Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

By

Chenxi Cai

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Abstract

The capacity of milk to provide adequate iron may be critical to breastfed infants as human milk is often their sole source of iron during early infancy. Extremely low iron content in human milk has been documented, yet whether the iron in breastmilk is adequate for exclusively breastfed infants and the underlying mechanism responsible for low iron in human milk are still unclarified. This study was designed to investigate the adequacy of iron intake in breastfed infants and the iron trafficking pathway in lactating human epithelial cells.

First, through the meta-analysis of randomised controlled feeding trials (RCT) it was determined that iron supplementation to healthy exclusively breastfed infants improves their iron status and cognitive development, but may delay their physical growth. There was no evidence to suggest iron supplementation could cause other adverse effects.

In order to address why iron concentrations are low in human milk, the expression of iron membrane transporters and iron-binding proteins genes in human breast milk samples was determined. RNA extracted from the breast milk fat globular fraction originates exclusively from epithelial cells when mothers are healthy. Transcript for the transferrin receptor 1 (TFRC), divalent metal transporter 1 (DMT1, SLC11A2), transferrin (TF) and lactoferrin (LTF) were present. However, the transcript encoding the iron transmembrane release protein ferroportin (FPN),
encoded by the *SLC40A1* gene, was absent in human milk. This contradicts existing reports of its presence in the rat mammary glands. A comparison of the iron transporter genes expressed in the mouse epithelial cell line HC11 to human milk confirms the presence of the transferrin receptor 1 and the divalent metal transporter 1 in lactating epithelial cells in both species.

In further analyses it was confirmed that RNA extracted from human milk fat is originated from epithelial cells, while RNA extracted from cells isolated from the breastmilk originated from both epithelial cells and leukocytes.

The transcripts encoding the transferrin receptor 1, divalent metal transporter 1, transferrin and lactoferrin (*LTF*), transferrin receptor 2 were present in RNA isolated from both the milk fat and the milk cells. Transcripts for two other transporters, the “Natural Resistance-Associated Macrophage Protein” (encoded by *SLC11A1*) and ferroportin (encoded by *SLC40A1*) were only found in the RNA isolated from milk cells, and might therefore not play a role in iron secretion through the mammary epithelial cell.

Overall, these findings suggest that RNA from milk fat rather than cells isolated from milk could be a novel tool to investigate the biological functions of lactating epithelial cells. The lack of iron exporter in lactating epithelial cells suggests that iron transmembrane export may not be used during human lactation.
Acknowledgments

I would like to express my deepest gratitude to my supervisor, Dr. James Friel, for his continuous guidance and kindness encouragement during this thesis. He not only taught me how to be excellent in the research but also how to be a better person. He offered me great opportunities to work with government, company, and hospital staff. During the same time, he taught me how to deal with different situations and help me growth in this profession. He is a role model to me and I cannot thank him enough for his kindness help.

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I am thankful to graduate peers, technicians, staffs at Department of Human Nutritional Sciences and Richardson Centre for Functional Foods and Nutraceuticals for their support and assistance. I also thankful to the participants in this project, this research would not have been possible without them.

Finally, I cannot thank enough my family for their unending love and continuous support.
Forward

This thesis was prepared following a group manuscript style, and it is composed of three manuscripts. Manuscript I (Chapter III) was accepted by the Journal of Breastfeeding Medicine. Manuscript II (Chapter IV) was published in the Journal of Pediatric Gastroenterology and Nutrition. While, manuscript III (Chapter V) was prepared to submit to the Journal of Pediatric Gastroenterology and Nutrition.
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Abbreviations

CD45  Protein tyrosine phosphatase
DCYTB  Duodenal cytochrome b
DMT1  Divalent metal transporter
DRIs  Dietary reference intakes
FPN  Ferroportin
HCP1  Heme carrier protein 1
HEPH  Hephaestin
Hb  Hemoglobin
ID  Iron deficiency
IDA  Iron deficiency anemia
LTF  Lactoferrin
MUC1  Mucin 1
NANOG  Nanog homeobox
NRAMP1  Natural resistance-associated macrophage protein 1
OCT4  POU class 5 homeobox
RCTs  Randomized controlled trials
RT-PCR  Real-time polymerase chain reaction
SOX2  Sex determining region Y-box2
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TFRC</td>
<td>Transferrin receptor 1</td>
</tr>
<tr>
<td>TFR2</td>
<td>Transferrin receptor 2</td>
</tr>
<tr>
<td>TFs</td>
<td>Transcription factors</td>
</tr>
</tbody>
</table>
Chapter I. Introduction

1.1 Background

Iron (Fe) is an essential trace mineral that plays an important role in the body; it is involved in erythropoiesis and the synthesis and functioning of Fe-requiring enzymes. Therefore, humans require a certain amount of dietary Fe to maintain biochemical functions of the body. The capacity of milk to provide adequate Fe can be critical for breastfed infants because it may be their sole source of Fe for most of infancy. Regulation of adequate amounts of Fe is not only important to normal mammary gland physiology, it is also critical to the lactating process. The Fe content in human breast milk is around 0.3mg/L, which is considered low in comparison to maternal serum Fe. The Fe status of exclusively breastfed infants remains a controversial topic in neonatal nutrition, with some voices supporting a Fe supplementation while others regard this as harmful due to potential Fe overload.

The mechanism of Fe transfer from maternal serum into the breast milk is still unknown for humans. Current available data have only been obtained from a rat model, which may not be a good model for human because the Fe-binding proteins in milk and the concentration of Fe in milk in human and rats are different. Thus, understanding the underlying mechanisms of Fe transport by the human mammary
glands had a much broader appeal. Due to ethical and technical problems, lactating human mammary gland tissue is difficult to obtain for research use. In this project, RNA from breastmilk will be used as a non-invasive source. This work will illuminate on this issue by determining the Fe transporters in lactating epithelial cells. The studies were designed to compare human and mouse as a means to extrapolate on existing rodent data.

1.2 Hypothesis and Objectives

1.2.1 Hypothesis
Iron transporters in the human mammary gland are responsible for low iron concentration in human breast milk.

1.2.2 Objectives
1. Investigate the evidence for the benefits and risks of daily oral Fe supplementation in exclusively breastfed infants.

2. Determine the Fe transporters expressed in a mouse mammary epithelial cell line before and after prolactin stimulation.

3. Determine the Fe transporters expressed in mRNA from human milk fat.


5. Compare the Fe transporters expressed in mRNA from human milk fat and that from human milk cells.
Chapter II. Literature Review

2.1 Iron Metabolism in the Human Body

2.1.1 Intestinal iron absorption

Iron (Fe) uptake by enterocytes depends on the forms of the Fe (Figure 2-1). The major sources of Fe include free non-heme Fe, heme and ferritin. It has been proposed that heme Fe is transported by an intestinal heme transporter, heme carrier protein 1 (HCP1) (1), or another transporter (2). However, this process has not been fully determined yet. Ferritin enters the enterocytes via an unknown mechanism, and it is then degraded in the lysosomes (3). Non-heme ferric Fe must be reduced to ferrous Fe by duodenal cytochrome b (DCYTB) (4) or other cell surface ferrireductases first (5), then it is transported into the enterocytes via divalent metal transporter (DMT1) (6). Once it enters the cell, cellular Fe can either be stored as ferritin, used by the intracellular compartments, or exported into the blood for the use by other tissues in the body (3). Intracellular ferrous Fe is transferred across the basolateral membrane by ferroportin (FPN) (3), and then it is oxidized to ferric Fe by hephaestin (HEPH), a membrane-anchored, multicopper ferroxidase (7). Then ferric Fe binds to transferrin (TF) in the interstitial fluids and circulates throughout the body.
Figure 2-1 Model of iron transport through the duodenal enterocytes. Heme Fe is transported by the heme carrier protein 1 (HCP1) or another transporter. Ferritin enters the enterocytes via an unknown mechanism, and it is then degraded in the lysosomes. Non-heme Fe is transported into the enterocytes via the divalent metal transporter (DMT1). Intracellular ferrous Fe is transferred across the basolateral membrane by ferroportin (FPN). Then ferric Fe binds to transferrin (TF) in the interstitial fluids and circulates throughout the body.

2.1.2 Cellular iron uptake: The transferrin cycle

The prevalent expression of transferrin receptor 1 (TFRC) reveals that it facilitates cellular Fe uptake in most cells (8) (Figure 2-2). TF-bound Fe enters the cells is mediated by TFRC (9). Fe-TF binds TFRC on the cell surface and enters the cell as the Fe-TF-TFRC complex (10), which is endocytosed into the endosome. Due to
the acidity of endosomes (11, 12), Fe is released from the complex and the TF-TFRC is returned to the cell surface. The released Fe is possibly reduced from ferric to ferrous by the ferrireductase, STEAP3 (13), and it is transported out of the endosome by DMT1. Fe is either located to the mitochondria or stored as ferritin in the cytosol (14).

Figure 2-2 Cellular iron uptake via the TF cycle

*Fe-TF binds TFRC on the cell surface and enters the cell as the Fe-TF-TFRC complex, which is endocytosed into the endosome. Due to the acidity of endosomes, Fe is released from the complex and the TF-TFRC is returned to the cell surface. The released Fe is transported out of the endosome by DMT1. Cytosolic Fe is either located to the mitochondria or stored as ferritin.*
2.1.3 *Iron metabolism in macrophage*

Macrophages play an important role in recycling Fe in the body (15). Senescent red blood cells are recognized and internalized by macrophages in the reticuloendothelial system (Figure 2-3). Fe is released from hemoglobin (Hb) in the phagolysosome and then transported to the cytosol by natural resistance-associated macrophage protein 1 (Nramp1). Then, it is either stored in ferritin or transported out of the cell by FPN (16). Heme is transported from the phagolysosome to the cytosol by HRG1 (a conserved heme-transporting permease) (17), and it may be effluxed from the cell by FLVCR1, the heme exporter (16).

*Figure 2-3 Iron metabolism in macrophage*

*Fe is released from Hb in the phagolysosome and then transported to the cytosol by Nramp1. Then, it is either stored in ferritin or transported out of the cell by*
FPN. Heme is transported from the phagolysosome to the cytosol by HRG1 and it may be effluxed from the cell by FLVCR1.

2.1.4 Potential iron transport mechanism in mammary epithelial cells

To date, no data is available on Fe transport mechanism in human lactating epithelial cells. TFR1, DMT-1 and FPN orthologues have been identified in the mammary tissues of lactating rats (18, 19). Leong and Lonneral (2005) investigated DMT1 and FPN in rat mammary glands at different stages of lactation and found that milk Fe, DMT1, and FPN decreased throughout lactation. Thus, they proposed that DMT-1 and FPN are possibly involved in the transfer of Fe to milk (19).

