
171. Timm NL. Maternal use of oxycodone resulting in opioid intoxication in her breastfed neonate. J Pediatr. 2013;162(2):421-2.
172. Milone MC. Laboratory testing for prescription opioids. J Med Toxicol. 2012;8(4):408-16.
173. Puet B, DePriest A, Knight J, Heltsley R, Black DL, Caplan YH, et al. Urine drug testing of chronic pain patients. V. Prevalence of propoxyphene following its withdrawal from the United States market. J Anal Toxicol. 2013;37(1):1-4.
174. Williams EL, Hammer LD. Breastfeeding attitudes and knowledge of pediatricians-in-training. Am J Prev Med. 1995;11(1):26-33.
175. Svendby HR, Løland BF, Omtvedt M, Holmsen ST, Lagerløv P. Norwegian general practitioners' knowledge and beliefs about breastfeeding, and their self-rated ability as breastfeeding counsellor. Scand J Prim Health Care. 2016;34(2):122-9.
176. Finneran B, Murphy K. Breast is best for GPs--or is it? Breastfeeding attitudes and practice of general practitioners in the Mid-West of Ireland. Ir Med J. 2004;97(9):268-70.
177. Manzoor F, Wei L, Hussain A, Asif M, Shah SIA. Patient Satisfaction with Health Care Services; An Application of Physician's Behavior as a Moderator. Int J Environ Res Public Health. 2019;16(18).
178. Oladapo OT, Iyaniwura CA, Sule-Odu AO. Quality of antenatal services at the primary care level in southwest Nigeria. Afr J Reprod Health. 2008;12(3):71-92.
179. Mazzi MA, Rimondini M, van der Zee E, Boerma W, Zimmermann C, Bensing J. Which patient and doctor behaviours make a medical consultation more effective from a patient point of view. Results from a European multicentre study in 31 countries. Patient Educ Couns. 2018;101(10):1795-803.
180. Larsen LA, Ito S, Koren G. Prediction of milk/plasma concentration ratio of drugs. Ann Pharmacother. 2003;37(9):1299-306.
181. Theodoulou FL, Kerr ID. ABC transporter research: going strong 40 years on. Biochem Soc Trans. 2015;43(5):1033-40.
182. Bai MR, Sun DL, Jiang HD, Zheng CH. [Expression and regulation of drug transporters in placenta]. Yao Xue Xue Bao. 2016;51(6):879-85.
183. Urquhart BL, Kim RB. Blood-brain barrier transporters and response to CNS-active drugs. Eur J Clin Pharmacol. 2009;65(11):1063-70.
184. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat Rev Cancer. 2018;18(7):452-64.

185. Durmus S, Hendrikx JJ, Schinkel AH. Apical ABC transporters and cancer chemotherapeutic drug disposition. Adv Cancer Res. 2015;125:1-41.
186. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. Aaps j. 2015;17(1):65-82.
187. Huang G, Wang L, Zhang X. Involvement of ABC transporters in the efflux and toxicity of MPA-COOH-CdTe quantum dots in human breast cancer SK-BR-3 cells. J Biochem Mol Toxicol. 2019;33(8):e22343.
188. Fletcher JI, Williams RT, Henderson MJ, Norris MD, Haber M. ABC transporters as mediators of drug resistance and contributors to cancer cell biology. Drug Resist Updat. 2016;26:1-9.
189. Allen LH. B vitamins in breast milk: relative importance of maternal status and intake, and effects on infant status and function. Advances in nutrition (Bethesda, Md). 2012;3(3):362-9.
190. Schafrank LA, Washabaugh JR, Hoke MK. An examination of breastmilk composition among high altitude Peruvian women. Am J Hum Biol. 2020:e23412.
191. Hahn WH, Jeong T, Park S, Song S, Kang NM. Content fat and calorie of human milk is affected by interactions between maternal age and body mass index. J Matern Fetal Neonatal Med. 2018;31(10):1385-8.
192. Bravi F, Wiens F, Decarli A, Dal Pont A, Agostoni C, Ferraroni M. Impact of maternal nutrition on breast-milk composition: a systematic review. Am J Clin Nutr. 2016;104(3):646-62.
193. Keikha M, Bahreynian M, Saleki M, Kelishadi R. Macro- and Micronutrients of Human Milk Composition: Are They Related to Maternal Diet? A Comprehensive Systematic Review. Breastfeed Med. 2017;12(9):517-27.
194. Alexander SPH, Kelly E, Mathie A, Peters JA, Veale EL, Armstrong JF, et al. THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Transporters. Br J Pharmacol. 2019;176 Suppl 1(Suppl 1):S397-S493.
195. Rives ML, Javitch JA, Wickenden AD. Potentiating SLC transporter activity: Emerging drug discovery opportunities. Biochem Pharmacol. 2017;135:1-11.
196. Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol. 2006;25(4):231-59.
197. van Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW, Schinkel AH. Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. Carcinogenesis. 2006;27(1):123-30.
198. Liu X. ABC Family Transporters. Adv Exp Med Biol. 2019;1141:13-100.
199. Jedlitschky G, Hoffmann U, Kroemer HK. Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition. Expert Opin Drug Metab Toxicol. 2006;2(3):351-66.
200. Murakami T, Takano M. Intestinal efflux transporters and drug absorption. Expert Opin Drug Metab Toxicol. 2008;4(7):923-39.
201. Schinkel AH. P-Glycoprotein, a gatekeeper in the blood-brain barrier. Advanced drug delivery reviews. 1999;36(2-3):179-94.
202. Hladky SB, Barrand MA. Elimination of substances from the brain parenchyma: efflux via perivascular pathways and via the blood-brain barrier. Fluids and barriers of the CNS. 2018;15(1):30-.
203. Dinić J, Podolski-Renić A, Jeremić M, Pešić M. Potential of Natural-Based Anticancer Compounds for P-Glycoprotein Inhibition. Curr Pharm Des. 2018;24(36):4334-54.
204. Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. Clin Cancer Res. 2001;7(6):1798-804.
205. Burger H, Foekens JA, Look MP, Meijer-van Gelder ME, Klijn JG, Wiemer EA, et al. RNA expression of breast cancer resistance protein, lung resistance-related protein,

multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response. Clin Cancer Res. 2003;9(2):827-36.
206. Liu X, Yue X, Chen S, Chen J, Li R. Significance of the expression of MRP1 and MRP2 in peripheral blood mononuclear cells of children with intractable epilepsy. Exp Ther Med. 2015;10(5):1784-8.
207. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol. 2005;204(3):216-37.
208. Hassiotou F, Geddes DT, Hartmann PE. Cells in human milk: state of the science. J Hum Lact. 2013;29(2):171-82.
209. Ito S, Alcorn J. Xenobiotic transporter expression and function in the human mammary gland. Adv Drug Deliv Rev. 2003;55(5):653-65.
210. Virkel G, Ballent M, Lanusse C, Lifschitz A. Role of ABC Transporters in Veterinary Medicine: Pharmaco- Toxicological Implications. Curr Med Chem. 2019;26(7):1251-69.
211. Schrickx JA, Fink-Gremmels J. Implications of ABC transporters on the disposition of typical veterinary medicinal products. Eur J Pharmacol. 2008;585(2-3):510-9.
212. Mahnke H, Ballent M, Baumann S, Imperiale F, von Bergen M, Lanusse C, et al. The ABCG2 Efflux Transporter in the Mammary Gland Mediates Veterinary Drug Secretion across the Blood-Milk Barrier into Milk of Dairy Cows. Drug Metab Dispos. 2016;44(5):700-8.
213. Sharma A, Aggarwal J, Sodhi M, Kishore A, Mishra BP, Mohanty AK, et al. Stage specific expression of ATP-binding cassette and solute carrier superfamily of transporter genes in mammary gland of riverine buffalo (Bubalus bubalis). Anim Biotechnol. 2014;25(3):200-9.
214. Juliano R, Ling V, Graves J. Drug-resistant mutants of chinese hamster ovary cells possess an altered cell surface carbohydrate component. Journal of supramolecular structure. 1976;4(4):521-6.
215. Akamine Y, Yasui-Furukori N, Uno T. Drug-Drug Interactions of P-gp Substrates Unrelated to CYP Metabolism. Curr Drug Metab. 2019;20(2):124-9.
216. Summerfield SG, Zhang Y, Liu H. Examining the Uptake of Central Nervous System Drugs and Candidates across the Blood-Brain Barrier. The Journal of pharmacology and experimental therapeutics. 2016;358(2):294-305.
217. Davidson AL, Chen J. ATP-binding cassette transporters in bacteria. Annual review of biochemistry. 2004;73(1):241-68.
218. Agarwal S, Hartz AM, Elmquist WF, Bauer B. Breast cancer resistance protein and P-glycoprotein in brain cancer: two gatekeepers team up. Curr Pharm Des. 2011;17(26):2793-802.
219. Patel J, Spencer EP, Flanagan RJ. HPLC of sertraline and norsertraline in plasma or serum. Biomed Chromatogr. 1996;10(6):351-4.
220. Edwards JE, Alcorn J, Savolainen J, Anderson BD, McNamara PJ. Role of P-glycoprotein in distribution of nelfinavir across the blood-mammary tissue barrier and blood-brain barrier. Antimicrob Agents Chemother. 2005;49(4):1626-8.
221. Chen Z, Shi T, Zhang L, Zhu P, Deng M, Huang C, et al. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. Cancer Lett. 2016;370(1):153-64.
222. Luna-Tortos C, Fedrowitz M, Loscher W. Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. Neuropharmacology. 2010;58(7):1019-32.
223. Luna-Tortós C, Fedrowitz M, Löscher W. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology. 2008;55(8):1364-75.
224. Rivers F, O'Brien TJ, Callaghan R. Exploring the possible interaction between anti-epilepsy drugs and multidrug efflux pumps; in vitro observations. Eur J Pharmacol. 2008;598(1-3):1-8.

225. Fei T, Shao Y, Yan Z, Zhu L, Li S, Pan J, et al. The effects of P-gp and CYP450 modulated by rifampicin on the steroid-induced osteonecrosis of the femoral head. J Bone Miner Metab. 2017;35(5):504-12.
226. Stöllberger C, Finsterer J. Relevance of P-glycoprotein in stroke prevention with dabigatran, rivaroxaban, and apixaban. Herz. 2015;40 Suppl 2:140-5.
227. Nakanishi T, Ross DD. Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression. Chin J Cancer. 2012;31(2):73-99.
228. Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, et al. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem. 1996;271(25):15091-8.
229. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol Biol. 2012;3(1):1-27.
230. Jani M, Ambrus C, Magnan R, Jakab KT, Beery E, Zolnerciks JK, et al. Structure and function of BCRP, a broad specificity transporter of xenobiotics and endobiotics. Arch Toxicol. 2014;88(6):1205-48.
231. Elmeliegy M, Vourvahis M, Guo C, Wang DD. Effect of P-glycoprotein (P-gp) Inducers on Exposure of P-gp Substrates: Review of Clinical Drug-Drug Interaction Studies. Clin Pharmacokinet. 2020;59(6):699-714.
232. Yagdiran Y, Oskarsson A, Knight CH, Tallkvist J. ABC- and SLC-Transporters in Murine and Bovine Mammary Epithelium--Effects of Prochloraz. PLoS One. 2016;11(3):e0151904.
233. Safar Z, Kis E, Erdo F, Zolnerciks JK, Krajcsi P. ABCG2/BCRP: variants, transporter interaction profile of substrates and inhibitors. Expert Opin Drug Metab Toxicol. 2019;15(4):313-28.
234. Su W, Pasternak GW. The role of multidrug resistance-associated protein in the blood-brain barrier and opioid analgesia. Synapse. 2013;67(9):609-19.
235. Cole SP. Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future. Annu Rev Pharmacol Toxicol. 2014;54:95-117.
236. Lee SH, Lee MS, Lee JH, Kim SW, Kang RH, Choi MJ, et al. MRP1 polymorphisms associated with citalopram response in patients with major depression. J Clin Psychopharmacol. 2010;30(2):116-25.
237. Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, et al. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. Circ Res. 2010;106(2):297-306.
238. Green BR, Bain LJ. Mrp2 is involved in the efflux and disposition of fosinopril. J Appl Toxicol. 2013;33(6):458-65.
239. Yagdiran Y, Oskarsson A, Knight CH, Tallkvist J. ABC- and SLC-Transporters in Murine and Bovine Mammary Epithelium--Effects of Prochloraz. PLoS One. 2016;11(3):e0151904-e.
240. Oskarsson A, Yagdiran Y, Nazemi S, Tallkvist J, Knight CH. Short communication: Staphylococcus aureus infection modulates expression of drug transporters and inflammatory biomarkers in mouse mammary gland. J Dairy Sci. 2017;100(3):2375-80.
241. Blazquez AMG, Macias RIR, Cives-Losada C, de la Iglesia A, Marin JJG, Monte MJ. Lactation during cholestasis: Role of ABC proteins in bile acid traffic across the mammary gland. Sci Rep. 2017;7(1):7475-.
242. Chan LM, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur J Pharm Sci. 2004;21(1):25-51.
243. Baumgartel KL, Conley YP. The utility of breastmilk for genetic or genomic studies: a systematic review. Breastfeed Med. 2013;8(3):249-56.
244. Twigger AJ, Hepworth AR, Lai CT, Chetwynd E, Stuebe AM, Blancafort P, et al. Gene expression in breastmilk cells is associated with maternal and infant characteristics. Sci Rep. 2015;5:12933.

245. Hassiotou F, Beltran A, Chetwynd E, Stuebe AM, Twigger A-J, Metzger P, et al. Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem cells (Dayton, Ohio). 2012;30(10):2164-74.
246. Hassiotou F, Hartmann PE. At the Dawn of a New Discovery: The Potential of Breast Milk Stem Cells. Adv Nutr. 2014;5(6):770-8.
247. Casey TM, Khan JM, Bringans SD, Koudelka T, Takle PS, Downs RA, et al. Analysis of Reproducibility of Proteome Coverage and Quantitation Using Isobaric Mass Tags (iTRAQ and TMT). J Proteome Res. 2017;16(2):384-92.
248. Weizmann Institute of Science. Gene Cards: The human gene database 2020 [Available from: https://www.genecards.org/.
249. Boutinaud M, Jammes H. Potential uses of milk epithelial cells: a review. Reprod Nutr Dev. 2002;42(2):133-47.
250. Yang M, Cao X, Wu R, Liu B, Ye W, Yue X, et al. Comparative proteomic exploration of whey proteins in human and bovine colostrum and mature milk using iTRAQ-coupled LC-MS/MS. Int J Food Sci Nutr. 2017;68(6):671-81.
251. Wiese S, Reidegeld KA, Meyer HE, Warscheid B. Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. Proteomics. 2007;7(3):340-50.
252. Thingholm TE, Palmisano G, Kjeldsen F, Larsen MR. Undesirable charge-enhancement of isobaric tagged phosphopeptides leads to reduced identification efficiency. J Proteome Res. 2010;9(8):4045-52.
253. Chen Z, Wang Q, Lin L, Tang Q, Edwards JL, Li S, et al. Comparative evaluation of two isobaric labeling tags, DiART and iTRAQ. Anal Chem. 2012;84(6):2908-15.
254. Beck KL, Weber D, Phinney BS, Smilowitz JT, Hinde K, Lonnerdal B, et al. Comparative Proteomics of Human and Macaque Milk Reveals Species-Specific Nutrition during Postnatal Development. J Proteome Res. 2015;14(5):2143-57.
255. Zhang L, de Waard M, Verheijen H, Boeren S, Hageman JA, van Hooijdonk T, et al. Changes over lactation in breast milk serum proteins involved in the maturation of immune and digestive system of the infant. Data Brief. 2016;7:362-5.
256. Lonnerdal B. Human milk proteins: key components for the biological activity of human milk. Adv Exp Med Biol. 2004;554:11-25.
257. Lonnerdal B, Erdmann P, Thakkar SK, Sauser J, Destaillats F. Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective. J Nutr Biochem. 2017;41:1-11.
258. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. Pediatr Clin North Am. 2013;60(1):49-74.
259. Kreissl A, Zwiauer V, Repa A, Binder C, Thanhaeuser M, Jilma B, et al. Human Milk Analyser shows that the lactation period affects protein levels in preterm breastmilk. Acta Paediatr. 2016;105(6):635-40.
260. Stevens CR, Millar TM, Clinch JG, Kanczler JM, Bodamyali T, Blake DR. Antibacterial properties of xanthine oxidase in human milk. Lancet. 2000;356(9232):829-30.
261. Hancock JT, Salisbury V, Ovejero-Boglione MC, Cherry R, Hoare C, Eisenthal R, et al. Antimicrobial properties of milk: dependence on presence of xanthine oxidase and nitrite. Antimicrob Agents Chemother. 2002;46(10):3308-10.
262. Zhang Q, Cundiff JK, Maria SD, McMahon RJ, Woo JG, Davidson BS, et al. Quantitative Analysis of the Human Milk Whey Proteome Reveals Developing Milk and Mammary-Gland Functions across the First Year of Lactation. Proteomes. 2013;1(2):128-58.
263. Prentice A, Prentice AM, Cole TJ, Paul AA, Whitehead RG. Breast-milk antimicrobial factors of rural Gambian mothers. I. Influence of stage of lactation and maternal plane of nutrition. Acta Paediatr Scand. 1984;73(6):796-802.
264. Montagne P, Cuilliere ML, Mole C, Bene MC, Faure G. Changes in lactoferrin and lysozyme levels in human milk during the first twelve weeks of lactation. Adv Exp Med Biol. 2001;501:241-7.

