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Are active efflux transporters contributing to infant drug exposure via breastmilk? A longitudinal study

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Abstract

Although most drugs are considered safe and compatible with breastfeeding, cases of toxic drug exposure have been reported. Active efflux transporters have been implicated as a mechanism in the transfer of drugs from mother to baby via breastmilk. Using breastmilk as a source of human mammary epithelial cells, this novel longitudinal study investigated the expression of four active transporters, namely, MDR1, MRP1, MRP2 and BCRP in the lactating human breast. BCRP gene was found to be strongly overexpressed with levels peaking at 5 months postpartum, potentially indicating a time where a breastfed infant may be at risk of inadvertent exposure to BCRP substrates. Serum albumin, a major component of human breastmilk was increasingly downregulated as lactation progresses. Xanthine oxidase/dehydrogenase, an enzyme in breastmilk attributed to a reduced risk of gastroenteritis caused by *Escherichia coli* and *Salmonella enteritides*, was downregulated. Lysozyme and fatty acid synthase are progressively upregulated. This study also shows that breastmilk-derived epithelial cells, when propagated in culture, exhibit characteristics significantly different to those derived directly from breastmilk. This serves to warn that in vitro studies are not a true representation of in vivo processes in the lactating breast; hence, application of in vitro data should be conducted with caution.

KEYWORDS
active transporters, breastmilk, efflux transporters, infant drug exposuremammary gland

1 | INTRODUCTION

Breastfeeding mothers and health professionals often hold concerns about inadvertently exposing breastfed infants to xenobiotics through breastmilk. Although most drugs are considered safe and compatible with breastfeeding, cases of toxic drug exposure have been reported.[1–5] More importantly, there is often a lack of clear and conclusive information about the safety of medicines in lactation resulting in the unnecessary discontinuation of breastfeeding or suboptimal treatment of maternal medical conditions with less effective alternatives.[2] Drug properties and pharmacokinetic parameters such as drug lipophilicity, protein binding and *p*Ka are used to determine milk to plasma ratio (M: P), a measurement of ratios of the areas under the curves of milk and plasma drug concentrations.[6] As most drugs enter breastmilk via passive diffusion, M:P ratio provides a

reliable indication of drug transferability into breastmilk. However, there are substances such as lead, amisulpride, nitrofurantoin, aciclovir and cimetidine where observed M:P has been significantly higher than the predicted M:P based on standard pharmacokinetic calculations.[7–17] This has been attributed to active transport mechanisms whereby transport proteins such as the ATP binding cassette (ABC) transporters on the membrane of mammary epithelial cells as demonstrated in Figure 1, actively pump these drugs into breastmilk.[8,18,19]

Transporters are cell surface proteins that allow endogenous molecules and xenobiotics to enter and exit cells via carrier mediated mechanisms.[20] Active transporters utilize the energy generated from ATP hydrolysis to move molecules across a cell membrane.[21] The expression of transport proteins such as those belonging to the ABC and SLC (Solute Carrier) superfamilies of transporters has been shown to vary greatly between lactating and non-lactating tissues in humans.[22] Animal studies have also shown and confirmed the distinct variation in the expression of these transporters between the lactating and non-lactating mammary gland of animals. The influence of lactation stage leading to an increase in their expression with the increasing duration of lactation and consequently influencing the amount of the drug or toxin that is excreted in the milk has also been demonstrated.[23–25]

Due to the potential negative human health impact that accumulation of veterinary drugs in milk, a human food source, efflux transporters have been widely studied in the dairy industry.[11,26,27] However, little is known about their expression pattern in the lactating human mammary gland where vulnerable breastfed infants can be inadvertently exposed to potentially toxic levels of medicines taken by the mother. 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, accidentally putting a breastfed infant at risk of toxicity.

This longitudinal study sought to investigate the expression profile of four active transporters in the human mammary gland that are implicated in drug disposition to ascertain if these could impact the transfer of their xenobiotic, drug and toxin substrates into breastmilk. The studied transporters were MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2) and BCRP (ABCG2). Breastmilk was used as a source of epithelial cells which are sloughed as milk is removed from the breast by the suckling infant or a breast pump. Breastmilk-derived cells were also propagated in culture medium to investigate if they retained their expression characteristics when cultured. This was done to determine if human breastmilk can be used as a non-invasive, reliable, personalized and cost-effective model to predict the transferability of actively transported drugs into breastmilk.