Based on experiments on rats, a possible Fe transport mechanism in lactating mammary epithelial cells has been proposed (Figure 2-4): Fe in serum is bound to TF. Fe-TF binds to the TFRC at the mammary epithelial cell surface and enters the cell as the Fe-TF-TFRC complex. In the endosome, Fe is released from the complex due to the acidic environment, and then the TF-TFRC complex is recycled to the plasma membrane (20). Fe is most likely exported from the endosome by DMT1 (19). Fe released from the endosome can be either used by the mitochondria, stored as ferritin, or be secreted across the luminal membrane into milk. Export of Fe from the mammary gland is most likely achieved by FPN, which is the only known transport mechanism that mediates cellular Fe efflux (20).
However, it should be noted that milk Fe concentrations in rat milk are at least 25 times higher than that in humans (21). In addition, unlike the mouse and human, in rats, TF is the Fe-binding protein in milk (22). Thus, the Fe transport mechanism in lactating rats could be different from the mechanism in lactating humans.

*Figure 2-4* Potential iron transport mechanism in rat mammary epithelial cell (23) Fe in serum is bound to TF. Fe-TF binds to the TFRC at the mammary epithelial cell surface and enters the cell as the Fe-TF-TFRC complex. In the endosome, Fe is released from the complex due to the acidic environment, and then the TF-TFRC complex is recycled to the plasma membrane. Fe is most likely exported from the endosome by DMT1. Export of Fe from the mammary gland is most likely achieved by FPN.
2.2 Iron Transporters and Iron Binding Proteins

Fe trafficking in mammalian systems depends on Fe transporters. A summary of these proteins is provided in Table 2-1, each of which will be discussed in more detail below.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Predominant tissue distribution</th>
<th>Major subcellular localization</th>
<th>Function</th>
<th>Link to disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFRC</td>
<td>Transferrin receptor 1</td>
<td>bone marrow, lymph node, brain, retina, cerebellum, spinal cord, heart, skeletal muscle, colon, liver, lung, thyroid, breast, skin, placenta, prostate</td>
<td>endosome, extracellular, plasma membrane, cytosol, mitochondrion</td>
<td>cellular iron uptake, erythropoiesis, neurologic development</td>
<td>immunodeficiency and gastric adenosquamous carcinoma</td>
</tr>
<tr>
<td>TfR2</td>
<td>Transferrin receptor 2</td>
<td>brain, retina, liver, thyroid, breast, skin, prostate</td>
<td>plasma membrane</td>
<td>cellular uptake of transferrin-bound iron</td>
<td>hereditary hemochromatosis type iii</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>NRAMP1</td>
<td>brain, retina, cerebellum, spinal cord, breast, skin, prostate</td>
<td>endosome, plasma membrane, lysosome, vacuole</td>
<td>iron metabolism; host resistance to certain pathogens</td>
<td>tuberculosis, leprosy, rheumatoid arthritis and Crohn disease</td>
</tr>
<tr>
<td>SLC11A2</td>
<td>DMT1</td>
<td>bone marrow, brain, retina, cerebellum, spinal cord, heart, kidney, pancreas, thyroid, breast, skin, placenta, prostate</td>
<td>endosome, Golgi apparatus, lysosome, nucleus, plasma membrane, vacuole</td>
<td>transports divalent metals; iron absorption</td>
<td>hypochromic microcytic anemia</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>Ferroportin</td>
<td>bone marrow, lymph node, retina, spinal cord, heart, skeletal muscle, colon, kidney, liver, lung, pancreas, thyroid, breast, skin, placenta, prostate</td>
<td>plasma membrane, endosome</td>
<td>iron export from duodenal epithelial cells</td>
<td>hemochromatosis type 4</td>
</tr>
<tr>
<td><strong>TF</strong></td>
<td>Transferrin</td>
<td>brain, lymph, cortex, retina, spinal cord, liver, pancreas, breast, skin</td>
<td>endosome, extracellular, plasma membrane</td>
<td>transport iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body</td>
<td>familial hypotransferrinemia, iron overload,</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------------------------------------------------------------------------</td>
<td>--------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>LTF</strong></td>
<td>Lactoferrin</td>
<td>whole blood, white blood cell, kidney, lung, stomach, salivary gland, breast, prostate, testis</td>
<td>extracellular, nucleus, lysosome, plasma membrane</td>
<td>major iron-binding protein in milk; body secretions with an antimicrobial activity</td>
<td>dental caries, superficial siderosis of the central nervous system</td>
</tr>
</tbody>
</table>

Footnotes:

2.2.1 Transferrin receptor 1 (TFRC)

Fe entry into most cells mainly occurs through transferrin receptors. To date, two transferrin receptors have been identified: TFRC and transferrin receptor 2 (TFR2). TFRC binds the circulating transferrin at the cellular membrane surface and it enters the cell as the TFRC-TF complex (24). TFRC consists of 760 amino acids, and its mRNA expression is highly regulated by its Fe status. The human TFRC gene is localized to the long arm of chromosome 3 (3q29) (Figure 2-5). TFRC expression is down-regulated in Fe replete conditions and up-regulated in iron deficient conditions (25). A mutation of TFRC is associated with degrading the development and functioning of erythroid tissues (26).

**Figure 2-5 Gene localization of TFRC**

*The human TFRC gene is localized to the long arm of chromosome 3 (3q29).*
2.2.2 Transferrin receptor 2 (TFR2)

The sequence homology of TFR2 is similar to TFRC, which can also bind and transport transferrin-bound Fe, but with a much lower affinity (27). The human TFR2 gene is localized to the long arm of chromosome 7 (7q22.1) (Figure 2-6). Its mRNA expression seems dose not to be regulated by Fe status; rather, it is up-regulated by elevated levels of di-ferric transferrin (28, 29). Unlike TFRC, TFR2 is not ubiquitously expressed. It is mostly expressed in the liver (30). A homozygous nonsense mutation of TFR2 is associated with severe hepatic Fe overload (31).

Figure 2-6 Gene localization of TFR2
The human TFR2 gene is localized to the long arm of chromosome 7 (7q22.1).

2.2.3 NRAMP1 (SLC11A1)

The SLC11 family of proteins has two mammalian members: NRAMP1 (SLC11A1) and DMT1 (SLC11A2). SLC11A1 is a 90–100 kDa integral
membrane phosphoglycoprotein located at the lysosomal compartment of macrophages and in the tertiary granules of neutrophils, which have a 64% amino acid sequence identity and 78% similarity with SLC11A2 (32-34). It is expressed abundantly in phagocytes, and its expression is stimulated by mycobacterial infection in macrophages (35, 36). It has been suggested that SLC11A1 assists the macrophageal antimicrobial function by moving divalent metal ions (Mn$^{2+}$, Fe$^{2+}$) from the phagolysosome; without these essential cofactors for bacteria-derived enzymes may stop the growth of bacteria (37). The human SLC11A1 gene is localized to the long arm of chromosome 2 (2q35) (Figure 2-7). The mutations of this gene are associated with some infectious diseases and inflammatory diseases, such as tuberculosis, leprosy, rheumatoid arthritis and Crohn’s disease (38).

Figure 2-7 Gene localization of SLC11A1
The human SLC11A1 gene is localized to the long arm of chromosome 2 (2q35).
2.2.4 DMT1 (SLC11A2)

The other SLC11 family member, SLC11A2, is a divalent metal transporter (DMT1) that is known for transporting ferrous Fe through the apical membrane of the intestinal epithelial cells (39, 40). DMT1 is also needed for the release of transferrin-bound Fe from endosomes to the cytosol during transferrin receptor-mediated cellular Fe uptake. Experiments in mice have shown that DMT1 plays an important role in both intestinal Fe absorption and erythropoiesis (41). The SLC11A2 protein contains about 560-570 amino acids, and its size is approximately 65kDa in size (42). The human SLC11A2 gene is localized to the long arm of chromosome 12 (12q13.12) (Figure 2-8). SLC11A2 is widely expressed, especially in the proximal duodenum, immature erythroid cells and the placenta. A lack of SLC11A2 can cause severe IDA (42).

Figure 2-8 Gene localization of SLC11A2
The human SLC11A2 gene is localized to the long arm of chromosome 12 (12q13.12).
2.2.5 *Ferroportin (SLC40A1)*

To date, SLC40A1 (FPN) is the only known membrane exporter mediating Fe release (42). The human *SLC40A1* gene is localized to chromosome 2q, it encodes a protein of 570 amino acids with a size of 62 kDa (Figure 2-9). *SLC40A1* is expressed at high levels on the surface of cells, especially macrophages, enterocytes, hepatocytes and the placental syncytiotrophoblasts, but it is also present on almost all cells (42). In humans with reduced FPN function, iron quickly accumulates in the macrophages, Fe concentrations increase in the parenchymal cells, Fe absorption decreases in the intestine and Fe levels decrease in the plasma (42). Mutations of human *FPN* have been associated with Fe loading syndrome, which could lead to either type IV hemochromatosis or FPN disease (43).

*Figure 2-9 Gene localization of SLC40A1*

The human SLC40A1 gene is localized to the long arm of chromosome 2 (2q32.2).
2.2.5 Transferrin (TF)

The major members of the TF superfamily are TF and lactoferrin (LTF). Approximately 3-4 mg of plasma-circulating Fe is bound to TF. The principal function of serum TF is transporting Fe to the sites of utilization and storage within the body. TF is approximately 80 kDa in size, and it is synthesized primarily in the liver (43). The human TF gene is localized to the long arm of chromosome 3 (22.1) (Figure 2-10). Mutations of TF are associated with familial hypotransferrinemia and Fe overload.

Figure 2-10 Gene localization of TF

2.2.6 Lactoferrin (LTF)

LTF is the most abundant Fe-binding protein in human milk (44). It has been shown to have multiple protective functions, including antimicrobial/antiviral activity, immunomodulatory activity, anticancer and antioxidant activity (45-47). LTF has 711 amino acids, and it is approximately 80 kDa in size (48). The human
The human LTF gene is localized to the short arm of chromosome 3 (3q22.1) (Figure 2-11). LTF is highly abundant in mucosal secretions, and significant levels of it are also located to secondary neutrophil granules (48, 49). Mutations of this gene are associated with dental caries and superficial siderosis of the central nervous system (50).

Figure 2-11 Gene localization of LTF
The human LTF gene is localized to the short arm of chromosome 3 (3q22.1).
2.3 Importance of Fe in Infant Development

2.3.1 Biological functions of Fe

Fe is an essential trace mineral that plays an important role in the human body; it is involved in erythropoiesis and the synthesis and functioning of Fe-requiring enzymes. Hb in red blood cells is a major Fe-bounded protein in the body; it transfers oxygen from the lungs to the tissues (51). Myoglobin is another Fe-bounded protein that transports and stores oxygen in muscle cells (52). The oxygen transport and storage function of Hb and myoglobin is facilitated by the heme group. The heme group consists of a central Fe atom and a porphyrin ring. Fe is also an integral part of several functional enzymes, such as cytochromes, which transfer energy in the mitochondria during the oxidative metabolism. For example, Cytochrome P450 is a type of Fe-requiring enzyme in the synthesis pathway of cholesterol and conversion of cholesterol into steroids hormones and bile salts (53); it detoxifies foreign substances in the liver (54); and it is involved in signal controlling in some neurotransmitters, such as the dopamine and serotonin systems in the brain (55).