265. Zhu JJ, Luo J, Wang W, Yu K, Wang HB, Shi HB, et al. Inhibition of FASN reduces the synthesis of medium-chain fatty acids in goat mammary gland. Animal. 2014;8(9):1469-78.
266. Vanselow J, Yang W, Herrmann J, Zerbe H, Schuberth H-J, Petzl W, et al. DNA-remethylation around a STAT5-binding enhancer in the alphaS1-casein promoter is associated with abrupt shutdown of alphaS1-casein synthesis during acute mastitis. J Mol Endocrinol. 2006;37(3):463-77.
267. Zhou MM, Wu YM, Liu HY, Zhao K, Liu JX. Effects of tripeptides and lactogenic hormones on oligopeptide transporter 2 in bovine mammary gland. J Anim Physiol Anim Nutr (Berl). 2011;95(6):781-9.
268. McCullough SD, Bowers EC, On DM, Morgan DS, Dailey LA, Hines RN, et al. Baseline Chromatin Modification Levels May Predict Interindividual Variability in Ozone-Induced Gene Expression. Toxicological sciences : an official journal of the Society of Toxicology. 2016;150(1):216-24.
269. Hirota T, Tanaka T, Takesue H, Ieiri I. Epigenetic regulation of drug transporter expression in human tissues. Expert Opin Drug Metab Toxicol. 2017;13(1):19-30.
270. Ivanov M, Kacevska M, Ingelman-Sundberg M. Epigenomics and interindividual differences in drug response. Clin Pharmacol Ther. 2012;92(6):727-36.
271. Chen L, Manautou JE, Rasmussen TP, Zhong XB. Development of precision medicine approaches based on inter-individual variability of BCRP/ABCG2. Acta Pharm Sin B. 2019;9(4):659-74.
272. Reustle A, Fisel P, Renner O, Büttner F, Winter S, Rausch S, et al. Characterization of the breast cancer resistance protein (BCRP/ABCG2) in clear cell renal cell carcinoma. Int J Cancer. 2018;143(12):3181-93.
273. Mizuno T, Fukudo M, Terada T, Kamba T, Nakamura E, Ogawa O, et al. Impact of genetic variation in breast cancer resistance protein (BCRP/ABCG2) on sunitinib pharmacokinetics. Drug Metab Pharmacokinet. 2012;27(6):631-9.
274. Natarajan K, Xie Y, Baer MR, Ross DD. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. Biochem Pharmacol. 2012;83(8):1084-103.
275. Baxter DE, Kim B, Hanby AM, Verghese ET, Sims AH, Hughes TA. Neoadjuvant Endocrine Therapy in Breast Cancer Upregulates the Cytotoxic Drug Pump ABCG2/BCRP, and May Lead to Resistance to Subsequent Chemotherapy. Clin Breast Cancer. 2018;18(6):481-8.
276. Haschke F, Haiden N, Thakkar SK. Nutritive and Bioactive Proteins in Breastmilk. Ann Nutr Metab. 2016;69 Suppl 2:17-26.
277. Lee JK, Bloom J, Zubeldia-Plazaola A, Garbe JC, Stampfer MR, LaBarge MA. Different culture media modulate growth, heterogeneity, and senescence in human mammary epithelial cell cultures. PLoS One. 2018;13(10):e0204645.
278. Joshi PS, Modur V, Cheng J, Robinson K, Rao K. Characterization of immortalized human mammary epithelial cell line HMEC 2.6. Tumour Biol. 2017;39(10):1010428317724283.
279. Garbe JC, Vrba L, Sputova K, Fuchs L, Novak P, Brothman AR, et al. Immortalization of normal human mammary epithelial cells in two steps by direct targeting of senescence barriers does not require gross genomic alterations. Cell Cycle. 2014;13(21):3423-35.
280. Stampfer MR , Yaswen P, Taylor-Papadimitriou J. Culture of human mammary epithelial cells. In: Freshney I, Freshney MG, editors. Culture of epithelial cells. Wiley-Liss. 2nd ed. Glasgow Wiley-Liss; 2002.
281. Zhang M, Lee AV, Rosen JM. The Cellular Origin and Evolution of Breast Cancer. Cold Spring Harb Perspect Med. 2017;7(3).
282. Cregan MD, Fan Y, Appelbee A, Brown ML, Klopcic B, Koppen J, et al. Identification of nestin-positive putative mammary stem cells in human breastmilk. Cell Tissue Res. 2007;329(1):129-36.

283. Saxena S, Jain AK, Pandey KK, Dewan AK. Role of steroid hormone and growth factor receptors and proto-oncogenes in the behavior of human mammary epithelial cancer cells in vitro. Pathobiology. 1997;65(2):75-82.
284. Hosseini SM, Talaei-Khozani T, Sani M, Owrangi B. Differentiation of human breast-milk stem cells to neural stem cells and neurons. Neurol Res Int. 2014;2014:807896.
285. Hassiotou F, Beltran A, Chetwynd E, Stuebe AM, Twigger AJ, Metzger P, et al. Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem Cells. 2012;30(10):2164-74.
286. Pacheco CMR, Ferreira PE, Sacaki CS, Tannous LA, Zotarelli-Filho IJ, Guarita-Souza LC, et al. In vitro differentiation capacity of human breastmilk stem cells: A systematic review. World J Stem Cells. 2019;11(11):1005-19.
287. Hassiotou F, Hepworth AR, Metzger P, Tat Lai C, Trengove N, Hartmann PE, et al. Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. Clin Transl Immunology. 2013;2(4):e3.
288. Tang C, Zhou Q, Lu C, Xiong M, Lee S. Comparison and culturing different types of cells from fresh breast milk with different culture medium. Pediatric Medicine. 2019;2.
289. Sani M, Hosseini SM, Salmannejad M, Aleahmad F, Ebrahimi S, Jahanshahi S, et al. Origins of the breast milk-derived cells; an endeavor to find the cell sources. Cell Biol Int. 2015;39(5):611-8.
290. Pisco AO, Jackson DA, Huang S. Reduced Intracellular Drug Accumulation in Drug-Resistant Leukemia Cells is Not Only Solely Due to MDR-Mediated Efflux but also to Decreased Uptake. Front Oncol. 2014;4:306.
291. Politi PM, Sinha BK. Role of differential drug uptake, efflux, and binding of etoposide in sensitive and resistant human tumor cell lines: implications for the mechanisms of drug resistance. Mol Pharmacol. 1989;35(3):271-8.
292. Lonza. Clonetics® Normal Human Mammary Epithelial Cell Systems Lonza group; 2008 [Available from: https://resources.altius.org/publications/Science_Maurano_Humbert_et_al/data/vHMEC_Protocol.pdf.
293. Qian J, Chen T, Lu W, Wu S, Zhu J. Breast milk macro- and micronutrient composition in lactating mothers from suburban and urban Shanghai. J Paediatr Child Health. 2010;46(3):115-20.
294. Stampfer MR, Yaswen P. Culture models of human mammary epithelial cell transformation. J Mammary Gland Biol Neoplasia. 2000;5(4):365-78.
295. Dimri G, Band H, Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. Breast Cancer Res. 2005;7(4):171-9.
296. Senarathna SM, Crowe A. The influence of passage number for Caco2 cell models when evaluating P-gp mediated drug transport. Pharmazie. 2015;70(12):798-803.
297. Siissalo S, Laitinen L, Koljonen M, Vellonen KS, Kortejarvi H, Urtti A, et al. Effect of cell differentiation and passage number on the expression of efflux proteins in wild type and vinblastine-induced Caco-2 cell lines. Eur J Pharm Biopharm. 2007;67(2):548-54.
298. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol. 2007;8(10):839-45.
299. Antoni D, Burckel H, Josset E, Noel G. Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci. 2015;16(3):5517-27.

# Appendix A: Breastfeeding Survey and Longitudinal Study Adverts


**ARE YOU CURRENTLY BREASTFEEDING?**

**OR**

**HAVE YOU BREASTFED IN THE LAST 12 MONTHS?**

- Researchers at Curtin University are studying the defence mechanism that stops many medicines from entering breast milk which helps prevent the unnecessary exposure of a nursing infant to the medicines that their mother may take.
- Please help us understand more about your breastfeeding journey by completing a short online survey on **www.breastfeedingresearch.com.au**
- The survey is completely anonymous and will take approximately 10-15 minutes of your time.
- To obtain further information about this survey, please visit our website www.breastfeedingresearch.com.au

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This study titled "The role of efflux transporters in the transfer of drugs from maternal plasma to the breastfed infant" has been approved by the Curtin human research ethics committee. Approval # HR110/2012.

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## ARE YOU CURRENTLY BREASTFEEDING?

## OR

## ARE YOU EXPECTING A BABY SOON? DO YOU INTEND TO BREASTFEED YOUR BABY?

Advertisement for project titled "Role of efflux transporters in the transfer of drugs from maternal plasma to the breastfed infant"

**Researchers in the school of pharmacy at Curtin University are studying the mechanisms by which medicines may be transferred from a mother to their breast fed infant. This research will help us better understand the safety of medicines in breastfeeding. And thus help in preventing any unnecessary interruption in the treatment of the mother because of concerns for the safety of the breastfed infant.**

## PREGNANT AND BREASTFEEDING MOTHERS WANTED

We need ***samples of your milk*** and about half an hour of your time. *Volunteers are not required to take any drug.* Transport costs to Curtin University can be covered and electric breast pumps will be supplied to you to help with milk collection .

Please Contact:
Ms Hilai Ahmadzai
Mobile 0430509906 or 9259 5161
hilai.ahmadzai@postgrad.curtin.edu.au
Or
Associate Professor Lisa Tee
School of Pharmacy
Curtin University of Technology
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# Appendix B: Breastfeeding related ADRs reported to the TGA (2003-16) Case Line Listing and Summary

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 223599 | 29/11/2006 | | | F | 37 | Recovered | Motilium (Domperidone) -Suspected | | Gynaecomastia<br>Exposure during breast feeding<br>Breast abscess |
| 224369 | 03/01/2007 | | | U | ?? | Recovered | Mirena (Levonorgestrel) -Suspected | | Irritability<br>Exposure during breast feeding<br>Screaming |
| 235284 | 14/11/2007 | | | U | 0 | Recovered | Quetiapine -Suspected<br>Escitalopram -Other drug | | Exposure during breast feeding<br>Vomiting |
| 235901 | 04/12/2007 | | | M | 1 | Unknown | Codeine -Suspected | | Dystonia<br>Somnolence<br>Hypotonia<br>Exposure during breast feeding |
| 238592 | 04/03/2008 | | | F | 31 | Unknown | Micronor (Norethisterone) -Suspected | | Constipation<br>Selective eating disorder<br>Exposure during breast feeding |
| 242955 | 16/07/2008 | 05/07/2008 | | M | 0 | Not yet recovered | Priorix (Measles, Mumps, Rubella Vaccine) -Suspected | | Lymphadenopathy<br>Exposure during breast feeding<br>Irritability<br>Rash rubelliform<br>Lacrimation increased |
| 245139 | 03/10/2008 | 01/03/2003 | | F | 0 | Recovered with seque | Prozac (Fluoxetine Hydrochloride) -Suspected | 59 | Exposure during pregnancy<br>Exposure during breast feeding<br>Autism |



# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 193423 | 22/12/2003 | 23/11/2003 | | F | 29 | Recovered | Measles Mumps Rubella Vaccine (Measles, Mumps, Rubella Vaccine) -Suspected | 2 | Rash<br>Exposure during breast feeding<br>Pyrexia |
| 197090 | 07/05/2004 | 16/04/2004 | | F | 35 | Unknown | Amoxycillin Trihydrate -Suspected<br>Metronidazole -Suspected<br>Nifedipine -Suspected<br>Pizotifen Malate -Other drug | 0<br>0<br>0 | Selective eating disorder<br>Exposure during breast feeding |
| 197756 | 31/05/2004 | 27/04/2004 | | F | 1 | Unknown | Demazin Clear Syrup (Chlorpheniramine Maleate-phenylephrine Hydrochloride) -Suspected | 1 | Nasopharyngitis<br>Exposure during breast feeding<br>Rash neonatal |
| 199954 | 18/06/2004 | | | F | ?? | Unknown | Lamisil (Terbinafine) -Suspected | | Hypotonia<br>Exposure during breast feeding<br>Developmental delay |
| 217183 | 08/04/2006 | | | F | ?? | Unknown | Amoxil (Amoxycillin Trihydrate) -Suspected | | Exposure during breast feeding<br>Drug hypersensitivity |
| 219420 | 19/06/2006 | 01/01/2006 | | F | 35 | Recovered | Luvox (Fluvoxamine Maleate) -Suspected | | Exposure during breast feeding<br>Rash neonatal |
| 220430 | 28/07/2006 | | | M | 0 | Unknown | Cephalexin -Suspected<br>Cephalothin Sodium -Suspected<br>Probenecid -Suspected | | Exposure during breast feeding<br>Diarrhoea |
| 221540 | 11/09/2006 | | | F | ?? | Unknown | Centrum Tablets (Vitamin Preparation Compound) -Suspected | | Exposure during breast feeding |

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| **248292** | 04/02/2009 | | | M | 0 | Unknown | Fluoxetine Hydrochloride -Suspected<br>Morphine Sulphate -Suspected<br>Oxycodone Hydrochloride -Suspected<br>Quetiapine -Suspected | | Weight decreased<br>Drug dependence<br>Exposure during breast feeding<br>Exposure during pregnancy |
| **248915** | 27/02/2009 | 27/02/2007 | | F | 0 | Unknown | Prograf (Tacrolimus) -Suspected<br>Diltiazem (Diltiazem Hydrochloride) -Other drug<br>Prednisolone -Other drug | | Exposure during breast feeding |
| **254377** | 24/07/2009 | | | M | 1 | Recovered | Prozac (Fluoxetine Hydrochloride) -Suspected<br>Seroquel (Quetiapine) -Suspected | | Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| **258176** | 15/10/2009 | 13/10/2009 | VIC | F | 0 | Unknown | Panvax H1N1 influenza vaccine (Influenza virus haemagglutinin vaccine) -Suspected | 0 | Somnolence<br>Increased appetite<br>Exposure during breast feeding<br>Diarrhoea |
| **258437** | 19/10/2009 | 15/10/2009 | ACT | M | 0 | Recovered | Panvax H1N1 influenza vaccine (Influenza virus haemagglutinin vaccine) -Suspected | 0 | Local swelling<br>Exposure during breast feeding<br>Irritability |
| **258675** | 23/10/2009 | | QLD | M | 0 | Unknown | Panvax H1N1 influenza vaccine (Influenza virus haemagglutinin vaccine) -Suspected | | Rash erythematous<br>Exposure during breast feeding |



# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| **263198** | 26/02/2010 | 22/02/2010 | Not Knov | M | 0 | Unknown | Codral NF Cold and Flu -Suspected | 1 | Somnolence<br>Listless<br>Exposure during breast feeding<br>Vomiting<br>Lethargy |
| **278506** | 09/02/2011 | | | M | ?? | Unknown | Efexor-XR (Venlafaxine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Eczema<br>Selective eating disorder<br>Exposure during breast feeding<br>Premature baby |
| **279827** | 14/03/2011 | | QLD | U | 11 | Unknown | Zoloft (Sertraline Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Learning disability |
| **279911** | 15/03/2011 | | | U | 9 | Unknown | Zoloft (Sertraline Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Diabetes mellitus |
| **280315** | 24/03/2011 | | | F | ?? | Unknown | Robitussin Chesty Cough and Nasal Congestion PE (Guaiphenesin; Phenylephrine hydrochloride) -Suspected<br>Robitussin ME Chesty Cough Forte (Guaiphenesin; Bromhexine hydrochloride) -Suspected | | Exposure during breast feeding |

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 286659 | 29/07/2011 | 21/07/2011 | | F | ?? | Unknown | Dimetapp Cold & Flu Night Relief Liquid Caps (Dextromethorphan hydrobromide; Paracetamol; Pseudoephedrine hydrochloride; Doxylamine succinate) -Suspected | 0 | Exposure during breast feeding |
| 290193 | 10/10/2011 | 05/10/2011 | Not Knov | U | 12 | Unknown | Dimetapp Daytime/Nightime Liquid Caps (Dextromethorphan hydrobromide; Doxylamine succinate; Paracetamol) -Suspected | 0 | Exposure during breast feeding |
| 290686 | 19/10/2011 | 14/10/2011 | QLD | F | 27 | Unknown | Havrix (Hepatitis A Vaccine) -Suspected<br>Influvac (Influenza Vaccine Trivalent) -Suspected<br>Typhim VI (Typhoid Vaccine) -Suspected | 0<br>0<br>0 | Exposure during breast feeding<br>Vomiting |
| 291518 | 08/11/2011 | 02/11/2011 | | M | 0 | Unknown | Advil (Ibuprofen) -Suspected | 0 | Exposure during breast feeding |
| 293557 | 19/12/2011 | | | F | 0 | Not yet recovered | Cymbalta (Duloxetine hydrochloride) -Suspected<br>Elevit RDI (Vitamin Preparation Compound) -Other drug<br>Oral Contraceptive NOS -Other drug | | Growth retardation<br>Foetal exposure during pregnancy<br>Hypoglycaemia<br>Exposure during breast feeding<br>Blood bilirubin increased<br>Premature baby |
| 294896 | 23/01/2012 | | Not Knov | M | ?? | Unknown | Magnevist (Dimeglumine Gadopentetate) -Suspected | | Arachnoiditis<br>Exposure during breast feeding |
| 295511 | 06/02/2012 | 09/01/2012 | SA | M | 0 | Recovered | Zuclopenthixol Acetate -Suspected | 3 | Hypertonia<br>Exposure during breast feeding |
| 296771 | 02/03/2012 | | Not Knov | U | ?? | Unknown | Dimetapp DM Cough and Cold Elixir (Brompheniramine maleate; Dextromethorphan hydrobromide; Phenylephrine hydrochloride) -Suspected | | Exposure during breast feeding |



# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 297543 | 19/03/2012 | | | M | 0 | Not yet recovered | Magnevist (Dimeglumine Gadopentetate) -Suspected | | Conjunctivitis<br>Lymphadenopathy<br>Contusion<br>Rash<br>Exposure during breast feeding<br>Vomiting<br>Lethargy<br>Diarrhoea<br>Hepatic function abnormal |
| 299444 | 24/04/2012 | 19/04/2012 | ACT | F | 33 | Recovered | Influenza Vaccine -Suspected | 0 | Sleep disorder<br>Irritability<br>Exposure during breast feeding<br>Crying |
| 304081 | 24/07/2012 | | Not Knov | U | ?? | Unknown | Caltrate Plus with 400IU Vitamin D and Minerals Tablets (Vitamins and Minerals Combination) -Suspected | | Exposure during breast feeding |
| 304344 | 27/07/2012 | 19/05/2012 | VIC | F | 0 | Unknown | Fluvax (Influenza Vaccine Trivalent) -Suspected | 1 | Somnolence<br>Irritability<br>Exposure during breast feeding<br>Vomiting |
| 307482 | 25/09/2012 | | Not Knov | M | ?? | Unknown | Cannabis Preparation -Suspected<br>Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 307488 | 25/09/2012 | | Not Knov | F | ?? | Unknown | Benzodiazepine NOS -Suspected<br>Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 307498 | 25/09/2012 | 15/01/2010 | Not Knov | F | 0 | Unknown | Benzodiazepine NOS -Suspected<br>Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Infection<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 307503 | 25/09/2012 | | Not Knov | F | ?? | Unknown | Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 307506 | 25/09/2012 | | Not Knov | M | ?? | Unknown | Cannabis Preparation -Suspected<br>Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 307507 | 25/09/2012 | | Not Knov | M | ?? | Unknown | Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |



# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 307592 | 27/09/2012 | | | F | ?? | Unknown | Cannabis Preparation -Suspected<br>Subutex (Buprenorphine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 309034 | 29/10/2012 | 30/06/2000 | QLD | F | 0 | Not yet recovered | Paroxetine NOS -Suspected | | Speech disorder developmental<br>Foetal exposure during pregnancy<br>Auditory disorder<br>Exposure during breast feeding<br>Autism spectrum disorder<br>Dyslexia<br>Developmental delay<br>Intelligence test abnormal<br>Premature baby |
| 313082 | 29/01/2013 | 22/01/2013 | WA | U | 0 | Recovered | Rectogesic (Glyceryl Trinitrate) -Suspected | 0 | Exposure during breast feeding<br>Vomiting |
| 317922 | 16/04/2013 | | NSW | F | 0 | Unknown | Blackmores Pregnancy and Breast - Feeding Gold (Vitamins and Minerals Combination) -Suspected | | Product label issue<br>Insomnia<br>Aphonia<br>Exposure during breast feeding<br>Vomiting<br>Crying |

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 321722 | 19/06/2013 | | | F | 24 | Unknown | Buprenorphine Hydrochloride -Suspected<br>Luvox (Fluvoxamine Maleate) -Suspected | | Foetal exposure during pregnancy<br>Irritability<br>Exposure during breast feeding<br>Pyrexia |
| 326602 | 10/09/2013 | 29/08/2013 | | M | 0 | Unknown | Efexor (Venlafaxine Hydrochloride) -Suspected<br>Seroquel XR (Quetiapine) -Suspected | | Exposure during breast feeding<br>Drug withdrawal syndrome neonatal |
| 337575 | 31/03/2014 | 12/08/2013 | | F | 0 | Unknown | Distaph (Dicloxacillin Sodium) -Suspected | 0 | Somnolence<br>Exposure during breast feeding<br>Syncope |
| 338084 | 07/04/2014 | | Not Knov | M | 0 | Unknown | Salofalk Tablets (Mesalazine) -Suspected | | Bilirubin conjugated abnorma<br>Foetal exposure during pregnancy<br>International normalised ratio increased<br>Platelet count decreased<br>Exposure during breast feeding<br>Decreased appetite<br>Hepatic function abnormal<br>Jaundice |
| 338354 | 10/04/2014 | 10/02/2014 | | M | 0 | Recovered | Copaxone (Glatiramer Acetate) -Suspected | | Necrotising colitis<br>Exposure during breast feeding |



# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 339766 | 05/05/2014 | 15/03/2014 | | F | 37 | Unknown | Botox (Botulinum Toxin Type A) -Suspected<br>Thyroxine Sodium -Other drug | 8 | Off label use<br>Dysphagia<br>Dyspnoea<br>Exposure during breast feeding |
| 342970 | 02/07/2014 | 23/09/2010 | | U | 0 | Unknown | Soliris (Eculizumab) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Premature baby |
| 345944 | 14/08/2014 | 03/08/2014 | | F | 39 | Recovered | Curam Duo Forte 875/125 (Amoxycillin-potassium Clavulanate) -Suspected | 0 | Oropharyngeal pain<br>Pain<br>Exposure during breast feeding<br>Abdominal pain<br>Vomiting<br>Syncope<br>Diarrhoea |
| 351013 | 02/12/2014 | | | M | 0 | Recovered | Risperdal Consta (Risperidone) -Suspected<br>Seroquel XR (Quetiapine) -Suspected<br>Insulin Aspart -Other drug<br>Insulin Isophane -Other drug | | Poor sucking reflex<br>Exposure during breast feeding<br>Exposure during pregnancy<br>Large for dates baby<br>Drug withdrawal syndrome neonatal<br>Hypoglycaemia neonatal<br>Neonatal respiratory distress syndrome<br>Premature baby |

# Case Line Listing

**Cases Count: 65**

| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 355968 | 13/03/2015 | | | F | 28 | Unknown | Canesoral (Fluconazole) -Suspected | | Malaise<br>Poisoning<br>Exposure during breast feeding<br>Dizziness<br>Vomiting<br>Pyrexia |
| 357059 | 01/04/2015 | 15/07/2014 | | M | ?? | Unknown | Remicade (Infliximab) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Premature baby |
| 359656 | 18/05/2015 | 14/05/2015 | | M | ?? | Unknown | Alprim (Trimethoprim) -Suspected | 0 | Exposure during breast feeding<br>Pyrexia<br>Vomiting<br>Feeling abnormal |
| 371725 | 02/12/2015 | 24/11/2015 | SA | M | 0 | Not yet recovered | Measles Mumps Rubella Vaccine (Measles, Mumps, Rubella Vaccine) -Suspected | 22 | Rash morbilliform<br>Exposure during breast feeding<br>Concomitant disease progression |
| 375836 | 15/02/2016 | 05/01/2016 | Not Knov | F | ?? | Unknown | Zoloft (Sertraline Hydrochloride) -Suspected | | Exposure during breast feeding<br>Tachycardia |
| 377095 | 08/03/2016 | | Not Knov | F | ?? | Recovered | Caltrate Bone Health (Calcium carbonate-Cholecalciferol) -Suspected | | Haematochezia<br>Exposure during breast feeding |



# Case Line Listing

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| Case No. | Report Date | Onset Date | State | Sex | Age | Outcome | Medicine | Onset Time | Reaction |
|---|---|---|---|---|---|---|---|---|---|
| 386000 | 08/04/2016 | | Not Knov | F | 0 | Unknown | Venlafaxine Hydrochloride -Suspected<br>Olanzapine -Other drug<br>Quetiapine -Other drug<br>Temazepam -Other drug | | Foetal exposure during pregnancy<br>Toxicity to various agents<br>Exposure during breast feeding<br>Neonatal behavioural syndrome<br>Poor feeding infant<br>Failure to thrive |
| 388420 | 20/05/2016 | | | F | 0 | Unknown | Venlafaxine (Venlafaxine Hydrochloride) -Suspected | | Foetal exposure during pregnancy<br>Exposure during breast feeding<br>Neonatal behavioural syndrome<br>Failure to thrive |

Selection Parameters : Date Range: 01/01/1960 To 31/12/2059 Medicine Status: General marketing Unclear causality excluded Term1: Exposure during breast feeding

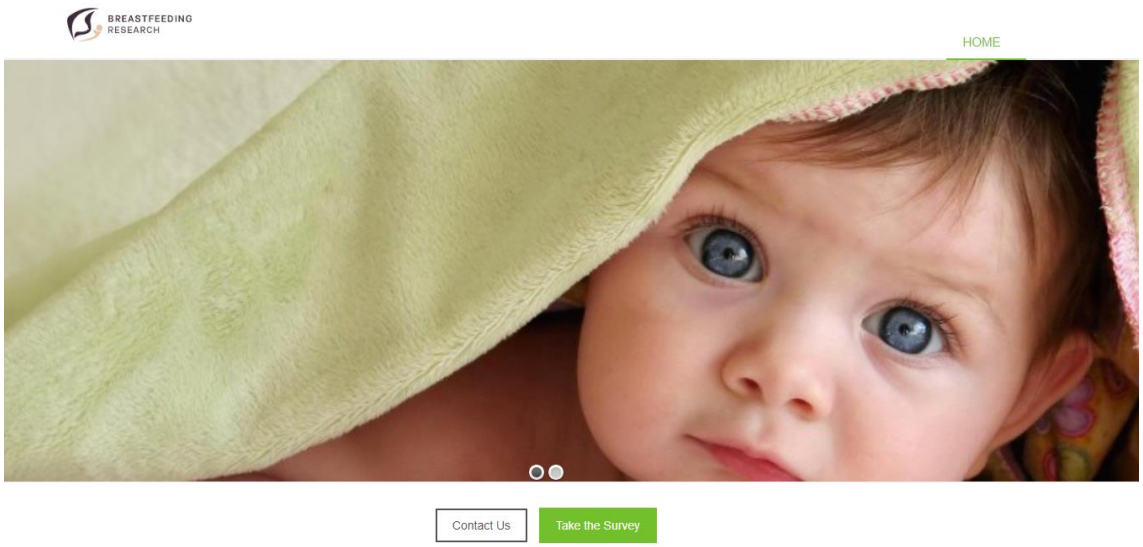
| 1 | Date | Reported by | Drug/offending agent | Description of reaction | Outcome | Baby's age at the time of | Impact on b/f or pharmacotherapy | Major/Minor | Comments |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 2003 | Health Departm | MMR vaccine | Rash, febrile and behavioural changes for 2 days | Rash resolved | 3.5 months | No effect | Major | |
| 3 | 2004 | Hospital | amoxycillin, metronidazole, nifedipine, pizotifen | ? | Selective eating disorder | Not stated | stopped all meds | Minor | |
| 4 | 2004 | Drug Company | Demazin clear syrup | Rash | Neonatal rash | 1yo | stopped medication | Major | |
| 5 | 2004 | Hospital | Lamisil | developmental delay | Significant hypotonia and developmental delay | Not stated | Not stated | Major | |
| 6 | 2006 | Pharmacist | Amoxicillin capsules 1500mg TDS | Diarrhoea | Symptoms disappeared when breastfeeding stopped and formula initiated. Symptoms reapperaed | Not stated | Not stated | Minor | ? Reported dose- likely 1500mg d in ddd |
| 7 | 2006 | General practit | Fluvoxamine 100mg | Rash | Rash resolved when luvox stopped | Not stated | stopped antidepressant | Major | |
| 8 | 2006 | Drug Company | Cephalexin | Severe Diarrhoea | Not stated | Not stated | Not stated | Major | |
| 9 | 2006 | General practit | Domperidone | Baby developed gynacomastia, breast abcess - while mother was prescribed domperidone to increase milk | Baby received Antibiotics | Not stated | Not stated | major | |
| 10 | 2007 | General practit | Mirena (Levonorgestrel) IUD | Very Unsettled and screaming baby | Mirena removed | Not stated | Not stated | Not stated | |
| 11 | 2007 | Doctor - Speci | Quetiapine 300mg nocte dose | baby projectile vomited | Quetiapine ceased | 39 days old | ceased Quetiapine | major | baby Hospitalise ddue to this reaction; Mother started taking this drug late pregnancy 8 weeks before due date; mum also on Escitalopram which was coninued |
| 12 | 2007 | Health Departm | Codeine based cough elixir | Intermittent jerky facial and body movements since breastfeeding post taking the elixir | Somnolece, hypotonia, Dystonia | Not stated | Not stated | Major | ? |
| 13 | 2008 | Patient | Micronor- norethisterone 350mg | increased behavioural issues - crying, distress, sleeplessness and irritability, constipation, selective eating | Symptoms disappeared on discontinuation of the drug | 5.5 months | ceased Norethisterone | major | Lots of information |
| 14 | 2008 | General practit | MMR vaccine | Rash rubelliform, irritability, Lymphadenopathy. Increased lacrimation | NIL- treated symptoms with paracetamol | 8 months | n/a | Minor | |
| 15 | 2008 | Drug Company | Fluoxetine | drug was taken during all trimesters and BF Autism diagnosed age three | N/A | 3 years | N/A | ? | Unsure if drug caused |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 16 | 2009 | Drug Company | Fluoxetine, Morphin and Quetiapine | Despite the combination, baby was reported to have normal growth and | Nil | 3 months | N/A | NIL | |
| 17 | 2009 | Drug Company | tacrolimus, Diltiazem, prednisolone | Not stated | Not stated | new born- 5 days | Nil | nil | |
| 18 | 2009 | Drug Company | Fluoxetine and Quetiapine | WitHealth Departmentrawal symptoms- jittery, loppy and febrile | Not stated | Not stated | Not stated | Not stated | |
| 19 | 2009 | Public | H1N1 Influenza vaccine | increased Somnolence, diarrhoea and increased appetite (possibly due to reduced milk supply) | Not stated | Not stated | Not stated | Not stated | |
| 20 | 2009 | Public | H1N1 Influenza vaccine | Swelling of baby's neck and irritability | Not stated | Not stated | Not stated | Not stated | |
| 21 | 2010 | Drug Company | Codral cold and flu tablets | lethargic, listless, groggy | baby vomited | 11 months | not stated | unsure | General practitioner unsure if the tablets caused the reaction as the baby also had a cold |
| 22 | 2011 | Drug Company | venlafaxine | selective eating disorder, premature baby, eczema | inutero exposure to venlafaxine | new born- 5 days | not stated | unsure | |
| 23 | 2011 | Drug Company | sertraline | learning disability, fetal exposure during pregnancy and breastfeeding | in utero exposure | 11 years | ? | major | |
| 24 | 2011 | Drug Company | sertraline | diabetes mellitus in child | in utero and breastmilk exposure | 9 years | ? | Major | |
| 25 | 2011 | Drug Company | Robitussin chesty cough syrup and PE | No adverse reaction reported | not stated | Not stated | Not stated | | |
| 26 | 2011 | Drug Company | Dimetapp cold and flu tablets night time | No adverse reaction reported | Not stated | Not stated | Not stated | ? | |
| 27 | 2011 | Pharmacist | Havrix, fluvaccine and Typhim vaccine | baby vomiting since mother's vaccinations | not stated | not stated | Not stated | | |
| 28 | 2011 | Drug Company | Ibuprofen (Advil) capsules | Not stated | Ibuprofen was withdrawn | Not stated | Ibuprofen was witheld | | |
| 29 | 2011 | Drug Company | Duloxetine, Elevit (multivitamins), Oral contarceptive pill (name not stated) | Hypoglycaemia, inutero exposure and via breast milk, premature baby, increased blood bilirubin, growth retardation | caused prolonged patient Hospitalisation | newborn | Not stated | major | |
| 30 | 2012 | Drug Company | gadolinuim based contrast agent for MRI (magnevist) | Arachnoiditis | Arachnoiditis | 6 weeks | Not stated | major | |
| 31 | 2012 | Specialist | Zuclopenthixol injection | Baby difficult to settle, irritable and stiff | breastfeeding ceased and baby | Not stated | Ceased breastfeeding | minor | |
| 32 | 2012 | Drug Company | Dimetapp DM cough and cold syrup | Not stated | Not stated- drug was used for 2 weeks | Not stated | dimetapp was ceased | | |
| 33 | 2012 | Public | gadolinuim based contrast agent for MRI (magnevist) | Conjunctivitis, easier to bruise, diarrhoea, lethargy, rashes, lymphadenopathy, | baby was 6 weeks old when mother was injected the dye and was continued to breastfeed for 6.5 months | 11 months | Not stated | major | |
| 34 | 2012 | Pharmacist | fluvax | increased irritability, crying and wakeful;nees for 12 hours post mothers fluvax vaccine | settled within 12 hours | Not stated | NIL | MINOR | |
| 35 | 2012 | Drug Company | CALTRATE PLUS | Not stated- just exposure via breast milk as mother was allergic to milk | Not stated | Not stated | Not stated | unsure | |
| 36 | 2012 | Public | fluvax | vomiting, unwell, Hospitalisation, drowsy | Not stated | 4 month | Not stated | minor | |
| 37 | 2012 | Drug Company | buprenorphine ( Subutex) | neonatal abstinence syndrome | baby's blood buprenorphine and norbuprenorphine levels were done - significant | Not stated | Not stated | major | baby's birthweight 2.97kg and when plasam levels done 5.5Kg |
| 38 | 2012 | Drug Company | buprenorphine ( Subutex) and bezodiazepines | neonatal abstinence syndrome | Not statedif the baby breastfed at all | new born | not stated | unsure - the outcome of this ad fx is unknown | breast milk concentration of buprenorphine and norbuprenorphine done |
| 39 | 2012 | Drug Company | Buprenorphine | Drug WitHealth Departmentrawal syndrome, foetal exposure during pregnacy and Bf | Not statedif the baby breastfed at all | new born | not stated | unsure | breast milk concentration of buprenorphine and norbuprenorphine done |
| 40 | 2012 | Drug Company | buprenorphine | breast milk exposure | no adverse reactions reported | new born | nil- as breastfeeding continued | n/a | breast milk concentration of buprenorphine and norbuprenorphine done |
| 41 | 2012 | Drug Company | buprenorphine and 8-tetrahydrocannabinol | neonatal abstinence syndrome and drug exposure via breast milk | NOT STATED | new born | not stated | N/A | |
| 42 | 2012 | Public | paroxetin | took paroxetin before, during and after pregnancy . Breastfed for 18 months. premature baby, delayed development, dyslexia, Auditory disorder, Autism spectrum disorder, foetal exposure durong pregnancy | premature baby, delayed development, dyslexia, Auditory disorder, Autism spectrum disorder, foetal exposure durong pregnancy | 12 years old | nil - as breastfeeding and pharmacotherapy was continued | Major | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 43 | 2013 | Pharmacist | Rectogesic suppositories and ointment | Mum used rectogesic ointment for haemhorroids- At first feed after ointment use, baby vomited heavily and reaction reccurred when ointment used a second time | baby vomited, increased crying and unsettled required a visit to the Dr. | 10 week old | Ceased pharmacotherapy | major | |
| 44 | 2013 | Public | Blackmores pregnanyc and breastfeeding Gold | increased crying, insomnia, vomiting, Aphonia | Mum Stopped taking the capsules and complained to Drug Company re geatin coating on the capsule which was omitted | 5 months | Ceased pharmacotherapy | major | |
| 45 | 2013 | Drug Company | buprenorphine and fluvoxamine | In utero developmental delays and irritability and fever on day 3 after birth | prolonged Hospital stay | newborn | not stated | | |
| 46 | 2013 | Drug Company | Quetiapine XR and venlafaxine | Drug wtHealth Departmentrawal syndrome - details not provided | Drug therapy was witHealth Departmentrawn | newborn | medications ceased- effect on mothers mental health not reported | The body of the report suggests that the reporting body is a doctor | |
| 47 | 2013 | Drug Company | dicloxacillin | Mother took four capsules and baby appeared relatively more sleepy than usual and fainted | Baby was taken to Hospital to be reviewed by cardiologist | 11 months | Not stated | The body of the report suggests that the reporting body is the baby's mother | |
| 48 | 2014 | Drug Company | mesalazine (salofalk) capsules | Exposure in utero and via breastmilk | reduced platelet count, increased INR, deranged LFTs, jaundice, Decreased appetite, increased bilirubin | 12 weeks | Breastfeeding ceased, prolonged Hospitalisation | The body of the report suggests that the reporting body is the baby's mother | |
| 49 | 2014 | Drug Company | glatiramer acetate | 10 day old baby was exposed to copaxone via breast milk. Baby was full term and delivered via C-section. On day ten, baby was diagnosed with necrotising colitis. BF was ceased and baby was admitted to Hospital for IV antibiotic therapy | Necrotising colitis | 10 days | Ceased Breastfeeding | Major | |
| 50 | 2014 | Drug Company | Botulinum toxin | NOT BF related- patient experienced breathing difficulties | NOT BF RELATED | newborn | Not stated | Not stated | |
| 51 | 2014 | Drug Company | Soliris ( eculizimab) | Transplacental as well as breastfeeding transfer | premature baby, delayed development, dyslexia, Auditory disorder, Autism spectrum disorder, foetal exposure during pregnancy and breastfeeding | new born | Not stated | Not stated | |
| 52 | 2014 | Drug Company | amoxycillin/clavulanic acid 875/125mg | not breastfeeding related- only appeared in this report as the patient was breastfeeding | not stated/relevant | not stated | nil | n/a | |
| 53 | 2014 | Drug Company | Risperidone consta 37.5mg, Quetiapine XR 50mg/day and at 32 weeks was started on s/c insulin (Isophane and aspart) therapy for gestational DM | mother was on these drugs during pregnancy; developed gestational T2DM, baby born at 36 weeks by C-Section | baby was breastfed for 2 weeks, but ceased due to poor suckling; baby was large had respiratory distress. Poor suckling led to cessation of Bf | new born | n/a | major | |
| 54 | 2015 | Drug Company | fluconazole | mother noticed foul odor of capsule but still took- felt nauseaus, vomited, feverish, dizzy; Baby also vomited and was diagnosed with dehydration | unwell baby- vomiting and dehydration | 10 month | n/a | minor | |
| 55 | 2015 | Drug Company | Infliximab | Transplacental transfer - on infliximab therapy until trimester 3 and continued after baby's birth | premature baby - outcome or impact of drug therapy on BF not stated | newborn | not stated | Not stated | |
| 56 | 2015 | Drug Company | trimethoprim 300mg | vomiting and fever in breastfed infant after two doses of antibiotic | Unwell baby- vomiting and pyrexia | not stated | not stated | minor | |
| 57 | 2015 | Public | MMR vaccine given to breastfed baby's mother and baby developed rash on the face - diagnosed as allergy (not measles) by General | Breastfed baby developed measle like rash on the face one week after mother was given MMR vaccine | Not measles | not stated | Not stated | minor | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 58 | 2015 | Drug Company | sertraline | mother overdosed on sertraline tablets (6x100mg = 6g) | baby developed tachycardia and was Hospitalised | not stated | Not stated | The body of the report suggests that the reporting body is a Pharmacist | |
| 59 | 2016 | Drug Company | caltrate bone health (calcium carbonate and cholecalciferol) | baby allergic to soy and cow's milk - mother took caltrate bone health and baby developed Haematochezia | symptoms disappeared 4 days after the cessation of this product | Not stated | Not stated | major | |
| 60 | 2016 | Drug Company | venlafaxine | drug exposure in utero and via breastmilk- Mother also took temazepam, quetiapine and olanzapine between 15 and 20 weeks. Venlafaxine as monotherapy from week 20 onwards | baby was born at term weighing 4.3kg (97th percentile). However, baby failed to thrive and gained weight. Baby was also excessively drowsy, neonatal behavioural | One month old | Ceased breastfeeding | major | |
| 61 | 2016 | Drug Company | venlafaxine | poor neonatal adaptation syndrome, witHealth Departmentrawal syndrome - inutero exposure and breastfeeding | Not stated | 3 days old | not stated | Not stated | |