2 | MATERIALS AND METHODS

This study was approved by the Human Research Ethics Committee of Curtin University (HR110/2012). The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[28] Healthy breastfeeding women not on any prescribed medicines (other than prenatal vitamins and supplements) who intended to breastfeed for 12 months were recruited during pregnancy or early

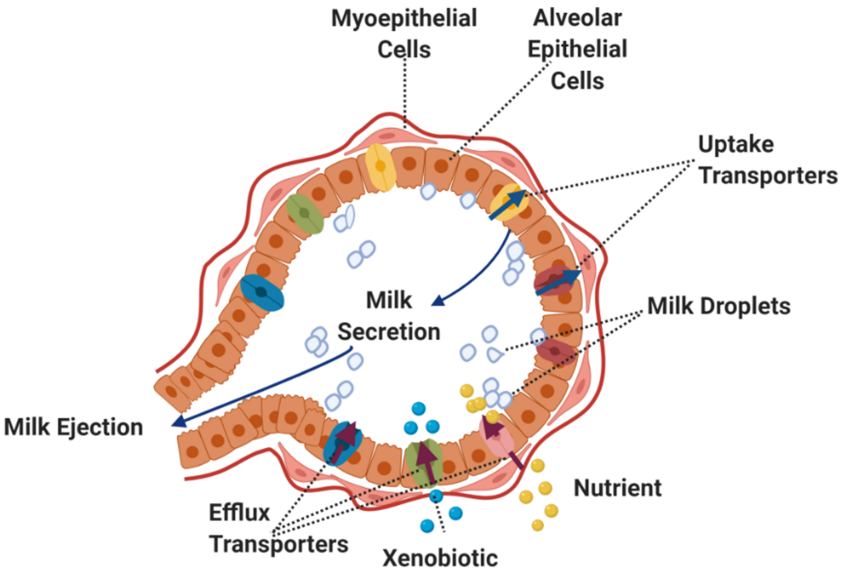


FIGURE 1 Schematic representation of active efflux transporters in the lactating mammary gland. In the lactating mammary gland active transporters are located in the basolateral and apical membranes of alveolar epithelial cells. These transporters influence the composition of breastmilk by concentrating vitamins, nutrients, xenobiotics, drugs and pesticides into milk by pushing these substances against their concentration gradient. (Figure created with Biorender.com)

postpartum through word of mouth and advertising through lactation consultants at two private maternity hospitals in Western Australia (St John of God Hospital Subiaco and St John of God Hospital Murdoch). Participants were excluded from the study if they or their newborn had developed medical conditions requiring long-term pharmacological treatment or if they had inadequate milk supply. Through this recruitment process, 22 healthy pregnant women were successfully enrolled in the study. All participants provided written informed consent and breastfed their infant for a minimum of 6 months. Participants with fewer than three donated samples were excluded from the longitudinal study.

2.1 | Breastmilk sample collection

Participants expressed breastmilk samples using a sterilized electric breast pump (Pigeon®). Samples were protected from light and transported to the laboratory at room temperature immediately after being expressed. Breastmilk samples were collected at five timepoints (T1–T5) representing increasing months post-partum at 1 (T1), 3 (T2), 5 (T3), 9 (T4) and 12 (T5) months. 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. Factors that may influence gene expression and/or breastmilk composition which include fore *versus* hind milk, maternal drug/alcohol use, maternal and infant infection, differences in pumps used (or manual expression), maternal diet and maternal general health were controlled.[29] 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.[30,31] Participants were provided with a Pigeon® electric breast pump to use the pump when expressing breastmilk for this study. Mothers were instructed to express milk after feeding the baby and at approximately 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, in which case collection dates were postponed until the dyad had fully recovered.

2.2 | 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 min 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 min and resuspended in PBS. Cell numbers and viability were 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.[31–33]

2.3 | Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with the mini-RNeasy extraction kit (Cat No. 74104; Qiagen, Valencia, CA, USA) following manufacturer's directions. Total RNA was reverse transcribed using the high-capacity cDNA kit (Applied Biosystems, Carlsbad, CA, USA) following manufacturer's directions. 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).] (Taqman®; Applied Biosystems) with the 7500 FAST RT-PCR system (supporting information Appendix A). Each sample was measured in triplicate or in a few cases in duplicate when the extracted RNA was inadequate. Genes were standardized to MCF10A (a normal human mammary epithelial cell [HMEC] line), and each sample was controlled with the housekeeping gene glyceraldehyde 3-phosphate 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.