2.3.2 Fe content in milk

For exclusive breastfed infants, breast milk is their sole source of Fe. The Fe concentration in human colostrum is approximately 0.8 µg/ml; in mature breast milk it is 0.2-0.4 µg/ml (56). In contrast, the Fe concentrations in the milk of most
other species are much higher (Table 2-2). The Fe concentration in mature milk in rat is 5-10 µg/ml, which is about 25 times higher than the Fe concentration in human milk. Although the Fe concentration is low in human milk, it is independent of the mother’s Fe status and it cannot be increased through maternal diet or Fe supplementation (57, 58).

Table 2-2 Iron concentration in milk of various species (56)

<table>
<thead>
<tr>
<th>Species</th>
<th>Iron concentration in mature milk(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.2-0.4</td>
</tr>
<tr>
<td>Rat</td>
<td>5-10</td>
</tr>
<tr>
<td>Mice</td>
<td>15</td>
</tr>
<tr>
<td>Dog</td>
<td>2-8</td>
</tr>
<tr>
<td>Pig</td>
<td>1-2</td>
</tr>
<tr>
<td>Cow</td>
<td>0.1-0.2</td>
</tr>
</tbody>
</table>

2.3.3 Fe requirement for infants

The literature reports conflicting results for the Fe requirement for infants. For newborn infants, most of the Fe in the body is found in Hb and some body stores. When a newborn is transferred from the uterus environment into an oxygen-rich atmosphere, the Hb level falls from 170 g/L to 120 g/L during the first 6 weeks of life because Hb synthesis is paused. For exclusively breastfed infants, the major source of Fe comes from the Fe stored in their own body because the Fe content in
breast milk is extremely low. Some researchers have assumed that a full-term infant’s body has a sufficient amount of Fe until about 4 to 6 months of age. Thus, based on the average Fe concentration in human milk, the Institute of Medicine recommends that Dietary Reference Intakes (DRIs) of Fe for infants before the age of 6 months is 0.27 mg/d (59). However, the American Academy of Pediatrics recommends that exclusively breast-fed, full-term infants receive 1 mg/kg per day of Fe supplement beginning at the age of 4 months (60).

2.3.4 Iron deficiency anemia

Maintaining an adequate level of Fe is critical to an infant’s physical and neurological development. Iron deficiency (ID) is commonly considered to develop in three stages: iron depletion, iron-deficient erythropoiesis, and iron deficiency anemia (IDA). IDA is a common cause of anemia in infants. The World Health Organization defines anemia as: ‘Anaemia is a condition in which the number of red blood cells or their oxygen-carrying capacity is insufficient to meet physiologic needs, which vary by age, sex, altitude, smoking, and pregnancy status’ (61). ID is usually defined by one of the accessible markers of Fe status, such as serum ferritin and blood Hb concentration. However, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee on Nutrition has noted that current reference ranges and cut-offs for the different Fe status biomarkers are inadequately defined in infants because of the great
physiological changes in Fe status and red cell morphology during the first year of life (62).

It is estimated that nearly 25% of preschool children develop IDA in the worldwide (63). Although no national study of ID in infants in Canada has been conducted over the past 30 years, the data from some studies have suggested that IDA is a health problem (64). A study conducted in Vancouver found that about 15% of breast-fed infants developed anemia at the age of 8 months (65). For preterm infants, the prevalence of IDA is higher (above 25%) during their infancy (66). Other studies have suggested that the prevalence of ID/IDA in breast-fed infants is higher than previously expected (67, 68).

2.3.5 Effects on neurodevelopment

Fe is critical for the rapid development of the central nervous system during infancy. Animal studies have shown that Fe is critical for brain development, such as myelination, monoamine neurotransmitter function, neuronal and glial energy metabolism (69). Some human studies have suggested that early ID is associated with later neurodevelopmental impairments (70). A longitudinal follow-up study in Costa Rica that assessed changes in cognitive functioning after ID in infancy by evaluating four subsequent follow-ups at 5, 11–14, 15–18, and 19 years of age. The results showed that the participants in the study group that had ID in infancy had
lower cognitive scores over time than the participants in the group with good Fe status (71-73). The results of these studies have increased concerns that early ID may irreversibly affect long-term neurodevelopment. A meta-analysis of 17 randomized clinical trials in children indicated that iron supplementation had a positive effect on mental development indices, which suggests that Fe supplements have positive cognitive effects in iron-deficient children (74). Consequently, early prevention of ID seems to be extremely important. In a double-masked, randomized study, Friel et al. demonstrated healthy, breast-fed babies that received Fe supplementation from 1 to 6 months had better scores in Bayley mental and psychomotor developmental indexes and visual acuity at 13 months of age than babies who received no Fe supplementation (75). However, whether or not Fe has positive neurodevelopment effects on exclusively breastfed infants still needs to be clarified.

2.3.6 Possible adverse effects of Fe

Some concerns have been voiced about Fe supplementation, as Fe is a potent pro-oxidant and it cannot be actively excreted by humans (76). Some studies have suggested that Fe supplementation may have adverse effects on iron-replete infants, such as increased risk of infections and impaired growth (77). Increased risk of severe infections seems to be restricted to malarious regions (78). However,
current evidence is not definitive. The negative effect of Fe supplements still needs to be confirmed in meta-analyses (79).
2.4 The Cellular Origin of Cells in Human Breastmilk

2.4.1 Anatomy of human lactating mammary glands

The important biological role of lactating mammary glands is to produce milk to nourish a baby. The anatomy of human lactating mammary gland is different from a resting mammary gland. Slightly below the center of the breast is the areola, which is a ring of pigmented skin that surrounds the nipple. Each mammary gland has 15-25 lobes that are padded and separated from each other by connecting tissues and fat. Mammary gland lobules are the small units within the lobes that contains the milk production glands, alveoli. Milk is synthesised in the epithelial cells and passes through the milk duct to the nipple (80).

Figure 2-12 Anatomy of human mammary glands

(Source: https://commons.wikimedia.org/wiki/File:Breast_anatomy.png)
Each mammary gland has 15-25 lobes (purple) that are padded and separated from each other by connecting tissues and fat (yellow). Mammary gland lobules are the small units within the lobes that contain the milk production glands, alveoli. Milk is synthesized in the epithelial cells and passes through the milk duct (blue) to the nipple.

2.4.2 Cell type distribution in human milk

Breast milk contains lipids, proteins, carbohydrates and bioactive molecules, such as vitamins and immunomodulatory factors, which provide immunological, developmental and nutritional support to the infant. In addition to its biochemical compounds, breast milk contains maternal cells, which can be retrieved noninvasively via expressed breastmilk (81). The cell content of human milk ranges approximately between 1x10⁴ and 1.3 x10⁷ cells/mL, with a wide range of individual variation; the cell viability is approximately 90%. Colostrum likely contains more cells than the mature milk (82). Breastmilk contains a heterogeneous mix of cells, including epithelial cells, leukocytes and stem cells (82). Cell distribution is diverse between women at the same lactation stage. When the mothers and infants are healthy, the dominant cells in the colostrum are the leukocytes while the dominant cells in mature milk are epithelial cells (83-85). However, with the incidence of infection, the population of the leukocytes increases in breast milk (85).
2.4.2.1 Epithelial cells

Epithelial cells contribute to the function and structure of lactating mammary glands. If the mother and infants are healthy, epithelial cells are to constitute up to 98% of the cells in breast milk (86). In the past, studies have suggested that epithelial cells in breast milk arose from the evacuation of dead cells from mammary glands. However, most recent studies have found that cells from freshly expressed human milk have high viability based on a trypan blue exclusion test. Moreover, they can be cultured and form functioning colonies (87, 88). This leads to the hypothesis that these epithelial cells in breast milk are detached from the ducts passively due to the milk producing process, such as milk synthesis, ejection and breast expansion (89). A few observation studies have suggested that the majority of the mature milk cells are lactocytes (milk secreting cells) (84, 90). In addition to these studies, Hassiotou et al. reported significant gene expression of β-casein, the milk containing protein in freshly expressed breast milk cells (82). However, until now, the majority of previous studies have examined the epithelial cells morphologically; thus, the function and character of breast milk epithelial cells requires further investigation using molecular analysis.

2.4.2.2 Leukocytes

Leukocytes in breast milk are most often studied by focusing on their immunology functions. Leukocytes are the dominant cell type in colostrum, but constitute less
than 2% of cells in mature breast milk when the mother and infant are healthy (91). It is believed that milk leukocytes originated in the blood and are transported into the milk via the paracellular pathway (92). They are recruited to the mammary glands when an infection occurs. Breast milk leukocytes include various types of cells, such as macrophages, lymphocytes and monocytes (44, 85, 93). Under a phase contrast microscope, the macrophages are mobile and activated (94). The bactericidal function of human milk leukocytes has been tested in vitro (95). In animal models, it has been reported that milk leukocytes can be transferred from the intestine to the blood circulation and different organs (96, 97). Recently, research in humans found that the human milk leukocyte count increased when a mother or an infant has an infection (85, 98). Overall, this evidence suggests the protective mechanisms of living human milk leukocytes to both mothers and infants.

2.4.2.3 Stem cells

The human body contains stem cells that can be differentiated into different cell types. Normally, stem cells are accessible from bone marrow, adipose tissue and blood (99). Recently, researchers have discovered that breastmilk contains stem cells with multi-lineage potential (88). Hassiotou et al. found that human milk stem cells expressed a set of pluripotency markers (88). These markers, including transcription factors (TFs) POU class 5 homeobox (OCT4), sex determining
region Y-box2 (SOX2) and nanog homeobox (NANOG), comprise the core self-renewal circuitry of human embryonic stem cells. Moreover, fresh isolated human milk stem cells can be differentiated under different in vitro environments. They can be differentiated into mammary gland cells, such as lactocytes and myoepithelial cells, as well as other cell types, such as neuron cells, mesodermal cells, and endodermal cells (88). The origin of human milk stem cells is still unknown. It has been suggested that mammary epithelium could be the origin of these milk stem cells because: 1) positive staining of pluripotency genes is present in both ductal and alveolar compartments, and 2) the gene is highly expressed during lactation (88, 100). The other possible origin of human milk stem cells is from the maternal bloodstream, since other parts of the body (such as bone marrow) express similar pluripotency genes (86, 101).

In animal models, stem cells from breastmilk can survive through the gastrointestinal tract of offspring, immigrate into the bloodstream and reach the organs of a neonate (102). This evidence suggests that the biological function of breastmilk stem cells is not only restricted to maternal breast remodeling; rather, it also impacts the offspring.
2.5 Use of Breastmilk in Gene Expression Studies

Due to technical and ethical difficulties, most studies use cell cultures and rodent models for mammary gland biology research. Although breast tissue could be obtained by surgery, healthy lactating tissue is rarely used in lactation research (103, 104). Alternately, breastmilk has been proposed as a non-invasive source of lactating epithelial cells for research. As discussed above, it is proposed that cells in breastmilk reflect the epithelium of the lactating mammary glands (82, 88). The mRNA extracted from breastmilk cells has been used to examine the gene expression of target proteins in different cell types of human lactating mammary glands. More recently, RNA from milk fat globules has been used to determine maternal genomic information. RNA in the cytoplasm of mammary epithelial cells is wrapped by fat globules, and it enters milk during the milk producing process (105, 106). Thus, the RNA in the fat globules of milk is expected to represent the transcriptome in milk-secretory cells.