# Appendix C: Survey website and questions




## Our Research

This survey is part of a research project conducted by the School of Pharmacy at Curtin University. This study investigates the implications of transfer of medications that a mother takes to their breastfed infant through breast milk. All women who are currently breastfeeding or have breastfed in the last 12 months are eligible to participate. The survey is open to all Australian residents. The information you provide will help us in establishing the occurrence of untoward or adverse reactions in a breastfed baby because of what the mother takes. This survey will take 10-15 minutes of your time and has been approved by the Curtin Human research ethics committee (approval number HR1110/2012) and the South Metropolitan Health Service Human Research Ethics Committee (approval number 2016-273). We appreciate your time and participation in this survey and for providing valuable insight into your breastfeeding journey.

For further information on our research, please read below under "More Information" or contact the investigators via email.

# Participant disclosure

- Your participation in this research is voluntary. By clicking the button below you are confirming that you are over 18 and are providing consent for the information that you provide to be used in the study.
- Once your completed form has been submitted, it will not be possible to withdraw from the study as there are no identifying questions on the form which allow the study team to find and remove your form.
- You will need an email address to log into the survey. This will allow you to pick up the survey from where you left in case you are unable to complete it in one sitting. The email will not be used for any other purpose.
- All data gained from this research will be stored in accordance with the Australian code for the responsible conduct of research.
- By completing the survey, you will be eligible to enter a draw for one of five $50 Coles Myer gift vouchers. If you wish to participate in the draw, please email your name and a contact number to **survey@breastfeedingresearch.com.au** with 'Survey Prize Draw' in the subject header. This will allow us to allocate you with a lottery number and enable us to contact you if you win in the draw. The lottery will be drawn on the 31st of December 2016. The names of the lottery winners will be published on this website. Should you wish your name not to be punblished, please advise us via email. Collection of your personal information will in no way be linked to your survey responses and will be stored confidentially on a password protected server until the lottery has been drawn. The lottery is currently restricted to residents of WA.
- **Please note that the lottery draw date has been changed to March 31, 2017.**

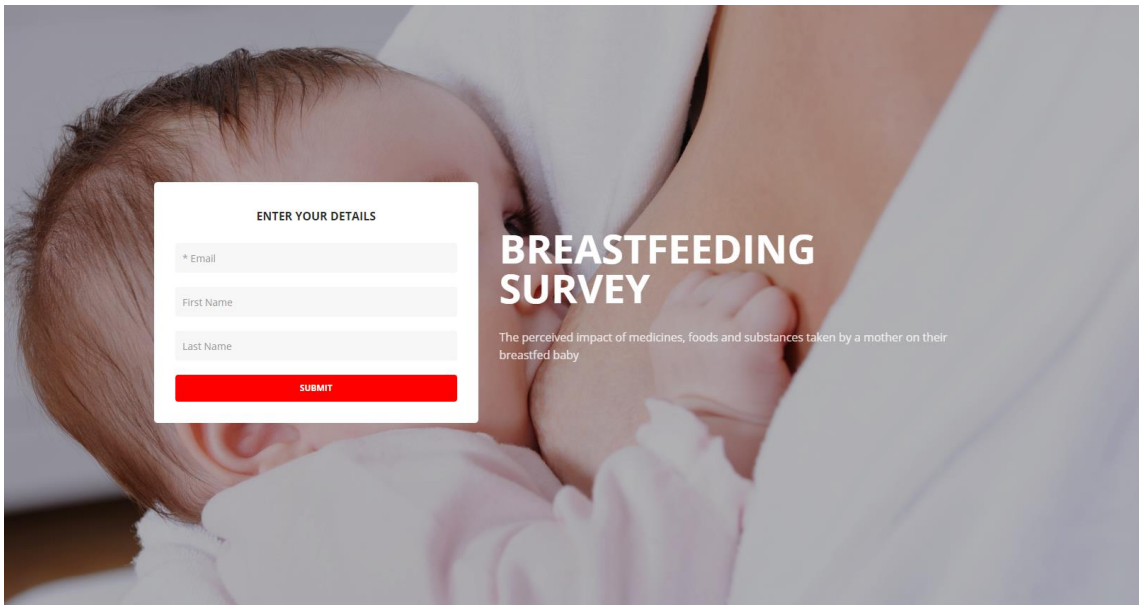
Take the Survey

## More Information

"Breastfeeding provides numerous significant benefits for the newborn infants and mothers. The Australian Breastfeeding Association, American Academy of Paediatrics and World Health Organization (WHO) all recommend and encourage exclusive breastfeeding during the first six months after birth. Breastfeeding provides tailored nourishment to the growing needs of infants, offering better nutrition, enhances immunity and neurological development amongst other benefits. However, there is always concern regarding the transfer of medications to the infant via breast milk if the mother has to take medications. Although most medications are safe to use during breastfeeding, there have been cases where high levels of certain medications have been detected in breast milk. Infant exposure to medications via breast milk, especially in the first six months when the infant is likely to be exclusively breastfed can have severe adverse effects possibly due to the underdeveloped infant organs systems. Hence, even small exposure to medication via breast milk may result in side effects in the infant. It is also important not to discontinue breastfeeding unnecessarily as there are many benefits associated with breastfeeding. A recent review of the literature has shown that adverse reactions in breastfed infants are notoriously under reported and this may be because the types and rate of adverse drug reactions experienced by breastfed infants whose mothers are taking medications has not been well defined.

Our research aims to provide insight from a mother's perspective into possible adverse reactions in their breastfed baby because of what they may have taken. Please help us get to know more about your breastfeeding experience by completing this short survey. For further information, please contact us on survey@breastfeedingresearch.com.au

Contact Us



1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products | 5. Adverse reaction probability | 6. Impact on breastfeeding

**1. State of Residence**

WA

**2. Are you currently breastfeeding?**

- Yes ( Go to question 4 )
- No

**3. How long has it been since you last breastfed?**

number of ... Months

**4. What is your baby's date of birth?**

**5. How long have you been breastfeeding or breastfed for?**

number of ... Days
number of ... Weeks
number of ... Months
number of ... Years

**6. What is the duration that you exclusively breastfed your baby i.e. the time period where your baby had ONLY breastmilk and NO solids or formula?**

number of ... Days
number of ... Weeks
number of ... Months

**7. What is your highest level of education ?**

- Primary school
- Secondary school
- Graduate diploma/TAFE/College
- Undergraduate degree
- Postgraduate degree

Previous Next

1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products | 5. Adverse reaction probability | 6. Impact on breastfeeding

**1. Do you suffer from any medical conditions?**

- ◯ Yes ( Go to question 2 )
- ◯ No ( Go to question 3 )

**2. Medical Conditions**

- ☐ Diabetes
- ☐ Epilepsy
- ☐ Depression
- ☐ Mood Disorder
- ☐ Skin condition
- ☐ Hypertension
- ☐ Migraines
- ☐ Chronic Pain
- ☐ Gastrointestinal disorders
- ☐ Other

**3. Do you currently take any regular medications? Please include any prescription, over-the-counter and complementary medications.**

- ◯ Yes - Please specify
- ◯ No

**4. Did you take any medications during pregnancy?**

- ◯ Yes - Please specify
- ◯ No

1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products | 5. Adverse reaction probability | 6. Impact on breastfeeding

**1. Did you start any NEW medications after the birth of your baby that you were not taking during pregnancy?**

- ◯ Yes - Please name the medication. PLEASE COMPLETE ALL QUESTIONS ON PAGE 5
- ◯ No ( Go to question 5 )

**2. Are you still taking this medication?**

- ◯ Yes ( Go to question 4 )
- ◯ No

**3. How long did you take this medication for?**

| | |
|---|---|
| number of ... | Days |
| number of ... | Weeks |
| number of ... | Months |
| number of ... | Years |

**4. Were you breastfeeding when you started taking this medication?**

- ◯ Yes
- ◯ No

**5. Were you at any stage concerned about the transfer of substances or medication to your baby through your breast milk?**

- ◯ Yes
- ◯ No

10. Please state the reaction

- [ ] Skin reactions eg rash
- [ ] Colic
- [ ] Breathing difficulties
- [ ] Vomiting or diarrhoea
- [ ] Behavioural changes such as increased crying, unsettled behaviour or loss of sleep
- [ ] Other - Please specify

Previous Next

6. Did you seek professional/medical advice for this concern?

- ( ) Yes
- ( ) No ( Go to question 8 )

7. Who did you consult?

- [ ] GP
- [ ] Pharmacist
- [ ] Hospital
- [ ] Lactation Consultant
- [ ] Internet
- [ ] Other - Please Specify

8. Did you at any stage notice any adverse or untoward reactions in your baby that you attributed to the medication that you took/used? NOTE: An adverse drug reaction is an unexpected reaction to a medication that may result in discomfort or toxicity and in extreme cases organ damage or death.

- ( ) Yes
- ( ) No ( Go to page 4 question 1 )

9. How old was your baby when this reaction occured?

| | |
|---|---|
| number of ... | Days |
| number of ... | Weeks |
| number of ... | Months |

1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products

5. Adverse reaction probability | 6. Impact on breastfeeding

1. Did you notice any untoward/adverse reaction in your baby after you ate a food product? NOTE: An adverse reaction is an unexpected reaction to a substance that may result in discomfort or toxicity.

- ○ Yes- Please specify the food product that caused the suspected reaction
- ○ No ( Go to page 5 question 1 )

2. Please state the reaction

- ☐ Skin reaction eg rash
- ☐ Colic
- ☐ Breathing diffculties
- ☐ Vomiting
- ☐ Behavioural changes such as increased crying, unsettled behaviour or loss of sleep
- ☐ Other - Please specify

3. Did this reaction have a negative impact on your breastfeeding experience?

- ○ Yes
- ○ No

Previous Next

1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products

5. Adverse reaction probability | 6. Impact on breastfeeding

1. Did you notice this adverse/untoward reaction in your baby after breastfeeding him/her after you took your medication?

- ○ Yes
- ○ No
- ○ Don't know

2. Are you aware of any similar reactions being reported in other babies?

- ○ Yes
- ○ No
- ○ Don't Know

3. How did you resolve this problem?

- ○ Stopped breastfeeding
- ○ Stopped medication
- ○ Changed the timing of feeds/medication
- ○ Other - please specify

4. Did your baby's symptoms improve when you did the above?

- ○ Yes
- ○ No
- ○ Don't Know

5. Did you try to re-expose your baby to the suspected medication by giving them more breast milk whilst you were still taking this drug?