2.4 | Protein quantitation by iTRAQ (isobaric tags for relative and absolute quantitation)

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. Timepoint 2 (T2) (third month post-partum) was deemed to be the most appropriate timepoint for exclusion from this analysis due to being close to two other timepoints (1 month post-partum and 5 months post-partum).

iTRAQ analysis was undertaken as described by Casey.[34] The sample with the least amount of protein (19.5 μg) was used as the standard mass for the eight samples at each timepoint. The eight samples were combined for each of the four timepoints, and the resultant four protein samples were analysed for protein concentration using the Direct Detect infrared method [Merck Millipore]. About 100 μg of each of the four samples was 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 ionization 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). About 4 μg of the pooled sample was loaded on the mass spectrometer. Spectral data were analysed against *Homo sapiens* peptide database using the SwissProt® database, facilitated by ProteinPilot™ 5.0 software.

2.5 | Cell culture

Breastmilk isolates were obtained from a total of seven participants ($n = 7$) who were at various stages of breastfeeding ranging from 1 week postpartum to 97 weeks. All milk samples were mature milk and contained both fore and hind milk. Breastmilk cells were isolated as described above in a sterile environment using RPMI medium instead of PBS for washing. Cells were divided into two parts, with one part stored at −80°C for RNA extraction and the remaining half were seeded in culture using modified HuMEC® Ready Medium (ThermoFisher Scientific). HuMEC® Ready Medium (ThermoFisher Scientific) is marketed by the manufacturing company as an optimized medium specifically for the growth of HMEC lines. To suit primary culture of HMEC, modification of this medium involved the addition of heat inactivated foetal bovine serum (15%) and antimicrobials (penicillin/streptomycin and amphotericin B) to prevent microbial contamination. In optimization studies with this medium, it was found that milk-derived primary HMEC were cultured successfully irrespective of the stage of lactation, reaching confluence at around day 22 (median 22.8 days: range 21–26 days). 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 with literature reports of between 7 and 50 days.[35]

The cells were prepared for harvest when the well/flask 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 5 min 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 5 min 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.

3 | RESULTS

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 1.

3.1 | *q*RT-PCR

Gene expression was determined by relative quantitation (Relative Quotient, RQ) compared with the control

TABLE 1 Demographic and breastmilk sample ($N = 88$) 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

MCF10A. PCR showed that the gene expression for BCRP was higher compared with the other three transporters as shown in Figure 2. The data for MRP1 and MRP2 showed a great degree of variation and consequently comprised a significant number of outliers.

BCRP was the most abundantly expressed transporter and thought to have the largest significance in transfer of xenobiotics from maternal plasma to breastmilk.[8] BCRP showed a statistically significant difference in expression over the five timepoints with peak levels occurring at T3 as demonstrated in Figure 3. As expected, interindividual differences were also significant.

3.2 | Breastmilk proteome

iTRAQ proteomic analysis detected 143 proteins (≥2 peptides with >95% CI), 17 of which were differentially expressed (Figures 4 and 5). The proteins that were differentially expressed vary in function with majority being enzymes and transport proteins (Table 2). Active transporter proteins were not detected by the iTRAQ analysis.

3.3 | Cell culture

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 6A, 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 6B.