Table 2-3 summarizes the gene expression studies that have used breastmilk mRNA. Most of the studies harvested the milk cells prior to mRNA extraction. Earlier studies use the guanidine thiocyanate method to extract the mRNA from milk cells (107, 108). More recently, commercially available extraction kits have been used to extract the RNA from milk cells (88, 91). However, the available extraction kit cannot be used to extract the RNA from milk fat globules directly. In
2007, Maningat et al. (109) reported that the RNeasy kit cannot isolate RNA from milk fat efficiently; the authors of that study determined that a TRIzol based extraction resulted in sufficient yields, which was confirmed in subsequent studies (110, 111).

Cells isolated from human breast milk had been used as a source of RNA to investigate gene expression. However, depending on the maternal health state the breast milk carries a varietal source of cells, posting challenges when the gene expression of the lactating epithelial cell is to be investigated. RNA extracted from the milk fat fraction seems to represent the transcriptome of the lactating epithelial cell, but extraction procedures could be optimized further.

Real-time polymerase chain reaction (RT-PCR) is the method most frequently used in these studies to determine the expression of target genes. These targeted genes include cytokines, glucose transporters, drug transporters and cell markers. In addition to RT-PCR, microarrays have been used to determine the transcriptome. RNA from breast milk was most often used to investigate the expression of immunological proteins and cell markers. Only one study investigated the nutrient transporter (glucose transporter). In future studies, RNA from breast milk could be used to investigate the nutrient transporters in lactating epithelium for a better understanding of lactation physiology. As more research on breast milk as a model is being conducted, studies use different methodologies and a comparison of their
methods is lacking. Consequently, future studies should optimize and standardize a milk RNA extraction procedure to advance the use of breast milk.
Table 2-3 Summary of gene expression studies using breastmilk

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample size</th>
<th>Breastmilk fraction</th>
<th>Method</th>
<th>Target genes</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Srivastava et al., 1996) (107)</td>
<td>N/A</td>
<td>Cells</td>
<td>Guanidine thiocyanate method</td>
<td>RT-PCR</td>
<td>Cells in breast milk expressed mRNA for cytokines</td>
</tr>
<tr>
<td>(Tunzi et al., 2000) (112)</td>
<td>6(individual)</td>
<td>Cells</td>
<td>TRIZOL</td>
<td>RT-PCR</td>
<td>Cells harvested from breast milk expressed hBD-1</td>
</tr>
<tr>
<td>(Obermeier et al., 2000) (108)</td>
<td>4(pooled)</td>
<td>Cells</td>
<td>Guanidine thiocyanate method</td>
<td>RT-PCR</td>
<td>SGLT1 and GLUT1</td>
</tr>
<tr>
<td>(Takahata et al., 2001) (113)</td>
<td>7(individual)</td>
<td>Cells</td>
<td>MagExtractor MFX-2000</td>
<td>semi-quantitative RT-PCR</td>
<td>IL-18</td>
</tr>
<tr>
<td>(Alcorn et al., 2002) (114)</td>
<td>6(pooled)</td>
<td>Cells</td>
<td>Immunomagnetic separation techniques + RNeasy mini kit</td>
<td>RT-PCR</td>
<td>Drug transporters expressed in lactating mammary epithelial cells</td>
</tr>
<tr>
<td>(Takahata et al., 2003) (115)</td>
<td>5(pooled)</td>
<td>Cells</td>
<td>ISOGEN</td>
<td>semi-quantitative RT-PCR</td>
<td>IP-10 and MIG gene expressed in milk cells</td>
</tr>
<tr>
<td>(Nagatomo et al., 2004) (116)</td>
<td>Pooled</td>
<td>Cells</td>
<td>ISOGEN</td>
<td>Microarray, RT-PCR</td>
<td>OPN gene highly expressed in milk cells</td>
</tr>
<tr>
<td>(Maningat et al., 2007) (109)</td>
<td>pooled</td>
<td>Fat globules</td>
<td>RNeasy, but later switch to Trizol</td>
<td>Microarray, RT-PCR</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>(Frankenberger et al., 2008) (117)</td>
<td>3 (pooled)</td>
<td>Cells</td>
<td>TRI-Reagent</td>
<td>RT-PCR</td>
<td>breast milk showed a very low level of M-Ficolin transcripts</td>
</tr>
<tr>
<td>Reference</td>
<td>Sample Size</td>
<td>Tissue/Cell Type</td>
<td>RNA Extraction Kit</td>
<td>RNA Analysis Method</td>
<td>Gene Expression Analysis</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>--------------------</td>
<td>---------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>(Maningat et al., 2009)</td>
<td>6 (pooled)</td>
<td>Fat globules</td>
<td>TRIzol</td>
<td>Microarray</td>
<td>mammary epithelial cell-specific gene</td>
</tr>
<tr>
<td>(Maningat et al., 2011)</td>
<td>6 (pooled)</td>
<td>Fat globules</td>
<td>TRIzol</td>
<td>Microarray</td>
<td>Global</td>
</tr>
<tr>
<td>(Hassiotou et al., 2012)</td>
<td>Pooled Cells</td>
<td>RNeasy extraction kit</td>
<td>RT-PCR</td>
<td>Pluripotency genes</td>
<td>Milk cells expressed pluripotency genes</td>
</tr>
<tr>
<td>(Twigger et al., 2015)</td>
<td>66 (individual) Cells</td>
<td>RNeasy extraction kit</td>
<td>RT-PCR</td>
<td>Various cell type markers</td>
<td>Stem cell markers myoepithelial marker, and lactocyte marker were highly expressed.</td>
</tr>
<tr>
<td>(Sharp et al., 2016)</td>
<td>Pooled Cells</td>
<td>QIAGEN RNeasy Micro kit</td>
<td>human Affymetrix array, RT-PCR</td>
<td>mammary epithelial cell-specific gene</td>
<td>Milk cells highly expressed milk synthesis related genes</td>
</tr>
</tbody>
</table>
2.6 Conclusion

Fe excreted through the breast milk is the sole source of intake in exclusively breastfed infants. Fe concentrations up to 0.4 µg/ml in mature milk (119) are relatively low in humans, often causing IDA in exclusively breastfed infants, and this could result in permanent neurodevelopmental impairments (71-73).

However, Fe supplementation for newborns and babies in early infancy is controversial, especially since current scientific evidence does not sufficiently support either of two major theories about the low amounts of Fe secreted into human breast milk: 1) the Fe contents of human milk are low in comparison to other mammals, warranting supplementation and 2) the Fe contents of human milk is low due to the need of “Fe withholding”, which protects against intestinal infections, implicating that an infant’s own stores of Fe are sufficient and Fe supplementation would be detrimental.

Until now, knowledge about the Fe secreting pathways in the milk-secretory mammary epithelial cells is lacking. Recent progress in molecular biology has shown that Fe trafficking in mammals depends on a) TF as a carrier protein in the blood (and intracellular storage protein), b) TFRC or TFR2 mediating endocytosis, c) the two membrane transporters, SLC11A1 (NRAMP1) and SLC11A2 (DMT1), mediating transmembrane uptake and d) SLC40A1 (FPN) mediating
transmembrane release. Importantly, in the absorbing intestinal epithelial cells, SLC11A2 functions as the proton-dependent apical transmembrane importer, while SLC40A1 mediates basolateral release into the blood stream.

The molecular basis of Fe excretion into the breast milk has not yet been determined, but no correlation has been found between maternal Fe intake and the Fe content in breast milk (57), supporting the theory that a fixed Fe excretion pathway exists in the secreting mammary epithelial cells. Therefore, we hypothesize that currently known proteins in the Fe trafficking pathway are involved in Fe excretion through the mammary epithelial cells of humans.

Due to ethical restrictions in the sampling of human lactating mammary tissue, most data on Fe transporters in the lactating mammary glands have been obtained from experiments on the rats, where the milk Fe concentrations are at least 25 times higher than that in humans (21). Moreover, the Fe-binding protein in rat milk (TF) is different than the Fe-binding protein in human milk (22). Therefore, the Fe excretion pathway is likely to be different in rats and humans, warranting specific investigations of human Fe excretion pathways.

Current research studies are in the process of establishing experimental settings and models to overcome the ethical restrictions about obtaining tissue samples from lactating human mammary glands. Studies have shown that RNA isolated
from human milk fat globules predominantly contains transcripts from the secreting epithelial cells. RNA isolated from breastmilk cells also contain maternal epithelium transcripts (103).

In addition to the previously described work, this project aims to further advance these issues by determining the Fe transporter genes expressed in lactating mammary epithelial cells using fresh human breast milk as a non-invasive biospecimen.
2.7 Reference


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Exclusive Breastfed Babies: Are They Getting Enough Iron?

Cai C, Morales M, Goldberg J, Friel JK.

Departments of Human Nutritional Sciences and Pediatrics,
University of Manitoba, Winnipeg, Canada, R3T 6C5

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An important question pertaining to the nutritional needs of human infants currently remains unanswered. Are exclusively breastfed infants, whose sole source of iron is breast milk, receiving an adequate amount of iron? The iron concentrations in the milk of other, non-human species are considerably higher than that of humans. The iron concentration in human colostrum is approximately 0.8 µg/ml, while that in mature human milk is 0.2-0.4 µg/ml. In contrast, the iron concentration in mature rat milk is 5-10 µg/ml, a value that is roughly 25 times higher (1). Although iron concentration in human milk is low, it is seemingly independent of the mother’s iron status. It therefore cannot be increased through alterations in the maternal diet, or by use of supplementation (2). Further, studies have shown that the iron concentration in mother’s milk is fairly similar in those with high and low intakes of iron (3, 4). There is still much controversy, as well as a lack of agreement among the scientific community with regards to what should be recommended for infants when it comes to iron. Do they need more iron, possibly provided through supplementation? Are they obtaining sufficient amounts of iron through the natural process of breast feeding? This article aims to show both the concerns and the relevant support for each side of this argument.

IDA is a common cause of anemia in infants. Early iron deficiency is associated with later neurodevelopmental impairment (5). A longitudinal study in Costa Rica assessed changes in cognitive functioning following iron deficiency in infancy.
This was done by scheduling 4 follow-up visits at the ages of 5, 11-14, 15-18, and 19 years old. The results showed that iron deficient infants had lower cognition scores compared to the control group, which included infants with good iron status (6-8). These findings increase the concern that early iron deficiency may lead to irreversible negative changes in long-term neurodevelopment, and accentuate the fact that maintaining adequate iron status is critical to the infant’s neurological development. Since iron is a critical nutrient involved in brain development, it is recommended by Health Canada that babies’ first complementary foods be iron-rich (9).