- ○ Yes ( Go to question 6 )
- ○ No ( Go to question 7 )

6. Did the problem reappear when your baby was re-exposed?

- Yes
- No
- Don't know
- Not done

7. Could there be anything else that you think may have caused this reaction in your baby?

- Yes
- No
- Don't Know

8. Did you try an alternative, non-medicated substance or a placebo to see if your baby reacted the same way as before?

- Yes, reacted the same way
- Yes, did NOT react the same way
- Did not try this

9. Were any medical/diagnostic tests such as blood tests or breast milk testing done?

- Yes ( Go to question 10 )
- No ( Go to question 11 )
- Don't know ( Go to question 10 )
- Not Done ( Go to question 11 )

10. Were you made aware of any concerns regarding such tests, for example high levels of the drug in your baby's blood or your breast milk sample?

- Yes
- No
- Don't know
- Not Done

11. Did you trial changing the dose of your medication?

- Yes ( Go to question 12 )
- No ( Go to question 13 )
- Not Done ( Go to question 13 )

12. Was the reaction more severe when the dose was increased or less severe when the dose was decreased?

- Yes, more severe when dose increased
- Yes, less severe when dose decreased
- No change at all
- Not done

13. Are you aware of your baby reacting to another drug in a similar way at a different time?

- Yes
- No
- Don't know

14. Were there any conclusive tests or diagnosis made by the treating medical professional that confirmed this adverse reaction in your baby was due to medication transfer in your breast milk?

- Yes
- No
- Not done
- Don't know

Previous Next

1. General details | 2. Mother's general health | 3. Medication use during breastfeeding | 4. Perceived reactions to food products | 5. Adverse reaction probability | 6. Impact on breastfeeding

1. Did you discontinue breastfeeding due to this adverse event?

- Yes
- No

2. Did this event make you change your medication or stop your treatment at all?

- Yes
- No

3. Did you report this adverse reaction to a healthcare professional?

- Yes
- No

4. Do you know if your healthcare professional (e.g doctor, lactation consultant, pharmacist, hospital, specialist) reported this adverse event to a regulatory body?

- Yes
- No
- Not done
- Don't know

Previous Submit

*Thank you for completing the survey.*

*You are now eligible to enter a draw for one of 5 $50 Coles Myer gift vouchers. If you wish to participate in the draw, please email your details to pharmacysurvey @breastfeedingresearch.com.au with 'Survey Prize Draw' in the subject header. This will allow us to allocate you with a lottery number and enable us to contact you if you win in the draw. This will be drawn on the 1st of December 2016.*

*Collection of your personal information will in no way be linked to your survey responses and will be stored confidentially on a password protected server until the lottery has been drawn. The lottery is currently restricted to residents of WA.*

# Appendix D: Ethics Approval Letters

Curtin University

Office of Research and Development

GPO Box U1987
Perth Western Australia 6845

**Telephone** +61 8 9266 7863
**Facsimile** +61 8 9266 3793
**Web** research.curtin.edu.au

25-Jul-2016

Name: Lisa Tee
Department/School: School of Pharmacy
Email: L.Tee@curtin.edu.au

Dear Lisa Tee

**RE: Amendment approval**
**Approval number: HR110/2012**

Thank you for submitting an amendment request to the Human Research Ethics Office for the project **The role of efflux transporters in the transfer of drug from maternal plasma to the breastfed infant**.

Your amendment request has been reviewed and the review outcome is: **Approved**

The amendment approval number is HR110/2012-01 approved on 25-Jul-2016.

The following amendments were approved:
Addition of a survey to the approved protocol as a new objective.

Any special conditions noted in the original approval letter still apply.

**Standard conditions of approval**

1. Research must be conducted according to the approved proposal
2. Report in a timely manner anything that might warrant review of ethical approval of the project including:
   - proposed changes to the approved proposal or conduct of the study
   - unanticipated problems that might affect continued ethical acceptability of the project
   - major deviations from the approved proposal and/or regulatory guidelines
   - serious adverse events
3. Amendments to the proposal must be approved by the Human Research Ethics Office before they are implemented (except where an amendment is undertaken to eliminate an immediate risk to participants)
4. An annual progress report must be submitted to the Human Research Ethics Office on or before the anniversary of approval and a completion report submitted on completion of the project
5. Personnel working on this project must be adequately qualified by education, training and experience for their role, or supervised
6. Personnel must disclose any actual or potential conflicts of interest, including any financial or other interest or affiliation, that bears on this project
7. Changes to personnel working on this project must be reported to the Human Research Ethics Office
8. Data and primary materials must be retained and stored in accordance with the Western Australian University Sector Disposal Authority

Government of **Western Australia**
**Child and Adolescent Health Service**

Dear Hilai Ahmadzai

**APPROVAL TO ACCESS CACH SITES TO RECRUIT PARTICIPANTS**

Please be advised that Child and Adolescent Community Health (CACH) supports the request to display of promotional posters and flyers at metropolitan Child Health Centres to to assist in the recruitment of children to the following project:

*The Impact of Medicines and Foods taken by the Mother on their Breastfed Baby.*

In order to complete the approval process, this letter of support must be submitted with the WA Health Access Request Form to the CAHS Research Governance Office.

When you receive final approval for this research project from CAHS Research Governance Office, please contact us by email at ResearchRequests.CACH@health.wa.gov.au to make arrangements for your posters to be displayed.

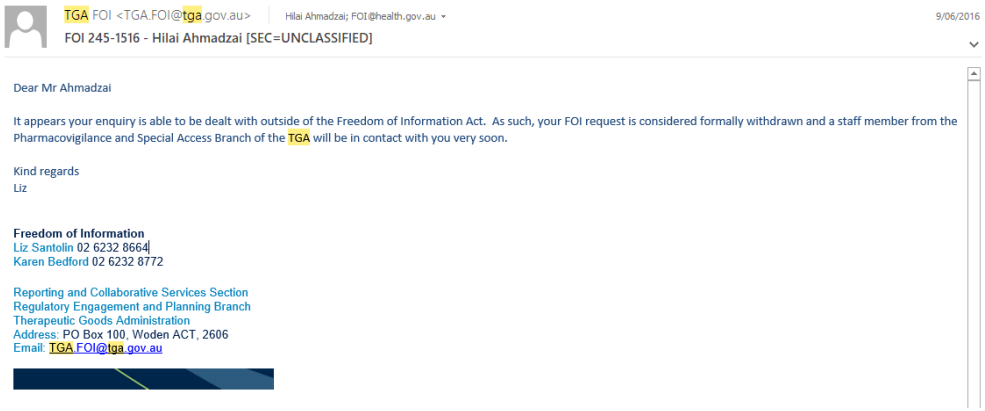
Yours faithfully

[redacted]

Lisa Brennan
**Executive Director**
**Child and Adolescent Community Health**

21 November 2016

Child and Adolescent Community Health
WASON Building
151 Wellington Street, Perth WA 6000
PO Box S1296 Perth WA 6845
Tel: (08) 9224 1625 Fax: (08) 9224 1612
www.cahs.health.wa.gov.au



TGA FOI <TGA.FOI@tga.gov.au> Hilai Ahmadzai; FOI@health.gov.au 9/06/2016

FOI 245-1516 - Hilai Ahmadzai [SEC=UNCLASSIFIED]

Dear Mr Ahmadzai

It appears your enquiry is able to be dealt with outside of the Freedom of Information Act. As such, your FOI request is considered formally withdrawn and a staff member from the Pharmacovigilance and Special Access Branch of the TGA will be in contact with you very soon.

Kind regards
Liz

**Freedom of Information**
Liz Santolin 02 6232 8664
Karen Bedford 02 6232 8772

Reporting and Collaborative Services Section
Regulatory Engagement and Planning Branch
Therapeutic Goods Administration
Address: PO Box 100, Woden ACT, 2606
Email: TGA.FOI@tga.gov.au

**From:** [redacted]
**Sent:** Thursday, 11 August 2016 10:22:03 AM
**To:** Hilai Ahmadzai
**Cc:** [redacted]
**Subject:** RE: Research collaboration- Curtin University

Hello Hilai

I'm pleased to advise that support of your research was endorsed at yesterday's Research Group meeting.

Please forward a copy of your finalised ethics approval asap.

Are you able to drop off some printed flyers that we can distribute to key services/centres?

**Sent:** Thursday, 1 September 2016 8:34:40 AM
**To:** Hilai Ahmadzai
**Subject:** RE: Research assistance and collaboration form

Hi Hilai,

Your research project *The role of efflux transporters in the transfer of drugs from maternal plasma to the breastfed infant* has been approved by ABA.

Wherever and whenever you promote your research you should include the following statement in full: **ABA Research Approval Number 2016-12.**

You will need to create a blurb for the ABA Forum which includes both the ABA research approval number and the university ethics number and the link to the survey. Once the blurb is written, send it to me and I will get the Forum admin to upload it.

See examples on the Forum already at:

http://forum.breastfeeding.asn.au/viewforum.php?f=14&sid=76d895e11e72b04addab4bb0af314027

Also, a reminder that, as part of the ABA research policy you have agreed to the following re publications:

Publication
All researchers whose requests are approved under this policy are encouraged to submit a synopsis/report of their research to Breastfeeding Review for consideration of publication
Research into ABA or the services offered by ABA will only be approved where it is intended to be published in Breastfeeding Review or another ABA publication.
Where approval is granted in accordance with this policy:
o ABA must be appropriately acknowledged in all research publications and presentations
o A copy of the research publication that involved ABA members, staff or resources must be sent to the Breastfeeding Information and Research team.

And remember to contact Tessa regarding paid advertisements in Essence and the ABA personal member enewsletter: tessa.gillespie@breastfeeding.asn.au

All the best,

[redacted]
Manager Breastfeeding Information and Research BSc PhD Dip Ed Dip Breastfeeding Management Cert IV TAE
Australian Breastfeeding Association
1818 Malvern Rd, Malvern East, VIC 3145
P: 03 9885 0855

# Appendix E: Supplementary Tables

**Table App 1. List of Taqman probes used for RT-PCR**

| Gene | Taqman probes |
|---|---|
| BCRP | Hs001053790_m1 |
| MDR1 | Hs00324085_m1 |
| MRP1 | Hs00910358_s1 |
| MRP2 | Hs01091188_m1 |
| GAPDH | Hs03929097_g1 |

**Table App 2. Antibody details used in immunostaining experiments**

| Transporter | Antibody | Concentration | Source |
|---|---|---|---|
| MRP1 | Mouse anti MRP1 | 1:100 | Santa Cruz |
| MRP2 | Mouse anti MRP2 | 1:200 | Santa Cruz |
| MDR1 | Mouse anti MDR1 | 1:100 | Santa Cruz |
| BCRP (ABCG2) | Mouse anti BCRP | 1:100 | Sapphire Bioscience |
| Secondary antibody | Goat antimouse conjugate AF 594 | 1:400 | Life Technologies |
| DAPI | Slowfade Gold antifade mountant with DAPI | - | Life Technologies |

**Table App 0.3 Longitudinal BCRP gene expression over duration of breastfeeding (T1 =1 month post-partum; T2 = 3 months post-partum, T3 = 5 months post-partum; T4 = 9 months post-partum; T5 = 12 months post-partum) in each participant.**

| **Fixed effects (Type III) p value =0.0063** | | | | |
|---|---|---|---|---|
| ***Tukey's multiple comparisons test*** | **Mean Diff.** | **95.00% CI of diff.** | **Summary** | **Adjusted P Value** |
| **P1** | | | | |
| **T1 vs. T2** | -1026 | -4299 to 2246 | not significant (ns) | 0.9102 |
| **T1 vs. T3** | -335.3 | -3608 to 2937 | ns | 0.9986 |
| **T2 vs. T3** | 691 | -2581 to 3963 | ns | 0.9778 |
| | | | | |
| **P2** | | | | |
| **T1 vs. T2** | -2403 | -5676 to 869.2 | ns | 0.2599 |
| **T1 vs. T3** | -8085 | -11358 to -4813 | **** | <0.0001 |
| **T2 vs. T3** | -5682 | -8954 to -2410 | **** | <0.0001 |
| | | | | |
| **P3** | | | | |
| **T1 vs. T2** | -5083 | -8355 to -1811 | *** | 0.0003 |
| **T1 vs. T3** | -5230 | -8502 to -1958 | *** | 0.0002 |
| **T2 vs. T3** | -147 | -3419 to 3125 | ns | >0.9999 |
| | | | | |
| **P4** | | | | |
| **T1 vs. T2** | -4915 | -8187 to -1643 | *** | 0.0005 |
| **T1 vs. T3** | 1185 | -2088 to 4457 | ns | 0.8571 |
| **T2 vs. T3** | 6100 | 2827 to 9372 | **** | <0.0001 |
| | | | | |
| **P5** | | | | |
| **T1 vs. T2** | -3299 | -6572 to -26.85 | * | 0.0471 |
| **T1 vs. T3** | -17746 | -21019 to -14474 | **** | <0.0001 |
| **T1 vs. T4** | -3994 | -7267 to -721.8 | ** | 0.0082 |
| **T1 vs. T5** | -534.3 | -3807 to 2738 | ns | 0.9915 |
| **T2 vs. T3** | -14447 | -17719 to -11175 | **** | <0.0001 |
| **T2 vs. T4** | -695 | -3967 to 2577 | ns | 0.9773 |
| **T2 vs. T5** | 2765 | -507.5 to 6037 | ns | 0.1412 |
| **T3 vs. T4** | 13752 | 10480 to 17024 | **** | <0.0001 |
| **T3 vs. T5** | 17212 | 13940 to 20484 | **** | <0.0001 |
| **T4 vs. T5** | 3460 | 187.5 to 6732 | * | 0.0324 |
| | | | | |
| **P6** | | | | |
| **T1 vs. T2** | 3161 | -111.8 to 6433 | ns | 0.064 |
| **T1 vs. T3** | 3548 | 275.5 to 6820 | * | 0.0262 |
| **T1 vs. T5** | 6087 | 2815 to 9359 | **** | <0.0001 |
| **T2 vs. T3** | 387.3 | -2885 to 3660 | ns | 0.9976 |
| **T2 vs. T5** | 2926 | -346.2 to 6199 | ns | 0.1038 |
| **T3 vs. T5** | 2539 | -733.5 to 5811 | ns | 0.2095 |

| | | | | |
|---|---|---|---|---|
| **P7** | | | | |
| **T1 vs. T2** | -3992 | -7264 to -719.5 | ** | 0.0082 |
| **T1 vs. T3** | -8107 | -11379 to -4835 | **** | <0.0001 |
| **T1 vs. T4** | -7364 | -10637 to -4092 | **** | <0.0001 |
| **T1 vs. T5** | -2876 | -6148 to 396.8 | ns | 0.1146 |
| **T2 vs. T3** | -4115 | -7387 to -842.5 | ** | 0.0058 |
| **T2 vs. T4** | -3372 | -6645 to -99.85 | * | 0.0398 |
| **T2 vs. T5** | 1116 | -2156 to 4389 | ns | 0.8817 |
| **T3 vs. T4** | 742.7 | -2530 to 4015 | ns | 0.9711 |
| **T3 vs. T5** | 5231 | 1959 to 8504 | *** | 0.0002 |
| **T4 vs. T5** | 4489 | 1216 to 7761 | ** | 0.0019 |
| | | | | |
| **P8** | | | | |
| **T1 vs. T2** | -2170 | -5442 to 1102 | ns | 0.3624 |
| **T1 vs. T3** | -1899 | -5171 to 1373 | ns | 0.5015 |
| **T1 vs. T4** | -1490 | -4762 to 1782 | ns | 0.7206 |
| **T1 vs. T5** | -1244 | -4516 to 2029 | ns | 0.8339 |
| **T2 vs. T3** | 271 | -3001 to 3543 | ns | 0.9994 |
| **T2 vs. T4** | 680 | -2592 to 3952 | ns | 0.9791 |
| **T2 vs. T5** | 926.3 | -2346 to 4199 | ns | 0.9366 |
| **T3 vs. T4** | 409 | -2863 to 3681 | ns | 0.997 |
| **T3 vs. T5** | 655.3 | -2617 to 3928 | ns | 0.9817 |
| **T4 vs. T5** | 246.3 | -3026 to 3519 | ns | 0.9996 |
| | | | | |
| **P9** | | | | |
| **T1 vs. T2** | -9502 | -12775 to -6230 | **** | <0.0001 |
| **T1 vs. T3** | -8040 | -11312 to -4768 | **** | <0.0001 |
| **T1 vs. T4** | 2626 | -646.2 to 5899 | ns | 0.1808 |
| **T2 vs. T3** | 1462 | -1810 to 4735 | ns | 0.7344 |
| **T2 vs. T4** | 12129 | 8856 to 15401 | **** | <0.0001 |
| **T3 vs. T4** | 10666 | 7394 to 13939 | **** | <0.0001 |
| | | | | |
| **P10** | | | | |
| **T1 vs. T2** | 7843 | 4571 to 11116 | **** | <0.0001 |
| **T1 vs. T4** | -698.3 | -3971 to 2574 | ns | 0.9769 |
| **T1 vs. T5** | 8740 | 5467 to 12012 | **** | <0.0001 |
| **T2 vs. T4** | -8542 | -11814 to -5269 | **** | <0.0001 |
| **T2 vs. T5** | 896.3 | -2376 to 4169 | ns | 0.9434 |
| **T4 vs. T5** | 9438 | 6166 to 12710 | **** | <0.0001 |
| | | | | |
| **P11** | | | | |
| **T2 vs. T3** | -6232 | -9505 to -2960 | **** | <0.0001 |
| **T2 vs. T4** | -216.7 | -3489 to 3056 | ns | 0.9998 |
| **T2 vs. T5** | -863 | -4135 to 2409 | ns | 0.9505 |