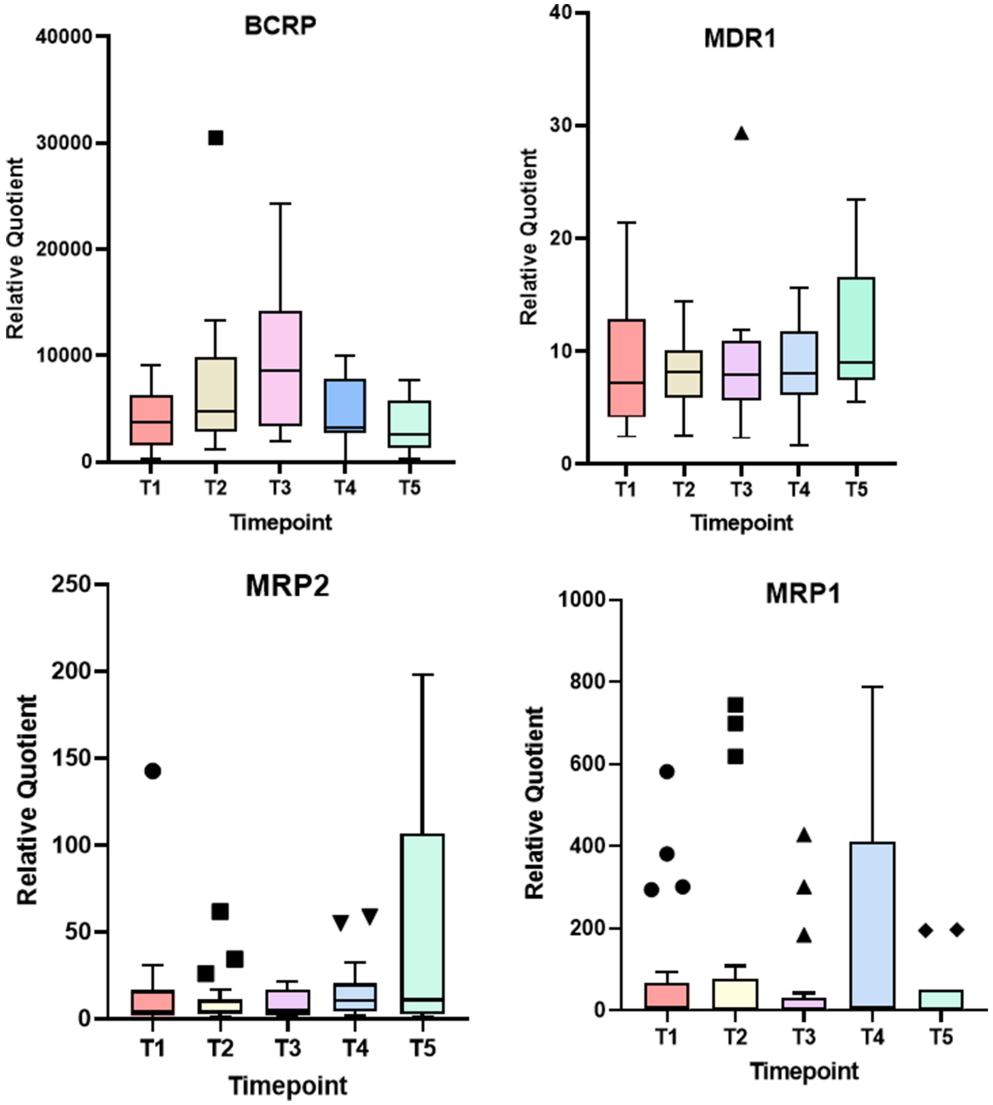
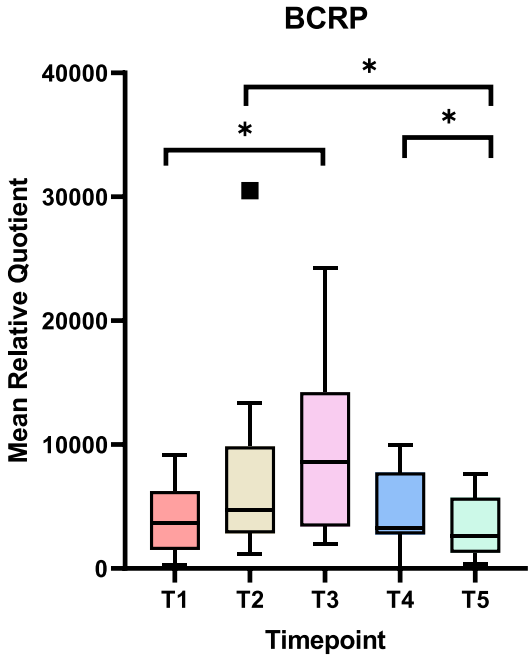


FIGURE 2 Distribution of gene expression of BCRP, MDR1, MRP2 and MRP1 by human breastmilk cells in 88 milk samples from 22 participants. Box plots represent gene expression distribution where tails show the minimum and maximum values (excluding outliers) and upper and lower interquartile ranges; middle line represents the median. (● = T1 outlier, ■ = T2 outlier, ▲ = T3 outlier, ▼ = T4 outlier, ◆ = T5 outlier). Individual PCR reactions were normalised against internal control (GAPDH) and relative to the expression level of MCF10A. Bars represent the mean ± SEM.