Although there is no current national data on iron deficiency in Canadian infants over the last 30 years, data from smaller studies suggests that iron deficiency anemia is a significant health problem (10). For example, a study in Vancouver found that approximately 15% of breast-fed infants developed anemia at 8 months of age (11). Further, the prevalence of iron deficiency anemia has been found to be even higher in preterm infants, and may be above 25% during their infancy (12). The rationale in Canada of waiting 6 months before introducing solids to provide iron is not convincing. First of all, it is unclear whether data from current animal models sufficiently represents human iron metabolism. The iron content of mother’s milk from species other than humans and cows is generally much higher (13). Additionally, iron intakes of animals in the a for mentioned studies were
much higher than the daily iron intake of exclusively breast-fed infants per bodyweight. Secondly, since lactoferrin expressed in human milk is only 5% to 8% saturated with iron, it would be expected to have antibacterial properties (14). Iron-binding proteins in human milk, including lactoferrin, may play an important part in resistance to infantile enteritis caused by E. coli (15, 16). This effect is abolished by saturating lactoferrin with iron. Therefore, the anti-pathogenic property of iron may be related to the binding forms of iron, rather than the amount of iron. Thirdly, early supplementation of iron may improve cognitive development of infants. In a double-blind, randomized study, Friel et al demonstrated that healthy, breast-fed babies given iron supplementation between the ages of 1 and 6 months had better scores on the Bayley mental and psychomotor developmental indexes, as well as better visual acuity at 13 months of age than did the babies in the non-iron supplement group (17). From these data, the American Academy of Pediatrics recommended that exclusively breast-fed full-term infants receive 1 mg/kg of iron supplementation per day, beginning at 4 months of age (18). This policy supports the concept that the small amount of iron in human milk may not be enough for the physical and neurological development of exclusively breast-fed infants.

Why did the human species evolve to rely, during the first months of life, on an iron endowment instead of a continuous source of this element in mother’s milk? In order to shed light on such an intriguing question, it must be analyzed from an
evolutionary perspective. It is hypothesized that the environment in which babies were raised, as well as their exposures during the first months of life, were quite different in ancient times as compared to current conditions in developed countries. However, these ancient conditions were not necessarily worse than those of the less fortunate populations in the present day. In ancient times, continual exposure to pathogens from the environment and from food, capable of causing infectious diseases and threatening survival, constantly put the continuation of the human species at risk. This exposure was unavoidable and likely played a key role in shaping several mechanisms for infection prevention and host defense (19, 20). Among these mechanisms, those involving iron obtainment, metabolism, and utilization are of special interest, since iron is an essential element for survival and present in every cell in the body. Iron is also required for intracellular reactions involving certain commensal and pathogenic microbes which reside on the mucosal surfaces of the intestine (21). Health Canada recommends that healthy, full-term infants should be breastfed until 6 months of age, at which point iron-rich complementary foods should be introduced. This recommendation is in accordance with the above hypothesis of auto-regulation in iron obtainment and utilization (9). It is known that human milk is low in iron, and that the iron content in human milk is independent of maternal iron status in all cases except those involving severe maternal anemia (22-24). This suggests regulation for transport of iron from the
mammary glands into breast milk (19). Interestingly, while iron in human milk declines during lactation (24), infants from 6 to 9 months develop an enhanced capacity to absorb iron, thereby avoiding iron deficiency (25). Overall, such mechanisms might suggest that too much iron is not desirable. In fact, there has been speculation as to whether unrestrained absorption and excessive iron in the intestinal lumen might be detrimental. This harmful effect could lead to iron accumulation in tissues, which may lead to damage (26), or the creation of a supportive environment for pathogenic, iron-requiring bacteria (21). Certainly, there are beneficial gut colonizing bacteria, such as lactobacilli and bifidobacteria, which do not rely on free iron. While lactobacilli are iron independent (27, 28), bifidobacteria harvest iron through scavenging compounds called siderophores (29) in order to thrive in the gut.

Thinking back to our ancestors, there are a few questions that become important. How did they cope with the low iron content in milk and the shrinking of the iron endowment between 4 and 6 months of life? Were they iron deficient? Were they anemic? One hypothesis is that the iron endowment used to be higher, owing to a greater transfer of maternal blood into the baby at birth. It is known that the modern day obstetric practice of early cord clamping after delivery reduces placental blood flow into the newborn, leading to an inferior iron status when compared to that of babies subject to delayed cord clamping (30, 31). As
previously mentioned, another possibility is that environmental exposure was much higher, and occurred earlier in life as compared to the modern world. It is speculated that iron from soil, as well as the introduction of iron rich foods, maybe pre-chewed by mothers (20), could have contributed to the prevention of iron deficiency conditions. Additionally, the ability to thrive in an environment in which exposure to pathogens was predominant may have led to the survival of the exposed individuals, allowing for their genes to spread.

From the studies discussed throughout this article, it is apparent that there is no clear answer to the proposed question. We know that our ancestors were able to thrive without any nutritional interventions, thereby suggesting that naturally acquired iron through mother’s milk is satisfactory. On the other hand, it has been shown that iron deficiency, commonly seen in exclusively breastfed babies, can lead to negative physical and neurological deficits. The question therefore, remains unanswered. Future research should focus on identifying the pros and cons to either iron supplementation for infants, or lack thereof. It is vital to human health to gain more clarity on this topic, with the hopes of setting more evidence-based guidelines on iron requirements for future generations.
Reference


Chapter III. Effect of daily iron supplementation in healthy exclusively breastfed infants: a systematic review with meta-analysis

Chenxi Cai¹, Matthew Granger¹, Peter Eck¹, James K Friel¹,²

Authors’ affiliations: 1. Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada. 2. Department of Pediatrics, University of Manitoba, Winnipeg, Canada.

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Citation:
3.1 Abstract

**Background:** The literature on the iron requirements of exclusively breastfed infants contains conflicting data and contrary views. The purpose of this study was to summarize the evidence for both benefits and risks of daily oral iron supplementation with regard to hematologic, growth, cognitive parameters and adverse effects in exclusively breastfed infants.

**Method:** Structured electronic searches were conducted using PubMed, Cochrane Library databases and Google Scholar for randomized controlled trials (RCTs) involving daily iron supplementation in full-term healthy exclusively breastfed infants. Random- and fixed-effects models were used for calculating the pooled estimates.

**Results:** Four RCTs with 511 infants were included in the meta-analysis. Iron interventions had no significant effect on iron deficiency or iron deficiency anemia, serum ferritin level, or hemoglobin level. Iron interventions did result in a significant increase in Bayley psychomotor developmental indices in later life (MD= 7.00, 95% CI, 0.99 to 13.01) and mean corpuscular volume (MD=2.17 fL; 95% CI, 0.99-3.35 fL). Iron supplementation was associated with slower growth during the exclusive breastfeeding period, but the long-term effect is unclear.
**Conclusion:** Limited available evidence suggests that daily iron supplementation has beneficial effects on hematologic parameters and cognitive development, but may delay physical growth in healthy exclusively breastfed infants. There was no evidence to suggest iron supplementation could cause other adverse effects.

**Key words:**

iron supplementation, breastfed infants, iron deficiency, mental development, breast milk

**Abbreviations used:**

ID, Iron deficiency; IDA, Iron deficiency anemia; RCT, randomized controlled trial
3.2 Introduction

Maintaining adequate iron status is critical to the infant’s physical and neurological development (1). Iron deficiency anemia (IDA) is a common cause of anemia in infants (2). The literature on iron requirements of exclusively breastfed infants contains conflicting data and contrary views (3). For exclusively breastfed infants, breast milk is their sole source of iron. The iron concentration in human breast milk is around 0.2 to 0.4 mg/L (4). Based on the average iron concentration in human milk, the Institute of Medicine recommended that the adequate intake of iron for term infants between 0-6 months is 0.27 mg/day (5). These recommendations assume that the small amount of iron in human milk is enough to maintain the growth of the exclusively breastfed infants to 6 months of age. Some researchers suggest that iron stores of full-term infants are sufficient till 6 months of age (6). In addition, it has been suggested that excessive iron intake may have adverse effects, including increased the risk of gastrointestinal infections (7).

Alternatively, concerns have been raised that iron deficiency (ID) is a common cause of anemia in infants which may affect their health and development. Some studies have suggested that early ID can lead to irreversible negative changes in long-term neurodevelopment (8-10). The American Academy of Pediatrics recommends that exclusively breast-fed full-term infants receive 1 mg/kg of iron supplementation per day, beginning at 4 months of age (11). This policy supports
the concept that the small amount of iron in human milk may not be enough for the physical and neurological development of exclusively breast-fed infants.

Although some studies have evaluated the health benefits and risks of iron-fortified food, it is important to determine whether these health effects and risks are associated with iron intake alone or are due to other nutrients (12, 13). Evidence for the benefits and risks of daily oral iron supplementation in exclusively breastfed infants has not been specifically evaluated in a systematic review.

The aim of this systematic review and meta-analysis is to summarize the evidence of the health effects of daily iron supplementation with respect to exclusively breastfed infants before 6 months of age. The data provided could assist policy makers and clinicians to understand the benefits and safety of daily iron supplementation in exclusively breastfed infants.
3.3 Methods

3.3.1 Search strategy

This systematic review was registered with PROSPERO (registration number: CRD42015026031). PubMed, Cochrane Library databases and Google Scholar were searched up to August 26, 2015. Reference lists from published reviews and meta-analyses were also reviewed. Search terms included: (iron or iron supplement or iron supplementation) and (infants or toddler or breast feeding or breastfed or breastfeeding). Studies were included if they fulfilled the following criteria: (1) randomized controlled trial; (2) assessing the effects of iron supplementation on health (iron status, physical growth, neurodevelopment, morbidity due to infectious disease); (3) with full-term healthy exclusively breastfed infants. Studies remained eligible when the infants were nearly exclusively breastfed (exclusive breastfeeding but were permitted to give small portions of foods). Studies were excluded if they included the infants that were low birth weight (<2500 g), or preterm (<37 weeks), or not nearly exclusively breastfed during the intervention, or supplementation with solid foods or formula, or combined iron with other nutrients as the intervention.

3.3.2 Data extraction

The following information was extracted from the articles: the first author, published year, study design, study population characteristics (origin and age),
feeding practice, iron form and dose, number of subjects, intervention time and outcome measurements. One author entered data and a second author checked entries for accuracy. Discrepancies were resolved through discussion.

3.3.3 Assessment of risk of bias in included studies

Two authors (Cai, C. and Granger, M.) independently assessed the quality of included RCTs in seven domains (sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting) by the Cochrane risk of bias tool (14). All the differences were resolved by discussion with the other two authors (Friel, J. and Eck, P).