| | | | | |
|---|---|---|---|---|
| **T3 vs. T4** | 6016 | 2743 to 9288 | **** | <0.0001 |
| **T3 vs. T5** | 5369 | 2097 to 8642 | *** | 0.0001 |
| **T4 vs. T5** | -646.3 | -3919 to 2626 | ns | 0.9827 |
| | | | | |
| **P12** | | | | |
| **T1 vs. T2** | 3379 | 106.2 to 6651 | * | 0.0392 |
| **T1 vs. T4** | 1749 | -1524 to 5021 | ns | 0.5832 |
| **T1 vs. T5** | 2309 | -963.2 to 5582 | ns | 0.2989 |
| **T2 vs. T4** | -1630 | -4902 to 1642 | ns | 0.6475 |
| **T2 vs. T5** | -1069 | -4342 to 2203 | ns | 0.8971 |
| **T4 vs. T5** | 560.7 | -2712 to 3833 | ns | 0.9898 |
| | | | | |
| **P13** | | | | |
| **T1 vs. T2** | -1483 | -4755 to 1790 | ns | 0.7242 |
| **T1 vs. T3** | -2281 | -5553 to 991.8 | ns | 0.3114 |
| **T1 vs. T4** | -6240 | -9512 to -2967 | **** | <0.0001 |
| **T1 vs. T5** | -4259 | -7531 to -986.5 | ** | 0.0038 |
| **T2 vs. T3** | -798 | -4070 to 2474 | ns | 0.9625 |
| **T2 vs. T4** | -4757 | -8029 to -1485 | *** | 0.0008 |
| **T2 vs. T5** | -2776 | -6049 to 496.2 | ns | 0.1383 |
| **T3 vs. T4** | -3959 | -7231 to -686.5 | ** | 0.009 |
| **T3 vs. T5** | -1978 | -5251 to 1294 | ns | 0.4592 |
| **T4 vs. T5** | 1981 | -1292 to 5253 | ns | 0.458 |
| | | | | |
| **P14** | | | | |
| **T1 vs. T2** | -3168 | -6440 to 104.5 | ns | 0.063 |
| **T1 vs. T3** | -573.3 | -3846 to 2699 | ns | 0.9889 |
| **T2 vs. T3** | 2595 | -677.8 to 5867 | ns | 0.1908 |
| | | | | |
| **P15** | | | | |
| **T1 vs. T2** | -1931 | -5203 to 1341 | ns | 0.4843 |
| **T1 vs. T3** | -2159 | -5431 to 1114 | ns | 0.3679 |
| **T1 vs. T4** | -518.3 | -3791 to 2754 | ns | 0.9925 |
| **T1 vs. T5** | -387.7 | -3660 to 2885 | ns | 0.9975 |
| **T2 vs. T3** | -227.7 | -3500 to 3045 | ns | 0.9997 |
| **T2 vs. T4** | 1413 | -1860 to 4685 | ns | 0.7587 |
| **T2 vs. T5** | 1543 | -1729 to 4816 | ns | 0.6932 |
| **T3 vs. T4** | 1640 | -1632 to 4913 | ns | 0.642 |
| **T3 vs. T5** | 1771 | -1501 to 5043 | ns | 0.571 |
| **T4 vs. T5** | 130.7 | -3142 to 3403 | ns | >0.9999 |
| | | | | |
| **P16** | | | | |
| **T1 vs. T2** | -4216 | -7489 to -943.8 | ** | 0.0044 |
| **T1 vs. T3** | -5773 | -9045 to -2500 | **** | <0.0001 |
| **T1 vs. T4** | 934.7 | -2338 to 4207 | ns | 0.9346 |

| | | | | |
|---|---|---|---|---|
| **T1 vs. T5** | -1669 | -4941 to 1603 | ns | 0.6265 |
| **T2 vs. T3** | -1556 | -4829 to 1716 | ns | 0.6865 |
| **T2 vs. T4** | 5151 | 1879 to 8423 | *** | 0.0002 |
| **T2 vs. T5** | 2547 | -725.2 to 5820 | ns | 0.2066 |
| **T3 vs. T4** | 6707 | 3435 to 9980 | **** | <0.0001 |
| **T3 vs. T5** | 4104 | 831.2 to 7376 | ** | 0.006 |
| **T4 vs. T5** | -2604 | -5876 to 668.8 | ns | 0.1879 |
| | | | | |
| **P17** | | | | |
| **T1 vs. T2** | 242 | -3030 to 3514 | ns | 0.9996 |
| **T1 vs. T3** | -11049 | -14321 to -7777 | **** | <0.0001 |
| **T1 vs. T4** | 7385 | 4113 to 10658 | **** | <0.0001 |
| **T1 vs. T5** | 7178 | 3906 to 10451 | **** | <0.0001 |
| **T2 vs. T3** | -11291 | -14563 to -8019 | **** | <0.0001 |
| **T2 vs. T4** | 7143 | 3871 to 10416 | **** | <0.0001 |
| **T2 vs. T5** | 6936 | 3664 to 10209 | **** | <0.0001 |
| **T3 vs. T4** | 18434 | 15162 to 21707 | **** | <0.0001 |
| **T3 vs. T5** | 18227 | 14955 to 21500 | **** | <0.0001 |
| **T4 vs. T5** | -207 | -3479 to 3065 | ns | 0.9998 |
| | | | | |
| **P18** | | | | |
| **T1 vs. T2** | -1857 | -5129 to 1416 | ns | 0.5244 |
| | | | | |
| **P19** | | | | |
| **T1 vs. T2** | 1167 | -2106 to 4439 | ns | 0.8638 |
| **T1 vs. T3** | 1258 | -2014 to 4531 | ns | 0.8279 |
| **T1 vs. T4** | 905 | -2367 to 4177 | ns | 0.9415 |
| **T1 vs. T5** | -1881 | -5153 to 1392 | ns | 0.5114 |
| **T2 vs. T3** | 91.67 | -3181 to 3364 | ns | >0.9999 |
| **T2 vs. T4** | -261.7 | -3534 to 3011 | ns | 0.9995 |
| **T2 vs. T5** | -3047 | -6320 to 225.2 | ns | 0.0813 |
| **T3 vs. T4** | -353.3 | -3626 to 2919 | ns | 0.9983 |
| **T3 vs. T5** | -3139 | -6411 to 133.5 | ns | 0.067 |
| **T4 vs. T5** | -2786 | -6058 to 486.8 | ns | 0.1359 |
| | | | | |
| **P20** | | | | |
| **T1 vs. T2** | -5360 | -8633 to -2088 | *** | 0.0001 |
| **T1 vs. T3** | -1721 | -4994 to 1551 | ns | 0.5981 |
| **T1 vs. T4** | 6021 | 2749 to 9294 | **** | <0.0001 |
| **T2 vs. T3** | 3639 | 366.5 to 6911 | * | 0.0209 |
| **T2 vs. T4** | 11382 | 8109 to 14654 | **** | <0.0001 |
| **T3 vs. T4** | 7743 | 4470 to 11015 | **** | <0.0001 |
| | | | | |
| **P21** | | | | |
| **T1 vs. T2** | -26719 | -29992 to -23447 | **** | <0.0001 |

| | | | | |
|---|---|---|---|---|
| **T1 vs. T3** | -17922 | -21194 to -14649 | **** | <0.0001 |
| **T1 vs. T4** | 529.7 | -2743 to 3802 | ns | 0.9918 |
| **T2 vs. T3** | 8798 | 5525 to 12070 | **** | <0.0001 |
| **T2 vs. T4** | 27249 | 23977 to 30521 | **** | <0.0001 |
| **T3 vs. T4** | 18451 | 15179 to 21724 | **** | <0.0001 |
| | | | | |
| **P22** | | | | |
| **T1 vs. T2** | 1396 | -1876 to 4669 | ns | 0.7664 |
| **T1 vs. T3** | -22886 | -26159 to -19614 | **** | <0.0001 |
| **T1 vs. T4** | -4251 | -7523 to -978.2 | ** | 0.0039 |
| **T2 vs. T3** | -24283 | -27555 to -21010 | **** | <0.0001 |
| **T2 vs. T4** | -5647 | -8919 to -2375 | **** | <0.0001 |
| **T3 vs. T4** | 18636 | 15363 to 21908 | **** | <0.0001 |

# Appendix F: iTRAQ Full Analysis Report

**Data analysis and protein identifications were done with ProteinPilot version 5.0**

**Database: SwissProt**

Your results were searched against the SwissProt database with taxonomy set to *Homo sapiens* (Human). It is a comprehensive, audited and curated database obtained from the UniProt Knowledgebase. It contains nonidentical protein sequence information.

**Unused (ProtScore)**

A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that have not already been completely "used" by higher scoring winning proteins. A "good" Unused ProtScore is one that corresponds to the level of confidence you require in your results. For 95% confidence, the required Unused ProtScore is 1.3.

**Total (ProtScore)**

A measure of the total amount of evidence for a detected protein. The Total ProtScore is calculated using all of the peptides detected for the protein. The Total ProtScore does not indicate the percent confidence for the identification of a protein.

**% Cov (Coverage)**

The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence.

**Ratio**

The average ratio for the protein, relative to the A (114).

**The p-value**

For each protein ratio reported the program calculates a p-value to help you assess whether changes in protein expression are real or not.
A p-value is a standard statistical metric in hypothesis testing. The p-value reports the probability that the null hypothesis "the observed value is different from unity by chance" is true. P-values range from 0 to 1.

**Colour coding**

The quantitative ratios of identified proteins are colour coded to indicate differential expression. Red indicates up-regulation and blue indicates down-regulation. The intensity of the colouring indicates the certainty of the differential expression, not the magnitude of the change. For example, the more certain the up-regulation, the more red the cells; the more certain the down-regulation, the more blue the cells.

**Note:** The coloring is only indicative of altered expression levels, and is determined by the p-value not by the size of the ratio.

**Global False Discovery Rate (FDR)**

The FDR was automatically calculated by the Proteomics System Performance Evaluation Pipeline (PSPEP) feature in the ProteinPilot™ software using the reversed version of the protein sequences contained in the search database. The software calculates both a local and a global FDR. The local FDR estimates the "local" error rate around a given identification, which indicates the likelihood that that the specific identification is incorrect. The global FDR estimates the error rate of the whole "global" set of answers defined by a threshold value. That is, the global FDR estimates the likely error rate of the entire set of identifications with scores as good as or better than the threshold.

| Colour codes | P-value | Ratio |
|---|---|---|
| Dark red | < 0.001 | > 1 |
| Medium red | 0.001 - < 0.01 | > 1 |
| Light red | 0.01 - < 0.05 | > 1 |
| No color | >= 0.05 | Any |
| Light blue | 0.01 - < 0.05 | < 1 |
| Medium blue | 0.001 - < 0.01 | < 1 |
| Dark blue | < 0.001 | < 1 |