	Adjusted *P* Value
T1 vs. T2	0.1908
T1 vs. T3	**0.0176**
T1 vs. T4	0.748
T1 vs. T5	0.9857
T2 vs. T3	0.2855
T2 vs. T4	0.9975
T2 vs. T5	**0.0406**
T3 vs. T4	0.9988
T3 vs. T5	**0.0509**
T4 vs. T5	**0.0176**

FIGURE 3 Longitudinal expression of BCRP over 12 months (Timepoints 1 to 5) of lactation in 22 participants (■ = T2 outlier; = *P* value <0.005)

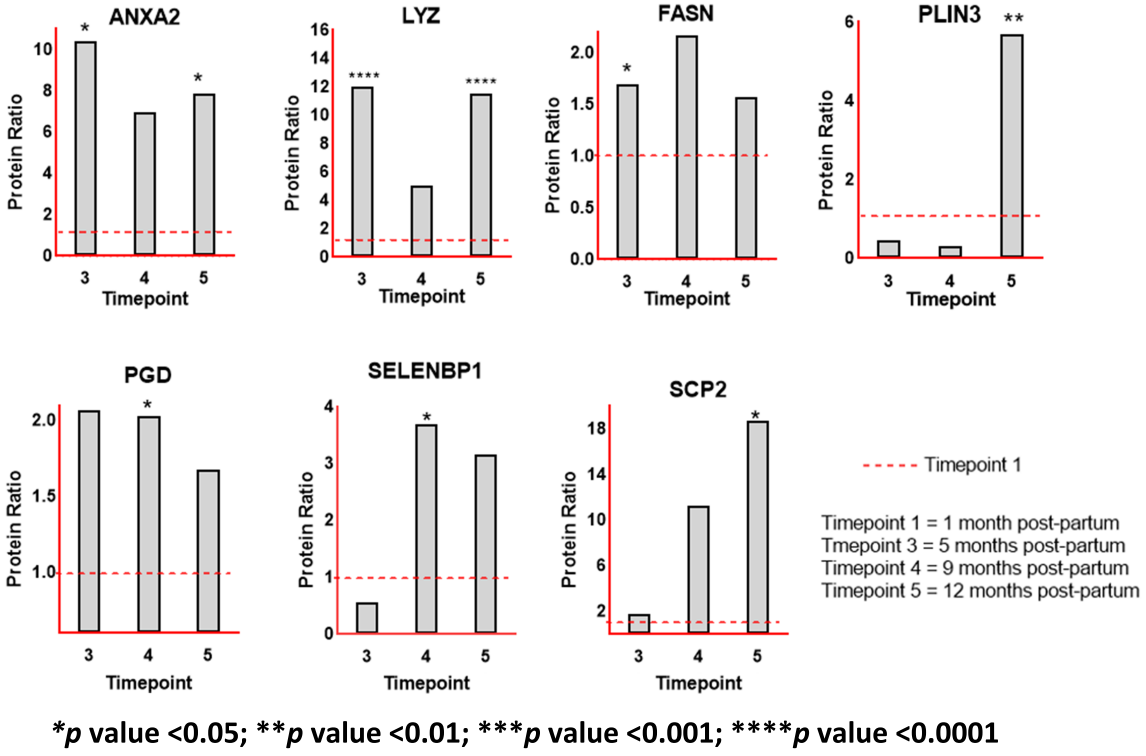


FIGURE 4 Upregulated proteins in lactation normalised to 1 month post-partum (Timepoint 1) set to a protein ratio of 1 as depicted by dashed line. (ANXA2, Annexin A2; LYZ = lysozyme; FASN, fatty acid synthase; PGD, 6-phosphogluconate dehydrogenase; PLIN3, perilipin; SCP2, non-specific lipid-transfer protein; SELENBP1, selenium-binding protein 1)

4 | STATISTICAL ANALYSIS

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. Data were analysed by fitting a mixed model. The mixed effects analysis showed that the expression of