3.3.4 Statistical analysis

The ID rate, diarrhea rate, weight gains, length gains, hemoglobin level, mean corpuscular volume, serum ferritin, mental development index and visual acuity Z-score were compared between breastfed infants who received iron supplementation and breastfed infants who did not received iron supplementation. The continuous data was extracted as mean ± standard deviation (SD), while the dichotomous data was extracted as even/total number. If the study reported two subgroups, the combination formulae were used to combine them into a single group (15). The continuous data were reported as weighted mean difference (WMD) while the
dichotomous data were presented as risk ratios (RR) with 95% confidence intervals (CIs). P < 0.050 was considered statistically significant. I² was used to evaluate the heterogeneity. A random-effects model was used if heterogeneity was detected (I² > 50%); otherwise, a fixed-effects model was used. All analyses were performed using Review Manager, Version 5.3 (Cochrane Collaboration, Oxford, UK).
3.4 Results

The initial search of the databases mentioned above identified 8456 publications which included 37 relevant articles for full-article review (Figure 3-1). An additional 7 studies were identified from previous reviews and meta-analyses. After excluding the studies that investigated the iron supplementation with solid food or formula or multivitamin mixtures, or if the infants were not nearly exclusively breastfed during the intervention, there remained RCTs (five articles) that were included in this systematic review and meta-analysis. A total 511 infants from Sweden, Honduras, Canada, Turkey and China were included in these four RCTs.
Figure 3-1 Flowchart of study selection.
3.4.1 Study characteristics

The characteristics of the included trials are summarized in Table 3-1. The source of iron included ferrous sulfate (16-19) and iron amino-acid chelate (20). The iron supplement intervention during the exclusively breastfeeding period lasted between two to two and half months. One RCT started the intervention at one month of age (17) while the other three RCTs started at four month of age (16, 18-20).
<table>
<thead>
<tr>
<th>Study</th>
<th>Origin</th>
<th>Study design</th>
<th>Sample Size</th>
<th>Age (month)</th>
<th>Intervention duration during the exclusively breastfed period</th>
<th>Definition of inclusion population</th>
<th>Intervention/control</th>
<th>Outcome measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewey, Domellof et al. 2002; Domellof, Cohen et al. 2001</td>
<td>Sweden, Honduras</td>
<td>RCT</td>
<td>101/131</td>
<td>4</td>
<td>2</td>
<td>All infants were exclusively or nearly exclusively breast-fed to 6 months</td>
<td>1 mg/kg/d ferrous sulfate Placebo: unknown</td>
<td>Growth Iron status Adverse effects</td>
</tr>
<tr>
<td>Friel, Aziz et al. 2003</td>
<td>Canada</td>
<td>RCT</td>
<td>77</td>
<td>1</td>
<td>2.5</td>
<td>All infants were exclusively or nearly exclusively breast-fed to 3.5 month</td>
<td>7.5 mg per day of ferrous sulfate Placebo: syrup</td>
<td>Growth Iron status Mental development Adverse effects</td>
</tr>
<tr>
<td>Yurdakok, Temiz et al. 2004</td>
<td>Turkey</td>
<td>RCT</td>
<td>79</td>
<td>4</td>
<td>2</td>
<td>Exclusively breast-fed to 6 months</td>
<td>1 mg/kg/d ferrous sulfate control: no iron</td>
<td>Iron status Adverse effects</td>
</tr>
<tr>
<td>Wang, Wu et al. 2012</td>
<td>China</td>
<td>RCT</td>
<td>123</td>
<td>4</td>
<td>2</td>
<td>Healthy term breast-fed infants exclusively breast-fed to 6 months</td>
<td>1 mg/kg/d Iron amino-acid chelate Control: no iron</td>
<td>Growth Iron status Adverse effects</td>
</tr>
</tbody>
</table>

Table 3-1 Summary of studies included in the meta-analysis
3.4.2 Risk of bias assessment

The quality of included RCTs were assessed by the Cochrane risk of bias tool (11). All included trials had certain methodological limitations (Figure 3-2). The major limitations were unclear allocation concealment, unclear blinding of outcome assessment and high risk of bias in selective reporting.
Figure 3-2 Assessment of bias risk.

(A) Summary of bias risk for each trial. (B) Risk of bias graph for all included trials. For each bias domain, information is either from trials with low risk of bias (green), or from trials with unclear risk of bias (yellow), or from trials with high risk of bias (red).
3.4.3 **Iron deficiency, iron deficiency anemia and hematologic parameters**

Three RCTs (n=251) were included in the analysis (Figure 3-3). There was no difference in the incidence of IDA or ID between the iron supplement group and control group (RR, 0.59; 95% CI, 0.27-1.30; P=0.19). Two RCTs reported extractable data on iron status parameters (Figure 3-4). Although serum hemoglobin levels (MD=1.78 g/L; 95% CI, -1.00-4.57 g/L; P=0.21) and ferritin levels (MD=-17.26 µg/L; 95% CI, -40.96-75.47 µg/L; P=0.56) were similar between both groups, mean corpuscular volume (MCV) was significantly higher in the group supplemented with iron (MD=2.17 fL; 95% CI, 0.99-3.35 fL; P=0.0003).

![Figure 3-3 Effect of iron supplementation on IDA or iron deficiency in exclusively breastfed infants. M-H=Fixed effects, Mantel-Haenszel, CI=confidence interval, IV=intravenous.](image-url)

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Iron Events</th>
<th>Control Events</th>
<th>Total</th>
<th>Weight M-H, Fixed, 95% CI</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dornell et al. 2001</td>
<td>5</td>
<td>78</td>
<td>8</td>
<td>53.1%</td>
<td>0.58 (0.21, 1.67)</td>
</tr>
<tr>
<td>Fri et al. 2003</td>
<td>1</td>
<td>30</td>
<td>2</td>
<td>14.7%</td>
<td>0.40 (0.04, 4.15)</td>
</tr>
<tr>
<td>Yurdakok et al. 2004</td>
<td>3</td>
<td>21</td>
<td>4</td>
<td>27.2%</td>
<td>0.71 (0.16, 2.80)</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>127</td>
<td><strong>124</strong></td>
<td>100.0%</td>
<td>0.59 (0.27, 1.30)</td>
<td></td>
</tr>
<tr>
<td><strong>Total events</strong></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity Chi² = 0.16, df = 2 (P = 0.91); I² = 0%

Test for overall effect: Z = 1.31 (P = 0.19)
3.4.4 Growth

Three studies addressed the effect of iron supplementation on growth in exclusively breastfed infants (Figure 3-5). Iron supplementation had no effect on length gain (MD= -0.01 cm; 95% CI, -0.08-0.06 cm; P= 0.77), but had a significant negative effect on weight gain (MD= -0.04 kg; 95% CI, -0.07- -0.01 kg; P= 0.003) and head circumference gain (MD= -0.14 cm; 95% CI, -0.18- -0.09 cm; P<0.00001).
Figure 3-5 Effects of iron supplementation on (A) Weight gain, (B) Length gain, and (C) Head circumference gain in exclusively breastfed infants. CI=confidence interval, IV=intravenous.

3.4.5 Mental and visual development

Mental and psychomotor developmental indices and visual acuity at 13 months of age were assessed using the Bayley Scales of Infant Development and the Teller visual acuity in the included RCT. Outcomes were analyzed by intention to treat. The Bayley psychomotor developmental index was significantly higher in the iron supplement group (MD 7.00, 95% CI 0.99 to 13.01; p= 0.02) (Figure 3-6). There were no significant difference in Bayley's mental development index (MD 0.00, 95%
CI -5.50 to 5.50; p= 1.00) and visual Z-scores (MD 0.52, 95% CI -0.16 to 1.20; p= 0.14) in infants receiving iron compared with control.

Figure 3-6 Effects of iron supplementation on (A) Mental developmental index, (B) Psychomotor developmental index, and (C) Visual acuity in exclusively breastfed infants. CI=confidence interval, IV=intravenous.

3.4.6 Adverse effects

Four studies presented adverse effects data, but only three presented extractable data. One RCT reported that there were no episodes of diarrhea related to iron supplementation (18), while the other two RCTs reported no infections related to iron supplementation (17, 20).
3.5 Discussion

This study is the first systematic review and meta-analysis to analyze the effect of daily iron supplementation in healthy exclusively breastfed infants. Previous systematic reviews evaluated the effects of daily iron supplementation in low birth weight infants (21) and 2 to 5 years old children (22). However, the specific effects of daily iron supplementation on healthy exclusively breastfed infants have not been addressed in other studies. Our study extends the literature in this specific population, which could be used by policy makers and clinicians.

Control group participants of included studies were at high risk of developing ID/IDA (12% prevalence of ID and IDA at endpoint in the control groups of the meta-analysis). Daily supplementation of ferrous sulfate at a dose of 1 mg/kg/d or 7.5 mg/day did not significantly reduce the incidence of ID/IDA (7% prevalence of ID and IDA at the endpoint in the iron supplementation groups of the meta-analysis). However, a 42% reduction in the prevalence of ID/IDA should merit the attention of clinicians since it has been shown that infants with ID/IDA can be subjected to irreversible negative cognitive development and altered neurological functioning in adulthood (8-10, 23).

Although there were no significant differences in ferritin or hemoglobin levels in both groups, those infants with iron supplementation had higher mean corpuscular
volume compared to infants in the control group. MCV is reduced in ID (24, 25). Taken together with the ID/IDA results, iron supplementation did improve iron status of breastfed infants.

This meta-analysis found that daily iron supplementation resulted in slower weight gain and head circumference gain over the duration of treatment. There was no effect on length gain. It has been suggested that iron supplementation may impair the growth of infants by inhibiting the absorption of other essential nutrients (26). Alternatively, while the connecting mechanism between growth deficit and iron is not entirely known (27), some recent studies suggest an association between obesity and ID in children (28-30). Taken together, it is unknown whether decreased weight gain and head circumference gain in infancy will impair long-term growth of infants or is an indicator for developing obesity. These data reinforce the need for a long-term iron supplementation trial in order to address these questions.

There was a statistically significant positive effect of iron supplementation on the Bayley psychomotor developmental index. This finding supports the rationale that iron is needed for the rapidly growing brain in the first year of life as it is involved in the development of brain and neural pathways (31).
The greatest concern of any supplemental intervention is that it may increase morbidity, however, there is no evidence that iron treatment results in an increase in infant morbidity from this meta-analysis or previous reports (22, 26).

3.6 Study Limitations

Limitations of this meta-analysis are that the sample sizes of the studies analyzed are small and only four RCTs were eligible for the meta-analysis. Among the eligible studies, the reported outcomes of the long-term effect of iron supplementation on mental development of breastfed infants are limited, and could restrict the outcome assessment.

3.7 Conclusions

This systematic review confirms that for healthy exclusively breastfed infants, daily iron supplementation results in increased MCV levels and has a beneficial effect on cognitive development. However, iron supplementation can slow the physical growth of infants. There was no evidence to suggest iron supplementation could cause other adverse effects.
Acknowledgments

Statement of authors’ contributions to manuscript

C.C, and F.J.K. designed research; C. C and G. M. conducted research; C. C, G. M, E.P and F.J.K analyzed data; and C. C wrote the paper. F.J.K. had primary responsibility for final content. All authors read and approved the final manuscript.
3.8 References


Bridge to Chapter IV

The previous chapters showed that Fe supplementation could improve the Fe status and psychomotor development of exclusively breastfed infants. However, Fe in human milk is independent of the mother’s iron status and cannot be increased through maternal diet or Fe supplementation. The composition of human milk is the result of complex transport processes across different extracellular and intracellular membranes, which are in great part mediated by solute carrier proteins. However, the identities of the proteins mediating the transfer of Fe into human milk remain to be determined. Hence, there is a lack of knowledge about the molecular mechanisms regulating Fe concentrations (1). Therefore, chapter IV comprises a manuscript that demonstrates the expression of Fe transporters in human lactating epithelial cells. This manuscript also illustrates the potential Fe transport pathway in human lactating epithelial cells.