| | |
|---|---|
| **Global FDR** | <0.1% |
| **Local FDR** | <0.1% |

| | Name | Peptides(95%) | B(115):A(114) | PVal B(115):A(114) | C(116):A(114) | PVal C(116):A(114) | D(117):A(114) | PVal D(117):A(114) |
|---|---|---|---|---|---|---|---|---|
| 2 | Non-specific lipid-transfer protein OS=Homo sapiens GN=SCP2 PE=1 SV=2 | 9 | 1.7219 | 0.1452 | 11.272 | 0.064 | 18.7068 | 0.0177 |
| 3 | Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2 | 7 | 10.3753 | 0.0275 | 6.9823 | 0.0213 | 7.8705 | 0.0796 |
| 4 | lic 10-formyltetrahydrofolate dehydrogenase OS=Homo sapiens GN=ALDH1L1 PE= | 4 | 2.1677 | 0.1033 | 5.2 | 0.0508 | 4.6989 | 0.0584 |
| 5 | nose-1-phosphate guanyltransferase alpha OS=Homo sapiens GN=GMPPA PE=1 S | 1 | 0.5916 | 0.2892 | 5.2 | 0.1478 | 1.1272 | 0.0832 |
| 6 | Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 | 4 | 12.0226 | 0.0615 | 5.0582 | 0 | 11.4815 | 0 |
| 7 | Neutrophil defensin 1 OS=Homo sapiens GN=DEFA1 PE=1 SV=1 | 2 | 2.884 | 0.373 | 4.2462 | 0.2727 | 0.955 | 0.2134 |
| 8 | Selenium-binding protein 1 OS=Homo sapiens GN=SELENBP1 PE=1 SV=2 | 11 | 0.5598 | 0.0733 | 3.6983 | 0.0229 | 3.1623 | 0.0568 |
| 9 | Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2 | 2 | 0.8872 | 0.2726 | 2.6303 | 0.0678 | 0.3908 | 0.044 |
| 10 | Fructose-1,6-bisphosphatase isozyme 2 OS=Homo sapiens GN=FBP2 PE=1 SV=2 | 1 | 0.5346 | 0.3586 | 2.4889 | 0.2548 | 3.8371 | 0.1792 |
| 11 | Fatty acid-binding protein, heart OS=Homo sapiens GN=FABP3 PE=1 SV=4 | 27 | 0.7178 | 0.5374 | 2.2909 | 0.8092 | 1.0765 | 0.8069 |
| 12 | Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3 | 117 | 1.6904 | 0.0725 | 2.1677 | 0.0122 | 1.5704 | 0.4104 |
| 13 | Nucleoside diphosphate kinase B OS=Homo sapiens GN=NME2 PE=1 SV=1 | 4 | 0.6982 | 0.2269 | 2.0893 | 0.1667 | 1.6596 | 0.0865 |
| 14 | sphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE= | 5 | 2.0701 | 0.0757 | 2.0324 | 0.0251 | 1.6749 | 0.166 |
| 15 | Proteasome activator complex subunit 1 OS=Homo sapiens GN=PSME1 PE=1 SV=1 | 3 | 1.1066 | 0.5914 | 1.9953 | 0.3098 | 0.871 | 0.2667 |
| 16 | Arylsulfatase A OS=Homo sapiens GN=ARSA PE=1 SV=3 | 1 | 0.4786 | 0.3127 | 1.977 | 0.1296 | 0.1675 | 0.0566 |
| 17 | Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2 | 1 | 0.6855 | 0.5218 | 1.9231 | 0.5226 | 0.3908 | 0.5634 |
| 18 | Malate dehydrogenase, cytoplasmic OS=Homo sapiens GN=MDH1 PE=1 SV=4 | 4 | 0.6138 | 0.2282 | 1.9231 | 0.0637 | 0.2051 | 0.0053 |
| 19 | 60S ribosomal protein L10a OS=Homo sapiens GN=RPL10A PE=1 SV=2 | 1 | 0.9908 | 0.8991 | 1.888 | 0.722 | 0.8091 | 0.6955 |
| 20 | rceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV | 28 | 0.7943 | 0.8812 | 1.7219 | 0.3805 | 1.6904 | 0.4119 |
| 21 | Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 | 7 | 0.7447 | 0.4015 | 1.6596 | 0.2331 | 0.8166 | 0.1663 |
| 22 | AP-1 complex subunit beta-1 OS=Homo sapiens GN=AP1B1 PE=1 SV=2 | 1 | 0.9204 | 0.8733 | 1.6144 | 0.5692 | 1.6444 | 0.6052 |
| 23 | Alcohol dehydrogenase [NADP(+)] OS=Homo sapiens GN=AKR1A1 PE=1 SV=3 | 6 | 0.912 | 0.7356 | 1.5704 | 0.2067 | 1.3305 | 0.1391 |
| 24 | rthrocyte band 7 integral membrane protein OS=Homo sapiens GN=STOM PE=1 SV | 1 | 0.787 | 0.6239 | 1.556 | 0.4538 | 0.8318 | 0.4692 |
| 25 | 40S ribosomal protein S2 OS=Homo sapiens GN=RPS2 PE=1 SV=2 | 1 | 0.912 | 0.6103 | 1.5276 | 0.4434 | 0.5012 | 0.4603 |
| 26 | Proteasome subunit beta type-4 OS=Homo sapiens GN=PSMB4 PE=1 SV=4 | 1 | 1.028 | 0.8822 | 1.4997 | 0.4969 | 0.4325 | 0.5234 |
| 27 | Protein S100-A1 OS=Homo sapiens GN=S100A1 PE=1 SV=2 | 1 | 1.1066 | 0.847 | 1.4454 | 0.5277 | 1.3552 | 0.569 |
| 28 | Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4 | 8 | 0.6081 | 0.5363 | 1.3932 | 0.4592 | 0.9462 | 0.4721 |
| 29 | T-complex protein 1 subunit zeta OS=Homo sapiens GN=CCT6A PE=1 SV=3 | 1 | 0.2831 | 0.1924 | 1.3804 | 0.6378 | 0.7798 | 0.6556 |
| 30 | Pyruvate kinase PKM OS=Homo sapiens GN=PKM PE=1 SV=4 | 5 | 0.6081 | 0.8367 | 1.3804 | 0.3392 | 0.5546 | 0.3388 |
| 31 | Aminopeptidase B OS=Homo sapiens GN=RNPEP PE=1 SV=2 | 1 | 1.4588 | 0.5 | 1.3804 | 0.3561 | 0.5248 | 0.351 |
| 32 | -aminoadipic semialdehyde dehydrogenase OS=Homo sapiens GN=ALDH7A1 PE= | 7 | 1.1169 | 0.1148 | 1.3677 | 0.3982 | 1.6596 | 0.416 |
| 33 | Adenine phosphoribosyltransferase OS=Homo sapiens GN=APRT PE=1 SV=2 | 3 | 1.3305 | 0.6539 | 1.3428 | 0.3058 | 1.8365 | 0.2481 |
| 34 | Proteasome subunit beta type-6 OS=Homo sapiens GN=PSMB6 PE=1 SV=4 | 1 | 0.3664 | 0.4355 | 1.3428 | 0.5799 | 1.2823 | 0.6171 |
| 35 | F-actin-capping protein subunit beta OS=Homo sapiens GN=CAPZB PE=1 SV=4 | 1 | 0.8872 | 0.8656 | 1.3428 | 0.3234 | 0.2559 | 0.2804 |
| 36 | T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8 PE=1 SV=4 | 1 | 1.5417 | 0.4613 | 1.3305 | 0.3536 | 0.7516 | 0.3506 |
| 37 | Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2 | 24 | 1.2474 | 0.9471 | 1.2942 | 0.5795 | 0.8091 | 0.614 |
| 38 | pcitrate dehydrogenase [NADP] cytoplasmic OS=Homo sapiens GN=IDH1 PE=1 SV= | 5 | 0.929 | 0.3772 | 1.2823 | 0.7577 | 0.863 | 0.7278 |
| 39 | L-lactate dehydrogenase A chain OS=Homo sapiens GN=LDHA PE=1 SV=2 | 5 | 1.3305 | 0.2618 | 1.2823 | 0.1437 | 0.3192 | 0.0779 |
| 40 | Poly(rC)-binding protein 1 OS=Homo sapiens GN=PCBP1 PE=1 SV=2 | 2 | 1.2942 | 0.4765 | 1.2706 | 0.4402 | 0.9817 | 0.4507 |
| 41 | Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3 | 8 | 1.4723 | 0.1917 | 1.2589 | 0.8777 | 0.7656 | 0.889 |
| 42 | T-complex protein 1 subunit eta OS=Homo sapiens GN=CCT7 PE=1 SV=2 | 2 | 1.1169 | 0.8161 | 1.2474 | 0.5127 | 0.6982 | 0.5366 |
| 43 | Kappa-casein OS=Homo sapiens GN=CSN3 PE=1 SV=3 | 6 | 1.4191 | 0.5308 | 1.2246 | 0.5856 | 0.8472 | 0.6195 |
| 44 | ndoplasmic reticulum resident protein 29 OS=Homo sapiens GN=ERP29 PE=1 SV= | 3 | 1.1588 | 0.2159 | 1.2134 | 0.6892 | 0.8872 | 0.6677 |
| 45 | Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4 | 7 | 0.912 | 0.8364 | 1.1803 | 0.631 | 1.0093 | 0.6346 |
| 46 | WD repeat-containing protein 1 OS=Homo sapiens GN=WDR1 PE=1 SV=4 | 2 | 1.556 | 0.4169 | 1.1803 | 0.3132 | 0.4169 | 0.2706 |
| 47 | latoxin B1 aldehyde reductase member 2 OS=Homo sapiens GN=AKR7A2 PE=1 SV | 1 | 0.955 | 0.7214 | 1.1695 | 0.2764 | 2.7542 | 0.2319 |
| 48 | 10 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPE1 PE=1 SV=2 | 1 | 1.2246 | 0.8282 | 1.1588 | 0.3314 | 1.9409 | 0.3346 |
| 49 | Fructose-1,6-bisphosphatase 1 OS=Homo sapiens GN=FBP1 PE=1 SV=5 | 2 | 0.7379 | 0.5872 | 1.1482 | 0.3788 | 1.0186 | 0.4052 |
| 50 | nembrane emp24 domain-containing protein 9 OS=Homo sapiens GN=TMED9 PE= | 1 | 1.0375 | 0.885 | 1.1482 | 0.4398 | 0.871 | 0.4479 |
| 51 | Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=6 | 1 | 1.1482 | 0.9856 | 1.1376 | 0.8211 | 1.2023 | 0.8216 |
| 52 | Endoplasmin OS=Homo sapiens GN=HSP90B1 PE=1 SV=1 | 14 | 1.0965 | 0.8126 | 1.1376 | 0.6747 | 1.1588 | 0.6613 |
| 53 | G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE | 2 | 0.9462 | 0.4655 | 1.1376 | 0.6088 | 0.9727 | 0.6291 |
| 54 | Peroxiredoxin-5, mitochondrial OS=Homo sapiens GN=PRDX5 PE=1 SV=4 | 3 | 1.0471 | 0.9776 | 1.1272 | 0.8417 | 0.929 | 0.8504 |
| 55 | 40S ribosomal protein S18 OS=Homo sapiens GN=RPS18 PE=1 SV=3 | 3 | 0.929 | 0.8663 | 1.1169 | 0.7626 | 1.0093 | 0.7385 |
| 56 | Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5 | 14 | 0.9376 | 0.8289 | 1.1169 | 0.5933 | 0.7656 | 0.624 |
| 57 | Aldose 1-epimerase OS=Homo sapiens GN=GALM PE=1 SV=1 | 1 | 1.0375 | 0.9289 | 1.1066 | 0.81 | 0.879 | 0.8111 |
| 58 | fatty acid synthase thioesterase, medium chain OS=Homo sapiens GN=OLAH PE= | 1 | 0.9817 | 0.8299 | 1.0965 | 0.5505 | 1.1066 | 0.5921 |
| 59 | osphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV | 12 | 1.2134 | 0.5735 | 1.0965 | 0.8135 | 1.0765 | 0.8162 |
| 60 | Acetyl-CoA acetyltransferase, cytosolic OS=Homo sapiens GN=ACAT2 PE=1 SV=2 | 4 | 1.1482 | 0.7473 | 1.0965 | 0.8083 | 1.0186 | 0.8022 |
| 61 | Alpha-lactalbumin OS=Homo sapiens GN=LALBA PE=1 SV=1 | 3 | 1.1272 | 0.4425 | 1.0965 | 0.6314 | 0.955 | 0.6373 |
| 62 | Proteasome subunit alpha type-6 OS=Homo sapiens GN=PSMA6 PE=1 SV=1 | 1 | 0.9376 | 0.8082 | 1.0965 | 0.8371 | 0.9204 | 0.8381 |
| 63 | utative elongation factor 1-alpha-like 3 OS=Homo sapiens GN=EEF1A1P5 PE=5 SV= | 21 | 0.9376 | 0.8978 | 1.0965 | 0.5333 | 0.3373 | 0.572 |
| 64 | Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2 | 3 | 1.1066 | 0.9363 | 1.0864 | 0.5271 | 2.466 | 0.5689 |
| 65 | Protein disulfide-isomerase A6 OS=Homo sapiens GN=PDIA6 PE=1 SV=1 | 1 | 1.1066 | 0.6183 | 1.0864 | 0.7676 | 1.2942 | 0.751 |
| 66 | terogeneous nuclear ribonucleoprotein Q OS=Homo sapiens GN=SYNCRIP PE=1 SV | 1 | 0.7516 | 0.6782 | 1.0864 | 0.8375 | 0.879 | 0.8408 |
| 67 | Histone H2A type 1-C OS=Homo sapiens GN=HIST1H2AC PE=1 SV=3 | 4 | 0.7586 | 0.9284 | 1.0765 | 0.1936 | 1.7219 | 0.0963 |
| 68 | 40S ribosomal protein S19 OS=Homo sapiens GN=RPS19 PE=1 SV=2 | 2 | 0.871 | 0.9525 | 1.0765 | 0.4884 | 1.2134 | 0.5012 |
| 69 | icotinamide phosphoribosyltransferase OS=Homo sapiens GN=NAMPT PE=1 SV= | 5 | 0.7447 | 0.7249 | 1.0765 | 0.4094 | 1.0471 | 0.424 |
| 70 | de-associated complex subunit alpha, muscle-specific form OS=Homo sapiens GI | 1 | 1.3552 | 0.5726 | 1.0765 | 0.7066 | 1.0471 | 0.6808 |

| 1 | Name | Peptides(95%) | B(115):A(114) | PVal B(115):A(114) | C(116):A(114) | PVal C(116):A(114) | D(117):A(114) | PVal D(117):A(114) |
|---|---|---|---|---|---|---|---|---|
| 71 | Protein disulfide-isomerase OS=Homo sapiens GN=P4HB PE=1 SV=3 | 12 | 0.9817 | 0.9527 | 1.0765 | 0.8678 | 1.0186 | 0.8794 |
| 72 | 60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1 | 2 | 1.0093 | 0.9504 | 1.0765 | 0.8485 | 0.9638 | 0.8524 |
| 73 | Peptidyl-prolyl cis-trans isomerase FKBP4 OS=Homo sapiens GN=FKBP4 PE=1 SV= | 4 | 1.0375 | 0.8173 | 1.0765 | 0.5857 | 0.3837 | 0.6232 |
| 74 | Macrophage-capping protein OS=Homo sapiens GN=CAPG PE=1 SV=2 | 3 | 1.7061 | 0.9397 | 1.0666 | 0.737 | 1.2134 | 0.7186 |
| 75 | Purine nucleoside phosphorylase OS=Homo sapiens GN=PNP PE=1 SV=2 | 1 | 1.6144 | 0.4621 | 1.0666 | 0.7686 | 0.9204 | 0.7769 |
| 76 | Tumor protein D52 OS=Homo sapiens GN=TPD52 PE=1 SV=2 | 3 | 1.2134 | 0.5775 | 1.0568 | 0.8999 | 1.0568 | 0.9064 |
| 77 | Peroxiredoxin-6 OS=Homo sapiens GN=PRDX6 PE=1 SV=3 | 1 | 1 | 0.8187 | 1.0568 | 0.8676 | 1.028 | 0.8735 |
| 78 | Acyl-protein thioesterase 1 OS=Homo sapiens GN=LYPLA1 PE=1 SV=1 | 2 | 0.3221 | 0.2136 | 1.0568 | 0.6072 | 0.7943 | 0.6251 |
| 79 | Malate dehydrogenase, mitochondrial OS=Homo sapiens GN=MDH2 PE=1 SV=3 | 2 | 1.2474 | 0.3782 | 1.0568 | 0.2273 | 0.5598 | 0.1562 |
| 80 | L-xylulose reductase OS=Homo sapiens GN=DCXR PE=1 SV=2 | 5 | 1.1169 | 0.8705 | 1.0471 | 0.8262 | 0.9376 | 0.8342 |
| 81 | Lactadherin OS=Homo sapiens GN=MFGE8 PE=1 SV=2 | 5 | 1.2942 | 0.1627 | 1.0375 | 0.7087 | 1.0666 | 0.684 |
| 82 | Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 | 31 | 0.6668 | 0.4673 | 1.0375 | 0.9564 | 1.0666 | 0.9462 |
| 83 | Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2 | 6 | 0.9817 | 0.8249 | 1.0375 | 0.7636 | 1.0568 | 0.7451 |
| 84 | Protein canopy homolog 2 OS=Homo sapiens GN=CNPY2 PE=1 SV=1 | 2 | 0.7447 | 0.5443 | 1.0375 | 0.7557 | 0.9727 | 0.7258 |
| 85 | nter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens GN=ITIH1 PE=1 SV= | 2 | 0.6855 | 0.5204 | 1.0375 | 0.5088 | 0.9638 | 0.5365 |
| 86 | ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=1 SV=3 | 4 | 0.5808 | 0.4277 | 1.0375 | 0.7555 | 0.9036 | 0.7249 |
| 87 | 14-3-3 protein epsilon OS=Homo sapiens GN=YWHAE PE=1 SV=1 | 5 | 1.0471 | 0.7771 | 1.0375 | 0.3285 | 0.5445 | 0.3033 |
| 88 | 40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=1 SV=2 | 1 | 0.912 | 0.7337 | 1.028 | 0.3011 | 1.5417 | 0.2449 |
| 89 | Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1 | 1 | 1.1066 | 0.8063 | 1.028 | 0.6511 | 1.1272 | 0.6558 |
| 90 | Chloride intracellular channel protein 1 OS=Homo sapiens GN=CLIC1 PE=1 SV=4 | 4 | 1.0864 | 0.6169 | 1.028 | 0.9548 | 0.955 | 0.936 |
| 91 | biquitin-conjugating enzyme E2 variant 2 OS=Homo sapiens GN=UBE2V2 PE=1 SV= | 3 | 1.028 | 0.967 | 1.028 | 0.9895 | 0.9376 | 0.9781 |
| 92 | Myeloid-derived growth factor OS=Homo sapiens GN=MYDGF PE=1 SV=1 | 1 | 0.1959 | 0.7163 | 1.0186 | 0.5498 | 3.1915 | 0.5894 |
| 93 | 60S acidic ribosomal protein P1 OS=Homo sapiens GN=RPLP1 PE=1 SV=1 | 2 | 1.028 | 0.2869 | 1.0186 | 0.7602 | 1.1803 | 0.7342 |
| 94 | Cytoskeleton-associated protein 4 OS=Homo sapiens GN=CKAP4 PE=1 SV=2 | 1 | 0.8472 | 0.7516 | 1.0093 | 0.4411 | 1.0375 | 0.4587 |
| 95 | Alpha-S1-casein OS=Homo sapiens GN=CSN1S1 PE=1 SV=1 | 9 | 0.6855 | 0.3431 | 1.0093 | 0.9592 | 1.028 | 0.9529 |
| 96 | 60S ribosomal protein L38 OS=Homo sapiens GN=RPL38 PE=1 SV=2 | 1 | 0.7379 | 0.5492 | 1.0093 | 0.888 | 0.9908 | 0.8907 |
| 97 | roteasome non-ATPase regulatory subunit 1 OS=Homo sapiens GN=PSMD1 PE=1 | 2 | 1.0666 | 0.937 | 1.0093 | 0.9897 | 0.5916 | 0.9838 |
| 98 | D-3-phosphoglycerate dehydrogenase OS=Homo sapiens GN=PHGDH PE=1 SV=4 | 2 | 1.6444 | 0.4026 | 1 | 0.4316 | 1.5849 | 0.4409 |
| 99 | Beta-casein OS=Homo sapiens GN=CSN2 PE=1 SV=4 | 36 | 1.2942 | 0.8719 | 1 | 0.5208 | 1.2589 | 0.5519 |
| 100 | Carbonyl reductase [NADPH] 1 OS=Homo sapiens GN=CBR1 PE=1 SV=3 | 2 | 0.7727 | 0.639 | 1 | 0.9201 | 1.0568 | 0.9296 |
| 101 | Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3 | 5 | 0.871 | 0.4732 | 1 | 0.9204 | 0.9462 | 0.93 |
| 102 | Clusterin OS=Homo sapiens GN=CLU PE=1 SV=1 | 3 | 0.912 | 0.7786 | 1 | 0.6876 | 0.7943 | 0.6669 |
| 103 | 60S ribosomal protein L6 OS=Homo sapiens GN=RPL6 PE=1 SV=3 | 1 | 1.2706 | 0.1964 | 1 | 0.1467 | 0.1854 | 0.0784 |
| 104 | uclease-sensitive element-binding protein 1 OS=Homo sapiens GN=YBX1 PE=1 SV | 1 | 1.1482 | 0.6824 | 0.9908 | 0.8721 | 1.7865 | 0.8854 |
| 105 | Glucosidase 2 subunit beta OS=Homo sapiens GN=PRKCSH PE=1 SV=2 | 1 | 0.9036 | 0.7234 | 0.9908 | 0.9243 | 0.9817 | 0.9325 |
| 106 | Farnesyl pyrophosphate synthase OS=Homo sapiens GN=FDPS PE=1 SV=4 | 1 | 0.787 | 0.5677 | 0.9908 | 0.6327 | 0.7727 | 0.6386 |
| 107 | 14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1 | 7 | 0.879 | 0.9003 | 0.9817 | 0.8189 | 1.4723 | 0.8209 |
| 108 | Trypsin-1 OS=Homo sapiens GN=PRSS1 PE=1 SV=1 | 5 | 1.2359 | 0.6857 | 0.9817 | 0.9765 | 1.0375 | 0.9618 |
| 109 | Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 | 39 | 0.5861 | 0.032 | 0.9817 | 0.8701 | 0.2858 | 0.0017 |
| 110 | 40S ribosomal protein S13 OS=Homo sapiens GN=RPS13 PE=1 SV=2 | 3 | 1.0471 | 0.8258 | 0.9727 | 0.9738 | 1.1066 | 0.9592 |
| 111 | Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2 | 5 | 0.492 | 0.197 | 0.9727 | 0.8639 | 1.0666 | 0.8717 |
| 112 | Proteasome subunit alpha type-4 OS=Homo sapiens GN=PSMA4 PE=1 SV=1 | 2 | 1.0186 | 0.8248 | 0.9727 | 0.861 | 1.028 | 0.8568 |
| 113 | CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1 | 1 | 2.2491 | 0.4752 | 0.9727 | 0.8167 | 1.0093 | 0.8167 |
| 114 | otein transport protein Sec61 subunit beta OS=Homo sapiens GN=SEC61B PE=1 SV | 1 | 0.8472 | 0.6957 | 0.9727 | 0.9893 | 1.0093 | 0.971 |
| 115 | Adenosylhomocysteinase OS=Homo sapiens GN=AHCY PE=1 SV=4 | 3 | 0.879 | 0.6264 | 0.9727 | 0.8972 | 0.9908 | 0.9008 |
| 116 | Cytosol aminopeptidase OS=Homo sapiens GN=LAP3 PE=1 SV=3 | 7 | 1.0568 | 0.4049 | 0.9727 | 0.6952 | 0.8954 | 0.6718 |
| 117 | Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=5 | 5 | 0.8551 | 0.6216 | 0.955 | 0.4279 | 1.2474 | 0.4371 |
| 118 | P--glucose-1-phosphate uridylyltransferase OS=Homo sapiens GN=UGP2 PE=1 SV | 21 | 0.2089 | 0.0014 | 0.955 | 0.9301 | 1.1588 | 0.2508 |
| 119 | Calreticulin OS=Homo sapiens GN=CALR PE=1 SV=1 | 5 | 0.8166 | 0.4666 | 0.955 | 0.7848 | 1.0186 | 0.7934 |
| 120 | 60S ribosomal protein L22 OS=Homo sapiens GN=RPL22 PE=1 SV=2 | 2 | 1.0471 | 0.7551 | 0.955 | 0.7226 | 0.9638 | 0.7094 |
| 121 | Destrin OS=Homo sapiens GN=DSTN PE=1 SV=3 | 3 | 0.673 | 0.5034 | 0.9376 | 0.7677 | 0.9817 | 0.7707 |
| 122 | 60S ribosomal protein L12 OS=Homo sapiens GN=RPL12 PE=1 SV=1 | 2 | 1.1272 | 0.4996 | 0.9376 | 0.7372 | 0.9638 | 0.7207 |
| 123 | uperoxide dismutase [Mn], mitochondrial OS=Homo sapiens GN=SOD2 PE=1 SV= | 1 | 0.7727 | 0.6421 | 0.9376 | 0.8692 | 0.929 | 0.8815 |
| 124 | Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2 | 2 | 0.7798 | 0.2553 | 0.929 | 0.7197 | 1.1376 | 0.6954 |
| 125 | ldo-keto reductase family 1 member C1 OS=Homo sapiens GN=AKR1C1 PE=1 SV= | 1 | 0.3631 | 0.4546 | 0.929 | 0.5678 | 1.0186 | 0.6035 |
| 126 | PRA1 family protein 3 OS=Homo sapiens GN=ARL6IP5 PE=1 SV=1 | 3 | 1.3062 | 0.5212 | 0.929 | 0.8938 | 0.9817 | 0.9004 |
| 127 | Transketolase OS=Homo sapiens GN=TKT PE=1 SV=3 | 5 | 0.9204 | 0.3171 | 0.929 | 0.3147 | 0.8017 | 0.2714 |
| 128 | e-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE= | 3 | 1.0093 | 0.9441 | 0.9204 | 0.9073 | 0.7447 | 0.9065 |
| 129 | Macrophage migration inhibitory factor OS=Homo sapiens GN=MIF PE=1 SV=4 | 4 | 0.52 | 0.4591 | 0.912 | 0.2755 | 1.9953 | 0.2291 |
| 130 | Histone H2B type 1-L OS=Homo sapiens GN=HIST1H2BL PE=1 SV=3 | 2 | 1.3428 | 0.4637 | 0.912 | 0.8596 | 1.3932 | 0.8553 |
| 131 | Nicotinate phosphoribosyltransferase OS=Homo sapiens GN=NAPRT PE=1 SV=2 | 2 | 0.9204 | 0.5776 | 0.912 | 0.8693 | 1.028 | 0.8827 |
| 132 | Transcription factor BTF3 OS=Homo sapiens GN=BTF3 PE=1 SV=1 | 1 | 0.5702 | 0.2558 | 0.9036 | 0.2718 | 1.6596 | 0.1966 |
| 133 | Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2 | 2 | 0.1138 | 0.329 | 0.9036 | 0.5204 | 0.631 | 0.5388 |
| 134 | Phosphoglucomutase-1 OS=Homo sapiens GN=PGM1 PE=1 SV=3 | 8 | 0.5916 | 0.6627 | 0.8954 | 0.8398 | 0.8872 | 0.8431 |
| 135 | Protein disulfide-isomerase A4 OS=Homo sapiens GN=PDIA4 PE=1 SV=2 | 6 | 0.6194 | 0.6007 | 0.8954 | 0.7672 | 0.7943 | 0.7459 |
| 136 | Small ubiquitin-related modifier 4 OS=Homo sapiens GN=SUMO4 PE=1 SV=2 | 1 | 0.6918 | 0.8484 | 0.8872 | 0.73 | 1.1482 | 0.7172 |
| 137 | Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2 | 3 | 1.1588 | 0.5552 | 0.8872 | 0.9316 | 1.028 | 0.933 |
| 138 | Rab GDP dissociation inhibitor beta OS=Homo sapiens GN=GDI2 PE=1 SV=2 | 2 | 0.4656 | 0.6536 | 0.8872 | 0.8243 | 0.9204 | 0.8287 |
| 139 | Cathepsin D OS=Homo sapiens GN=CTSD PE=1 SV=1 | 2 | 0.929 | 0.9916 | 0.8872 | 0.3985 | 0.4406 | 0.4163 |
| 140 | Butyrophilin subfamily 1 member A1 OS=Homo sapiens GN=BTN1A1 PE=1 SV=3 | 3 | 1.6293 | 0.3383 | 0.879 | 0.7745 | 1.1588 | 0.7881 |
| 141 | ethylaminobutyraldehyde dehydrogenase OS=Homo sapiens GN=ALDH9A1 PE=1 | 12 | 0.9204 | 0.8812 | 0.879 | 0.7633 | 1.0186 | 0.7437 |
| 142 | 40S ribosomal protein S25 OS=Homo sapiens GN=RPS25 PE=1 SV=1 | 3 | 0.9036 | 0.758 | 0.871 | 0.7269 | 1.1376 | 0.7115 |