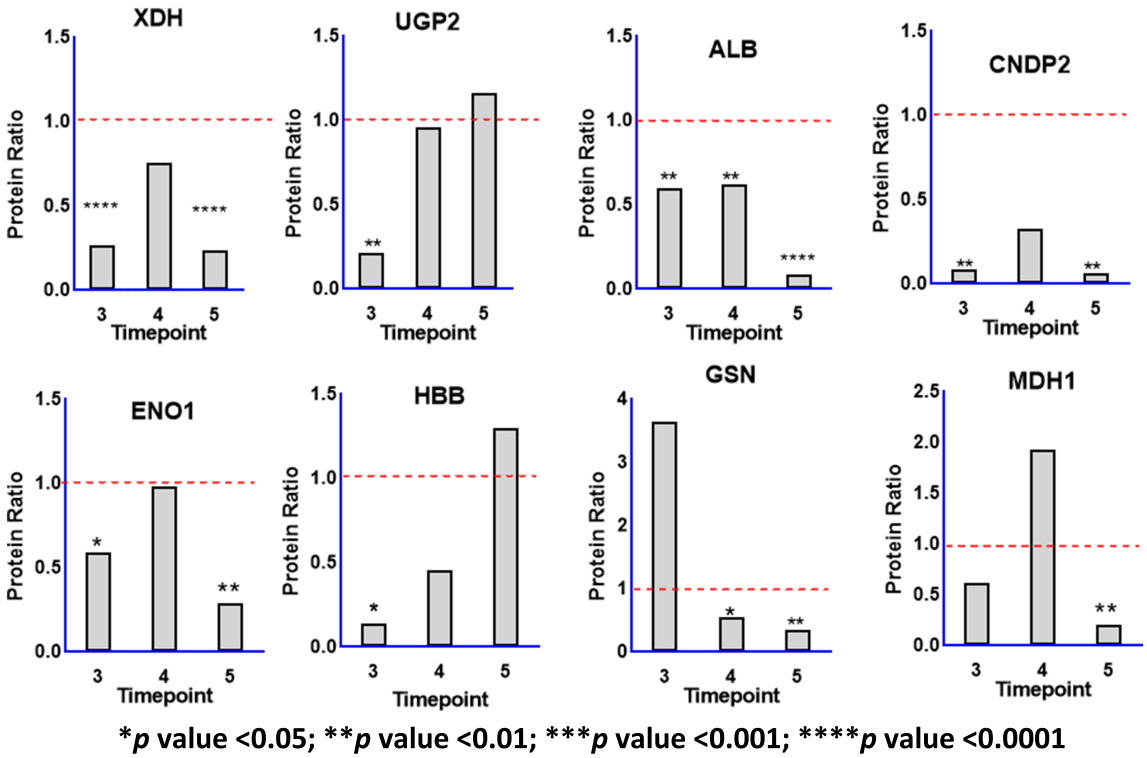


FIGURE 5 Downregulated proteins in lactation normalised to 1 month post-partum (Timepoint 1) set to a protein ratio of 1 as depicted by dashed line. (ALB, serum albumin; CNDP2, cytosolic non-specific dipeptidase; ENO1, alpha-enolase; GSN, gelsolin; HBB, haemoglobin subunit beta; MDH1, malate dehydrogenase; UGP2, UTP--glucose-1-phosphate uridylyl transferase; XDH, xanthine dehydrogenase/oxidase)

TABLE 2 List of differentially expressed proteins in cells isolated from human 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		

Note: Protein expression was compared with Timepoint 1 (1 month post-partum) as the baseline.
Abbreviations: ALB, serum albumin; ANXA2, annexin A2; ANXA5, annexin A5; CNDP2, cytosolic non-specific dipeptidase; ENO1, alpha-enolase; FASN, fatty acid synthase; GSN, gelsolin; HBB, haemoglobin subunit beta; LDHB, L-lactate dehydrogenase B chain; LYZ, lysozyme; MDH1, malate dehydrogenase; PGD, 6-phosphogluconate dehydrogenase; PLIN3, perilipin; SCP2, non-specific lipid-transfer protein; SELENBP1, selenium-binding protein 1; UGP2, UTP--glucose-1-phosphate uridylyl transferase; XDH, xanthine dehydrogenase/oxidase.