Reference:

Chapter IV. Gene expression profiles suggest iron transport pathway in the lactating human epithelial cell

Chenxi Cai\(^1\), Peter Eck\(^1\), and James K Friel\(^{1,2}\)

Authors’ affiliations: 1. Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada. 2. Department of Pediatrics, University of Manitoba, Winnipeg, Canada.

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Citation:

91
4.1 Abstract

**Background:** The molecular background of iron excretion into breast milk has not been determined in humans. The objective of this study was to examine the expression of known iron transporters in mRNA extracted from human milk fat globules to deduce which known transporters are responsible for iron excretion into human milk.

**Methods:** The expression of iron transporters in mRNA from human milk fat globules and mouse mammary epithelial cell lines were determined by quantitative Real-time polymerase chain reaction (qPCR).

**Results:** The expression of the transferrin receptor 1 (*TFRC*), divalent metal transporter 1 (*SLC11A2*), transferrin (*TF*) and lactoferrin (*LTF*) was confirmed in RNA isolated from the human milk fat globule. Similar expression was observed in the mouse mammary epithelial cell line HC11 in resting and lactating phenotypes. No iron export protein could be determined in the RNA isolated from fat globules in human breast milk and a human mammary epithelial cell line.

**Conclusion:** The lack of iron exporters in the human mammary epithelia, in conjunction with the presence of lactoferrin suggests that transmembrane transport is not a major route of iron excretion into human milk.

**Key words:** iron transporters; human milk; gene expression
4.2 Introduction

Maintaining adequate iron status is critical to the infant’s physical and neurological development. For exclusively breastfed infants, breast milk is their sole source of iron. The iron concentration in human colostrum is approximately 0.8 µg/mL and in mature milk is 0.2-0.4 µg/mL (1). The Institute of Medicine (IOM), based on the average iron concentration in human milk, suggested that the adequate intake of iron for term infants between 0-6 months is 0.27 mg/day (2). This recommendation assumes that the small amount of iron in human milk is enough to maintain the growth of exclusively breastfed infants to 6 months of age. However, the literature on iron requirements of full-term infants before 6 months of age contains conflicting data and divergent views. Although the iron concentration is low in human milk, it is independent of the mother’s iron status and cannot be increased through maternal diet or iron supplementation (3), since milk iron concentration is similar between mothers of high iron or low iron intakes (4, 5). To date, the molecular background of iron secretion into human milk has not been clarified.

Iron trafficking in mammalian systems depends on iron transporters. Currently, most data on iron transporters in the lactating mammary gland have been obtained principally from experiments on the rat, where milk iron concentrations are at least
25 times higher compared to humans (6). In addition, unlike the mouse and human, the iron binding protein in rat milk is transferrin (7). Although mouse milk contains higher iron than human milk, human and mouse milk both contain lactoferrin as an iron binding protein (8). Therefore, the iron secretion pathway of the rat is likely to be distinct from human and mouse, with the latter being a better rodent model for a comparative study (Iron transporter coding genes in human, mouse and rat were listed in Supplemental Digital Content Table S4-1).

Recently, breast milk has been studied as a source of mammary epithelial cell RNA (9, 10). The mRNA and other cytoplasmic remnants in epithelial cells are enveloped by the cell membrane and secreted into milk via fat globules (11). Thus, RNA extracted from breast milk may be used to reflect the biology of the lactating mammary epithelial cell (12). The gene expression of certain proteins in the mammary epithelial cell have been previously examined by extracting mRNA from breast milk (12). However, the gene expression of iron transporters in the human lactating epithelial cell has not been studied, to our knowledge. In the present study, we investigate known iron transporters’ expression in RNA extracted from human milk fat globules, a human breast cancer cell line and mouse mammary epithelial cell lines. We aimed to determine which iron transporters are responsible for iron secretion into the human lactating mammary gland and to compare the iron transporter profiles of different mammary epithelial cell lines.
4.3 Methods

4.3.1 Study design

This study was designed to determine the expression of known iron transporters in human and mouse models by quantitative Real-Time polymerase chain reaction (RT-qPCR) to deduce which transporters are responsible for iron excretion into breast milk.

Mouse mammary epithelial HC11 cells (a gift from Dr. Carrie Shemanko, University of Calgary, Canada) were the basis for the mouse assay, which were isolated from a midpregnant BALB/c mouse mammary gland. HC11 differentiation into a secretory phenotype can be induced by prolactin (13). The expression of iron transporter genes (Tfrc, Tfr2, Slc11a1, Slc11a2, Slc40a1) in the HC11 cell line before and after prolactin stimulation were assessed to compare the iron transporter profiles in both the non-lactating and lactating mouse epithelial cell lines.

RNA extracted from the fat fraction of human milk was used to investigate iron transporter expression in lactating epithelial cells.

The MCF-7 (American Type Culture Collection, Rockville, MD) carcinoma cell line was utilized as a proxy for iron transporters’ expression in non-lactating mammary cells.
The expressions of the seven human genes TFRC, TFR2, SLC11A1, SLC11A2, SLC40A1, TF, and LTF, which are involved in iron transport, were assessed by qPCR.

4.3.2 Human subjects

This study was approved by the University of Manitoba Bannatyne campus research ethics board and written informed consent was obtained from all participants. Fresh human milk samples were obtained from nine eligible lactating donors who volunteered to participate in this study. The inclusion criteria were that mothers must be successfully breastfeeding and the mothers and their infants were healthy at the time of the study. Exclusion criteria were not breastfeeding or receiving medication during breastfeeding.

4.3.3 Milk collection and RNA extraction

Fresh breast milk was collected simultaneously from both breasts via a standard breast pump (Medela AG, Baar, Switzerland) and immediately placed in a cooler surrounded by ice pads. Total mRNA of breast milk was prepared with a hybrid of the Trizol protocol and QIAGEN RNeasy Miniprep kit (Qiagen, Valencia, CA). Briefly, fifteen milliliters of breast milk were transferred into a sterile, RNase-free tube and then centrifuged at 1600 x g for 10 minutes. The supernatant fat layer was transferred to a new tube, using a sterile cell scraper. Ten milliliters of TRIzol
reagent (Invitrogen Life Technologies, Burlington, ON) were added to the fat layer. Then the homogenized samples were mixed with 2 mL of chloroform (Fisher scientific, Ottawa, ON) and centrifuged at 1600 x g for 5 minutes. The upper aqueous phase was transferred to a new tube and mixed with 1 volume of 70% ethanol. Total RNA was isolated from the mixture using a QIAGEN RNeasy Miniprep kit following the manufacturer’s suggested procedures. And total RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was measured by gel electrophoresis.

4.3.4 Cell culture and RNA extraction

The HC11 cells were grown in a Growth medium containing RPMI-1640 medium (Sigma, St. Louis, MO) with Penicillin (100 IU/mL)/Streptomycin (100 µg/mL), 10 ng/mL Epidermal Growth Factor, 5 µg/mL insulin and 10% Fetal Bovine Serum. To differentiate the HC11 cells into their secretory phenotypes, they were cultured in a Differentiation Medium which contained 5 µg/mL prolactin. The MCF-7 cells were grown in Eagle’s MEM (GIBCO Invitrogen, Carlsbad, CA) with 10% Fetal Bovine Serum, 10 µg/mL insulin and Penicillin (100 IU/mL) /Streptomycin (100 µg/mL). The cultured cells were washed twice with phosphate buffered saline and lysed by TRIzol reagent. Total mRNA was prepared with RNeasy Miniprep kit (Qiagen, Valencia, CA).
4.3.5 Gene expression analysis by qPCR

Total mRNA of each sample was reverse transcribed using SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The relative expressions of the iron transporters’ messenger RNA were determined by qPCR for \textit{TFRC}, \textit{TFR2}, \textit{SLC11A1}, \textit{SLC11A2}, \textit{SLC40A1}, \textit{TF} and \textit{LTF}. Assays were designed using the PrimeTimeqPCR design tool. Amplification reactions were performed with TaqMan Gene Expression Master Mix, 20X TaqMan Gene Expression Assay/PrimeTimeqPCR Prime Assay (Supplemental Digital Content, Table S4-1), and sample cDNA. Samples were analyzed by Applied Biosystems StepOnePlusTM Real-Time PCR System. Ten serial dilutions of standard cDNA (1:10 factor; from 10ng to $1 \times 10^{-5}$ pg) were used to construct a standard curve for each gene. For the endogenous control, \textit{18s rRNA} was used as the human housekeeping gene. \textit{Actb} was used as the mouse housekeeping gene. Expression of the transcript of interest was normalized to the respective housekeeping gene.

In order to confirm the source of mRNA in human milk, the expression of mRNAs encoding epithelial marker (\textit{MUC1}, mucin 1, cell surface associated) and mRNAs encoding leukocyte marker (\textit{CD45}, protein tyrosine phosphatase, receptor type, Cp) were examined by qPCR. qPCR primer sequences were listed in Supplemental Digital Content Table S4-2.
4.3.6 Statistical analysis

Data was expressed as mean ± SD and analyzed by Two-tailed unpaired t-test. Statistical analysis was conducted using GraphPad Prism 5.0. Significance is accepted at $P < 0.05$. 
4.4 Results

4.4.1 Subject characteristics

Nine healthy women between 25-40 years of age were recruited for the study. All the infants were healthy full-term infants and their ages were between 5-6 months (Supplemental Digital Content, Table S4-3). No maternal nor infant illness or infection was reported when the mothers provided the samples.

4.4.2 Origin of the mRNA extracted from the human milk fat globule

To determine the relative amount of mRNA from epithelial cells to that of white blood cells, we compared MUC1 to CD45 expression in the fresh breast milk, as epithelial marker gene and leukocyte marker gene, respectively. MUC1 was highly expressed in all of breast milk samples (Figure 4-1A). In contrast, very low levels of CD45 were found in five of the nine samples, indicating that the majority of mRNA extracted from the fat layer of human milk originated from the epithelial cells. The presence of none or very low CD45 transcripts indicates neglectable contributions from leukocytes during the extraction procedure (9).
Figure 4-1 Iron transporter gene expression in mRNA derived from human milk (N=9).

A, epithetical cell marker MUC1 is strongly expressed, while leucocyte marker CD45, is very low or absent in the breastmilk of nine individuals (S1-S9). B, transferrin receptor TFRC and iron transporter SLC11A2 as well as transferrin (TF) and lactoferrin (LTF) are present in the same breastmilk samples (S1-S9), while transferrin receptor 2 (TFR2) and the iron exporter SLC40A1 are absent.
4.3.4 Iron transporters transcripts in human milk fat globules

The presence of the TFRC and the SLC11A2, but not the SLC40A1 transcripts were confirmed in human milk fat globules (Figure 1B). Very small amounts of SLC11A1 were detected and coincided with CD45 expression, indicating a neglectable contribution of leukocytes transcripts. Transcripts for the iron binding proteins TF and LTF were also detected.