| 1 | Name | Peptides(95%) | B(115):A(114) | PVal B(115):A(114) | C(116):A(114) | PVal C(116):A(114) | D(117):A(114) | PVal D(117):A(114) |
|---|---|---|---|---|---|---|---|---|
| 143 | Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1 | 20 | 0.4093 | 0.1836 | 0.871 | 0.9607 | 1.0965 | 0.9588 |
| 144 | Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1 | 5 | 0.5598 | 0.3598 | 0.863 | 0.5764 | 1.1482 | 0.6129 |
| 145 | 40S ribosomal protein SA OS=Homo sapiens GN=RPSA PE=1 SV=4 | 7 | 0.8954 | 0.1468 | 0.8551 | 0.6106 | 1.2823 | 0.6339 |
| 146 | Dihydropyrimidinase-related protein 3 OS=Homo sapiens GN=DPYSL3 PE=1 SV=1 | 7 | 0.863 | 0.405 | 0.8551 | 0.7681 | 0.8551 | 0.7718 |
| 147 | Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1 | 9 | 1.4454 | 0.4156 | 0.8472 | 0.8568 | 1.0471 | 0.8548 |
| 148 | Proteasome subunit alpha type-1 OS=Homo sapiens GN=PSMA1 PE=1 SV=1 | 2 | 1.6444 | 0.1717 | 0.8472 | 0.6215 | 1.0186 | 0.6344 |
| 149 | 60S ribosomal protein L27a OS=Homo sapiens GN=RPL27A PE=1 SV=2 | 1 | 1.6904 | 0.3641 | 0.8318 | 0.8125 | 1 | 0.8121 |
| 150 | Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 | 6 | 1.556 | 0.3513 | 0.8318 | 0.4525 | 0.7047 | 0.4691 |
| 151 | Glyoxalase domain-containing protein 4 OS=Homo sapiens GN=GLOD4 PE=1 SV=1 | 2 | 1.0186 | 0.6375 | 0.8241 | 0.2041 | 2.1281 | 0.1166 |
| 152 | Ribosome-binding protein 1 OS=Homo sapiens GN=RRBP1 PE=1 SV=4 | 5 | 0.9638 | 0.3385 | 0.8166 | 0.547 | 1.0965 | 0.5844 |
| 153 | Proteasome subunit beta type-2 OS=Homo sapiens GN=PSMB2 PE=1 SV=1 | 3 | 0.8241 | 0.4252 | 0.8091 | 0.6983 | 0.5058 | 0.6794 |
| 154 | Omega-amidase NIT2 OS=Homo sapiens GN=NIT2 PE=1 SV=1 | 2 | 0.9376 | 0.9135 | 0.7943 | 0.46 | 1.2706 | 0.4781 |
| 155 | ukaryotic translation initiation factor 5A-2 OS=Homo sapiens GN=EIF5A2 PE=1 SV= | 1 | 0.6918 | 0.4876 | 0.787 | 0.4868 | 0.955 | 0.4943 |
| 156 | 60S acidic ribosomal protein P0 OS=Homo sapiens GN=RPLP0 PE=1 SV=1 | 4 | 1.0864 | 0.8324 | 0.7798 | 0.913 | 1.0864 | 0.9143 |
| 157 | Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3 PE=1 SV=2 | 2 | 1.1588 | 0.7594 | 0.7516 | 0.4511 | 0.8091 | 0.4666 |
| 158 | Xanthine dehydrogenase/oxidase OS=Homo sapiens GN=XDH PE=1 SV=4 | 30 | 0.2655 | 0 | 0.7516 | 0.1232 | 0.2355 | 0 |
| 159 | Immunoglobulin kappa light chain OS=Homo sapiens PE=1 SV=1 | 1 | 0.7244 | 0.6052 | 0.7447 | 0.5611 | 0.7447 | 0.6032 |
| 160 | Triosephosphate isomerase OS=Homo sapiens GN=TPI1 PE=1 SV=3 | 12 | 0.955 | 0.7754 | 0.7244 | 0.5197 | 0.4055 | 0.5378 |
| 161 | Myotrophin OS=Homo sapiens GN=MTPN PE=1 SV=2 | 1 | 0.912 | 0.3057 | 0.7112 | 0.4102 | 0.7656 | 0.4347 |
| 162 | 78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 | 20 | 0.6546 | 0.1339 | 0.7047 | 0.3563 | 2.9376 | 0.3601 |
| 163 | Peptidyl-prolyl cis-trans isomerase B OS=Homo sapiens GN=PPIB PE=1 SV=2 | 4 | 0.9817 | 0.7748 | 0.7047 | 0.5419 | 0.7311 | 0.5757 |
| 164 | Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1 | 10 | 1.0568 | 0.7278 | 0.6982 | 0.5322 | 1.0864 | 0.5697 |
| 165 | ptic vesicle membrane protein VAT-1 homolog OS=Homo sapiens GN=VAT1 PE=1 | 1 | 0.3664 | 0.4228 | 0.6982 | 0.3314 | 0.5248 | 0.3294 |
| 166 | Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2 | 4 | 0.2443 | 0.2706 | 0.673 | 0.5022 | 0.6982 | 0.5325 |
| 167 | 60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A PE=1 SV=2 | 1 | 0.4875 | 0.3536 | 0.6668 | 0.4956 | 0.7379 | 0.5205 |
| 168 | Immunoglobulin heavy constant alpha 1 OS=Homo sapiens GN=IGHA1 PE=1 SV=2 | 3 | 0.6427 | 0.3376 | 0.6546 | 0.6341 | 0.8017 | 0.646 |
| 169 | Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2 | 6 | 1.0186 | 0.9003 | 0.631 | 0.4481 | 1.3183 | 0.4645 |
| 170 | eptidyl-prolyl cis-trans isomerase FKBP1A OS=Homo sapiens GN=FKBP1A PE=1 SV | 1 | 1.3804 | 0.5553 | 0.6194 | 0.4451 | 0.7727 | 0.4632 |
| 171 | Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 | 14 | 0.597 | 0.0051 | 0.6194 | 0.0098 | 0.0847 | 0 |
| 172 | Putative 40S ribosomal protein S10-like OS=Homo sapiens GN=RPS10P5 PE=5 SV= | 1 | 0.7178 | 0.3769 | 0.6138 | 0.4355 | 0.9817 | 0.4446 |
| 173 | nnose-1-phosphate guanyltransferase beta OS=Homo sapiens GN=GMPPB PE=1 S | 3 | 0.7656 | 0.222 | 0.6081 | 0.4941 | 1.2706 | 0.5012 |
| 174 | LDLR chaperone MESD OS=Homo sapiens GN=MESDC2 PE=1 SV=2 | 2 | 1.4588 | 0.5032 | 0.5861 | 0.3673 | 1.4588 | 0.4025 |
| 175 | Protein disulfide-isomerase A3 OS=Homo sapiens GN=PDIA3 PE=1 SV=4 | 12 | 1.0186 | 0.8353 | 0.5754 | 0.7796 | 0.9204 | 0.7912 |
| 176 | Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4 | 8 | 1.2134 | 0.93 | 0.5649 | 0.3941 | 1.9409 | 0.4157 |
| 177 | Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2 | 8 | 1.4191 | 0.7174 | 0.5598 | 0.3859 | 1.0666 | 0.4155 |
| 178 | 6-phosphogluconolactonase OS=Homo sapiens GN=PGLS PE=1 SV=2 | 1 | 1.3062 | 0.6112 | 0.5546 | 0.323 | 2.6546 | 0.2771 |
| 179 | Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1 | 2 | 3.6308 | 0.092 | 0.5546 | 0.0258 | 0.3342 | 0.0049 |
| 180 | as GTPase-activating-like protein IQGAP1 OS=Homo sapiens GN=IQGAP1 PE=1 SV= | 1 | 0.8954 | 0.3207 | 0.5395 | 0.3631 | 0.5649 | 0.3862 |
| 181 | Annexin A4 OS=Homo sapiens GN=ANXA4 PE=1 SV=4 | 2 | 1.2474 | 0.5133 | 0.5105 | 0.6324 | 1.0765 | 0.6379 |
| 182 | Programmed cell death protein 4 OS=Homo sapiens GN=PDCD4 PE=1 SV=2 | 1 | 0.6918 | 0.5244 | 0.5058 | 0.3308 | 0.7727 | 0.3057 |
| 183 | Annexin A3 OS=Homo sapiens GN=ANXA3 PE=1 SV=3 | 3 | 0.7244 | 0.3704 | 0.4831 | 0.3193 | 2.2284 | 0.2747 |
| 184 | Proteasome subunit alpha type-5 OS=Homo sapiens GN=PSMA5 PE=1 SV=3 | 3 | 1.0375 | 0.6145 | 0.4656 | 0.5546 | 0.7379 | 0.5927 |
| 185 | Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2 | 3 | 0.138 | 0.0482 | 0.4571 | 0.1198 | 1.2942 | 0.522 |
| 186 | Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2 | 6 | 1.0186 | 0.7373 | 0.4406 | 0.1577 | 0.5297 | 0.0837 |
| 187 | LIM and SH3 domain protein 1 OS=Homo sapiens GN=LASP1 PE=1 SV=2 | 2 | 0.9462 | 0.7796 | 0.4325 | 0.1856 | 0.6252 | 0.0899 |
| 188 | Cytosolic non-specific dipeptidase OS=Homo sapiens GN=CNDP2 PE=1 SV=2 | 12 | 0.0871 | 0.006 | 0.3251 | 0.0681 | 0.0625 | 0.0032 |
| 189 | Perilipin-3 OS=Homo sapiens GN=PLIN3 PE=1 SV=3 | 12 | 0.4529 | 0.2279 | 0.3105 | 0.0619 | 5.7016 | 0.0023 |
| 190 | 14-3-3 protein beta/alpha OS=Homo sapiens GN=YWHAB PE=1 SV=3 | 6 | 1.1803 | 0.5137 | 0.2992 | 0.2008 | 0.3436 | 0.0987 |
| 191 | 60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=5 | 1 | 1.2246 | 0.9844 | 0.278 | 0.4189 | 0.6138 | 0.4348 |
| 192 | 60S ribosomal protein L8 OS=Homo sapiens GN=RPL8 PE=1 SV=2 | 1 | 0.871 | 0.8988 | 0.2333 | 0.1386 | 1.4859 | 0.0679 |
| 193 | L-lactate dehydrogenase B chain OS=Homo sapiens GN=LDHB PE=1 SV=2 | 11 | 1.2823 | 0.2265 | 0.2291 | 0.0668 | 0.1786 | 0.0338 |
| 194 | Protein DJ-1 OS=Homo sapiens GN=PARK7 PE=1 SV=2 | 3 | 0.5702 | 0.2881 | 0.1786 | 0.4778 | 1.0093 | 0.4784 |
| 195 | Inorganic pyrophosphatase OS=Homo sapiens GN=PPA1 PE=1 SV=2 | 1 | 1.0093 | 0.9865 | 0.1169 | 0.2205 | 1.7539 | 0.1509 |
| 196 | Tumor protein D54 OS=Homo sapiens GN=TPD52L2 PE=1 SV=2 | 2 | 1.3677 | 0.7912 | 0.0879 | 0.2358 | 0.631 | 0.1701 |
| 197 | Perilipin-2 OS=Homo sapiens GN=PLIN2 PE=1 SV=2 | 3 | | | | | | |
| 198 | rin repeat domain-containing protein 34A OS=Homo sapiens GN=ANKRD34A PE=2 | 1 | | | | | | |
| 199 | ike phospholipase domain-containing protein 2 OS=Homo sapiens GN=PNPLA2 P | 1 | | | | | | |
| 200 | Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1 | 1 | | | | | | |
| 201 | Peroxiredoxin-4 OS=Homo sapiens GN=PRDX4 PE=1 SV=1 | 2 | | | | | | |
| 202 | Glutathione S-transferase omega-1 OS=Homo sapiens GN=GSTO1 PE=1 SV=2 | 1 | | | | | | |
| 203 | Talin-1 OS=Homo sapiens GN=TLN1 PE=1 SV=3 | 2 | | | | | | |
| 204 | Elongation factor 1-beta OS=Homo sapiens GN=EEF1B2 PE=1 SV=3 | 2 | | | | | | |