BCRP was statistically significant between the different timepoints ($p = 0.0063$). Post hoc analysis was performed using Tukey's multiple comparison test which confirmed that a significant difference in the expression of BCRP over the five timepoints as shown in BCRP was the most abundantly expressed transporter and thought to have

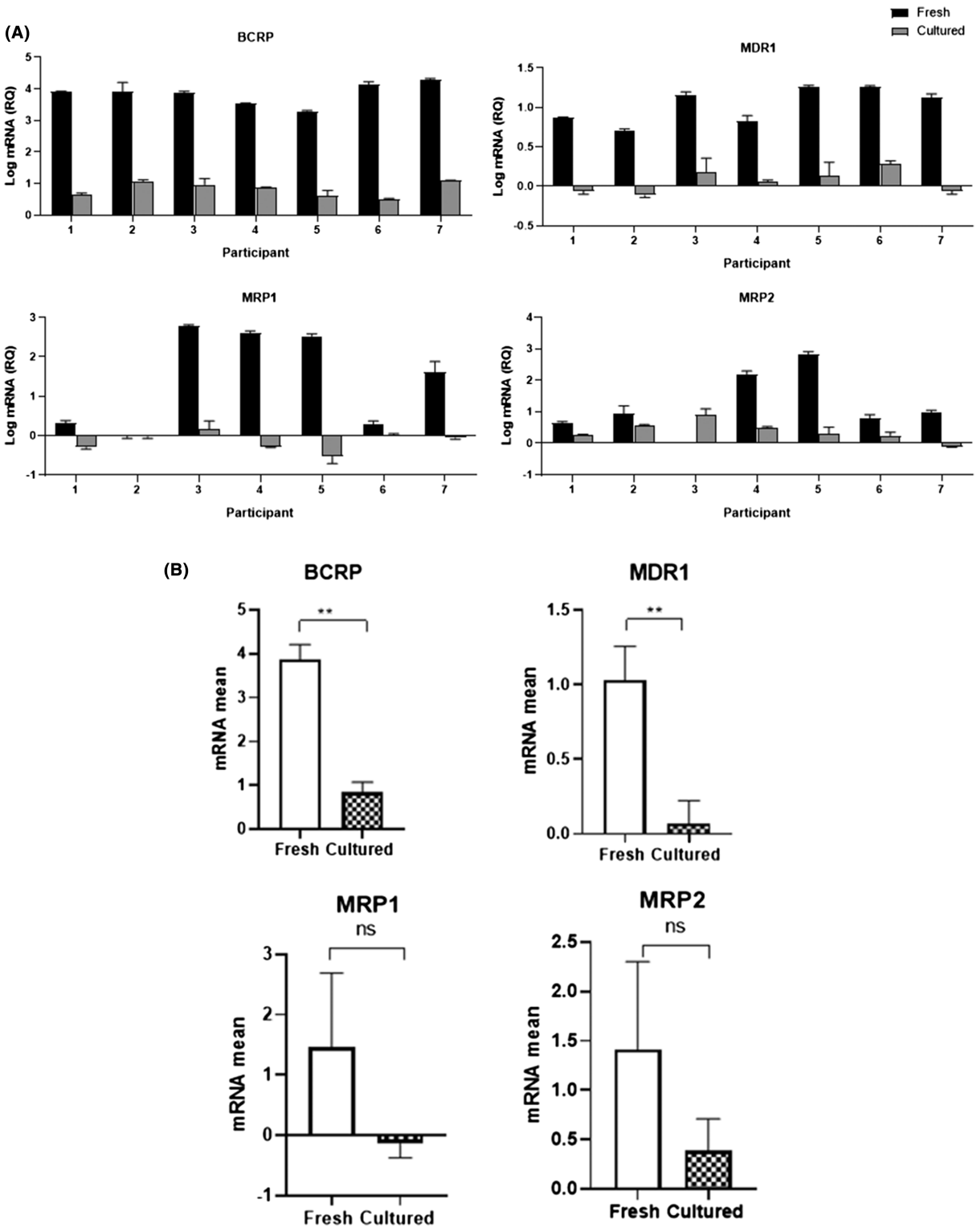


FIGURE 6 (A) Log mRNA (RQ, relative quotient) expression of BCRP, MDR1, MRP1 and MRP2 relative to MCF10A normalised to GAPDH in seven breastmilk-derived mammary epithelial cell samples. (B) Comparison of means of mRNA expression of BCRP (p, 0.0017), MDR1 (p, 0.0016), MRP1 (p, 0.0537) and MRP2 (p, 0.1094) in breastmilk derived cells (Fresh) with those grown in culture medium (Cultured)

the largest significance in transfer of xenobiotics from maternal plasma to breastmilk.[8] BCRP showed a statistically significant difference in expression over the five timepoints with peak levels occurring at T3 as demonstrated in Figure 3. Interindividual differences and changes in the expression of BCRP in each

woman over time was also significant (supporting information Appendix B). 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.

5 | DISCUSSION

RNA extracted from breastmilk cells is considered representative of gene expression in the mammary gland and provides an insight at a molecular level.[36] Using PCR, the presence of BCRP was confirmed. It was also shown 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 was also confirmed, their expression remained at much lower levels compared with BCRP and there was interindividual variability making it difficult to derive statistically significant conclusions. The role of BCRP in drug disposition in breastmilk was anticipated to be more prominent compared with the other three transporters (MDR1, MRP1 and MRP2) due to its relative mRNA overexpression in the lactating mammary gland. Furthermore, many studies have identified BCRP to be involved in regulating the composition of breastmilk.[8,37,38]

Isobaric tags for relative and absolute quantitation (iTRAQ) have been previously used to study active transporters such as MDR1, biological samples and the milk proteome across species.[39–41] 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.[42] For instance, iTRAQ labelling has been linked to a reduction in the number of identifiable proteins due to the introduction of undesirable charge enhancements.[43] 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 with 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 active 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.[44] For this study, due to resource limitations, only four timepoints could be added to a 4-plex assay. A total of 32 samples from eight participants were pooled for the four timepoints for iTRAQ analysis. Similar to PCR assay findings, there was a great degree of inter-individual variability between samples. This variability was possibly due to maternal and environmental factors that influence milk composition.[45] iTRAQ analysis of the 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, seven were downregulated over time as demonstrated in Table 2.

Serum albumin, a major component of human breastmilk, was found to be increasingly downregulated over time. Most milk proteins are synthesized within the mammary gland but a few such as serum albumin may be transferred from maternal blood.[46] The overall protein content of human milk is known to gradually reduce over time as infants 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.[47–49] However, one study showed an increase in the serum albumin content over a 6 months period which is in contradiction to these findings.[45]

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.[50,51] 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 T4 (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 (12 months post-partum) is very similar to the level at T3 (5 months post-partum). Gao and colleagues also showed that XDH is downregulated in lactation.[52] 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 as shown in Figure 4. This is in alignment with previous research showing consistent upregulation of lysozyme over the duration of lactation of up to 26 months.[53,54] Lysozyme has a bacteriolytic function and enhances the activity of immuno-agents 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[55] and this observation is now confirmed in humans.

As the first longitudinal human study of the expression of efflux transporters in the mammary gland, this study confirms 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.[56,57] 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 at −80°C prior to RNA extraction. Mothers were instructed to notify the researcher 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 intrasubjective factors.

Lactogenic hormones such as prolactin, insulin and hydrocortisone have an important role in modulating expression of transporters.[58,59] 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.[57,58] 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.[57,60] 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.[29,61] 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.[62] Genetic polymorphism related to the ABCG2 gene is attributed to the differences in response to chemotherapy in breast cancer.[63] 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,[64–68] 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.

This study being the first longitudinal study of efflux transporters in humans has demonstrated that from its mRNA levels, 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 possible that a nursing infant may be at risk of inadvertent exposure to BCRP substrates, particularly around the 5 to 6 months post-partum period owing to the upregulation of BCRP mRNA at this time. However, the lack of relevant conclusive protein expression data and interindividual variability prevents the potential role of BCRP in breastfed infant ADRs from being categorically confirmed.

The cell culture study highlighted the magnitude of the difference between fresh and cultured cells which ranged from 10- to 1000-fold in some cases, and in all cases the cultured cells had lower mRNA expression. It is known that 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.[69] 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.[70] This highlights that culturing the primary cells would not represent the patient's clinical situation given their vastly lower expression of the main transporters present in the

breast tissue. 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.

6 | CONCLUSION

This study is the first longitudinal study of efflux transporters in the lactating human mammary gland using breastmilk as a source of epithelial cells. BCRP, an active transporter implicated in drug disposition and consequently drug resistance, was found to be highly expressed during lactation with its peak expression occurring at 5 to 6 months post-partum. While the lack of relevant protein expression data and the observed interindividual variability prevent conclusive inferences, the high mRNA expression warrants further investigation to elucidate the extent of BCRP's involvement in the excretion of its substrates into breastmilk.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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