4.4.5 Iron transporters expressed in the lactating and dormant phenotypes of the mouse mammary epithelial cell line HC11

The mouse mammary epithelial cell line HC11 is the only existing cell model of an inducible lactating phenotype. Upon prolactin stimulation, a lactating phenotype is induced (13). In both states, Tfrc and Slc11a2 were expressed, while the presence of Tfr2, Slc11a1 and Slc40a1 could not be detected (Figure 4-2). The induction of the lactating phenotype by prolactin resulted in a significant downregulation of Tfrc and Slc11a2 transcripts (Tfrc/Actb ratio, before prolactin: 0.54±0.01; after prolactin: 0.49±0.01; P=0.014; Slc11a2/Actb ratio, before prolactin: 1.11±0.01; after prolactin: 1.02±0.01; P=0.005) (Figure 4-2).

An identical expression pattern was found between the mouse mammary epithelial model and mRNA extracted from human milk fat globules. TFRC/Tfrc and the SLC11A2/Slc11a2 are present, while TFR2/Tfr2, SLC11A2/Slc11a1 and
SLC40A1/Slc40a1 could not be detected. However, the ratio of TFRC/Tfrc to SLC11A2/Slc11a2 expression was reversed between mouse and human, resulting in a significantly higher expression of Slc11a2 compared to Tfrc in HC11 compared to that of human fat globular mRNA.

Figure 4-2 Iron transporters gene expression in HC11 cells with (grey bars) or without prolactin stimulation (white bars).

Transferrin receptor Tfrc and iron transporter Slc11a2 are expressed in both cellular states and upregulated upon prolactin stimulation. No expression of the iron exporters Slc11a2 and Slc40a1 as well as the transferrin receptor Tfr2 is observed. Data are presented as mean ± SD. *p < 0.05.
4.4.6 Iron transporters expressed in the human breast ductal carcinoma cell line MCF7

The expression of MUC1 was determined in the human breast cancer cell line MCF7, while CD45 was absent (Figure 4-3A), indicating its utility as a model for dormant mammary epithelial cells. In concordance with the above results, TFRC, SLC11A2 and TF were expressed in ratios comparable to the human milk samples, while SLC11A1 and SLC40A1 are absent (Figure 4-3B, compare to Figures 4-1B). In contrast, TFR2 is present, while LTF expression could not be confirmed.

Overall, the gene expression seems to be consistent with a differentiated mammary epithelial phenotype.
Figure 4-3 Iron transporters gene expression in MCF-7 cells.

A, expression of the epithetical cell marker *MUC1* and absence of the leucocyte marker *CD45* indicates the epithelial origin of the cancer cell line. B, transferrin receptors *TFRC* and *TFR2*, iron transporter *SLC11A2*, and transferrin (*TF*) are present, while the iron exporters *SLC40A1, SLC11A1* are absent. The notable absence of lactoferrin (*LTF*) indicates a non-lactating epithelia phenotype.
4.5 Discussion

To circumvent ethical and technical problems in obtaining tissue samples from the lactating human mammary gland, we explored alternative and readily available sources of RNA to determine transcript levels of iron transporters in the lactating and resting human mammary cells. Breast milk fat globules envelope cytoplasmic remnants during their secretion into the milk, and are therefore a valuable source of RNA from lactating human mammary cells, as confirmed in this work and by others (12). For our dataset, the high MUC1 expression, coinciding with only traces of CD45 in some samples, confirms that the determined gene expression represents RNA from lactating epithelial cells rather than white blood cells (9).

The molecular nature of iron excretion into the human milk remains undetermined. The presented transcriptome data from human milk fat globules suggest an iron transport pathway containing the transferrin receptor TFRC, transmembrane solute carrier DMT1, and the secreted iron binding proteins lactoferrin (LTF) and transferrin (TF). However, the iron transmembrane release protein, ferroportin, encoded by the SLC40A1 gene, is absent. Similar, another protein mediating cellular iron release, SLC11A1, is absent in fat globular samples solely representing lactating epithelial origin.
An expression pattern comparable to milk fat globules was observed for the MCF7 cell line, representing a non-lactating mammary carcinoma cell; however, lactoferrin was absent. We speculate that lactoferrin expression is activated upon lactation, and that it therefore might have a role in iron excretion only in the lactating state.

The presented data also indicate that the mouse might be a more suitable model organism for human lactation, compared to the rat, since our data confirm a lack of Slc40a1 expression in a mouse model of mammary epithelial cells (HC11). Slc40a1 expression had been reported for the lactating rat (14).

The expression patterns strongly indicate the “transferrin cycle” as a mechanism of iron uptake into the human mammary epithelial cell, as it is known for other cell types (15). In the “transferrin cycle”, the iron-transferrin complex in the plasma binds to membrane bound TFRC. Upon binding the transferrin-TFRC complex is endocytosed, followed by iron release in the endosome. The iron is carried across the endosome membrane into the cytosol via SLC11A2 (DMT1), where it can be utilized as enzymatic co-factor or stored bound to ferritin (Figure 4-4).
Figure 4-4 Hypothetical model of the iron transport pathways in human lactating epithelial cells according to the here presented expression data.

Extracellular iron (red dot) in serum is exclusively bound to transferrin (TF), transferrin binds to transferrin receptor (TFRC) on the basal side of the cell, and the transferrin-transferrin receptor complex is endocytosed. In the resulting endosome, the iron is released and transported across the endosomal membrane by SLC11A2, also known as DMT1. In the cytosol, iron is stored in a ferritin complex, or utilized as a co-factor for enzymatic reactions. A possible excretion pathway remains to be determined. One possible excretion mechanism could involve an iron-lactoferrin complex (LTF), since no iron exporter expression could be confirmed in this work. Alternatively, no iron excretion mechanism might exist.
No transcripts of known iron membrane exporters are present in breast milk; however, lactoferrin is expressed. This suggests that if any iron excretion into the human milk occurs, it might be mediated through a lactoferrin complex rather than direct transmembrane transport, supported by the fact that lactoferrin is the only known iron-binding protein in human milk (16). The lack of transmembrane release might explain the low iron concentrations of human milk, compared to rat, where the known iron exporter ferroportin, encoded by the SLC40A1 gene, is present in the lactating rat mammary gland (14).

Another hypothesis is simpler but more controversial. There is no mechanism for active iron secretion into breast milk. Only iron from milk cells is present in breast milk. The significance of this phenomenon will have to be validated; however, we speculate support for the theory of iron withholding for the infant as an evolutionary means to protect from intestinal infections. This would explain the absence of any cellular exporters. In support of this reasoning, it has also been suggested that lactoferrin, the iron-binding protein in human milk, can protect the infant from infection by depriving iron from pathogenic bacteria (17). The low iron content of lactoferrin may be a protective mechanism to provide a bacteriostatic function (18). In conclusion, we present transcript based evidence for the iron uptake and excretion pathways in the human and mouse mammary epithelial cell, suggesting known cellular uptake pathways, but a lack of capacity for
transmembrane excretion. The only means of secretion into the breast milk might be via an iron-lactoferrin complex. However, the data do not rule out a complete lack of iron excretion capacity into human milk. The current study has several limitations which should be acknowledged. First, the expression of iron transporters was determined at the mRNA level, further studies to demonstrate expression at the protein level is needed to confirm this finding. Moreover, localization and mechanistic studies are needed to further confirm the proposed pathway.
4.6 References


Input for authorship

Chenxi Cai, MSc.

- Substantial contributions to the conception and design of the work
- Acquisition and analysis of data for the work
- Drafting the work
- Taking primary responsibility for communication with the journal during the manuscript submission, peer review, and publication process, and typically ensures that all the journal’s administrative requirements

Peter Eck, Ph.D.

- Substantial contributions to the conception and design of the work
- Revising the work critically for important intellectual content
- Analysis of data for the work

James K Friel, Ph.D.

- Substantial contributions to the conception and design of the work
- Revising the work critically for important intellectual content
- Analysis of data for the work
## Supplementary material

Table S4-1 Comparison of the iron transporter coding genes from human, mouse and rat (information extracted from NCBI).

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Table S4-2 Taqman and IDT Primer sequences used in quantitative qPCR for iron transporters, housekeeping genes and markers.

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**Table S4-3** Characteristics of participates and their neonates.

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Bridge to Chapter V

The previous chapter describes evidence for the expression of Fe transporters in mRNA from milk fat. It remains unknown as to whether mRNA from milk fat and milk cells reflects similar expressions of Fe transporter. The following chapter comprises a manuscript that investigated the expression of known Fe transporters in RNA extracted from human milk fat and cells. It also aimed to determine which Fe transporters are present in human milk cells, and conducted a comparative analysis of Fe transporters expressed in the RNA extracted from human milk fat and cells.
Chapter V. A Comparative Analysis of Iron Transporters

Expressed in the mRNA Extracted from Human Milk Fat and Cells

Chenxi Cai\textsuperscript{1}, Zhengxiao Zhang\textsuperscript{3}, James K Friel\textsuperscript{1,2} and Peter Eck\textsuperscript{1}

Authors’ affiliations: 1. Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada. 2. Department of Pediatrics, University of Manitoba, Winnipeg, Canada. 3. Department of Animal Science, University of Manitoba

This manuscript is under submission.
5.1 Abstract

Objectives: The gene expression profiles of iron transporters in human lactating mammary glands are poorly understood. The present study was a comparative analysis of iron transporters expressed in the RNA extracted from human milk fat and cells.

Method: Human milk samples were collected from 15 eligible lactating women, and the expression of iron transporters in RNA from human milk fat globules and human milk cells was determined for each sample using quantitative real-time polymerase chain reaction.

Results: It was found that the human fat RNA originated from epithelial cells, while the human cell RNA originated from both epithelial cells and leukocytes. The expression of TFRC, TFR2, SLC11A2, TF and LTF was confirmed in RNA isolated from both the milk fat and milk cells. Two other transporters, SLC11A1 and SLC40A1, were found only in RNA isolated from milk cells.

Conclusion: RNA from milk fat and milk cells could be used as a novel tool to investigate the biological functions of lactating epithelial cells and lactating mammary glands respectively by different purposes. The lack of iron exporter in lactating epithelial cells suggests that a novel iron transport routine may be used during human lactation.
5.2 Introduction

Iron is an essential trace mineral that plays an important role in infant health; the capacity of human milk to provide an adequate amount iron is critical to exclusively breastfed infants. The iron concentration of human milk is approximately 0.3µg/mL, which is far lower than that found in the milk of other mammals, such as rats and mice (1, 2). Moreover, the iron content of human milk cannot be increased by maternal diet (3). Due to the ethical and technical issues, to date, the iron transporters in the lactating tissues studies have primarily been conducted in rat, and few human data are available.

Human milk has recently been proposed as a non-invasive source from which to obtain RNA from lactating mothers. In epithelial cells, mRNA is enveloped and secreted in milk via fat globules, so human milk fat could be a good source of mRNA (4). Further to this, milk cells are a rich source of mRNA. Human milk contains heterogeneous populations of maternal cells, which include epithelial cells, leukocytes and stem cells (4), and these cells are shed into breast milk during lactation. It has been suggested that if the mother and infant are healthy, then the domain cells are epithelial cells and that leukocytes compose less than 2% of the cell population (4).

We recently found a lack of the only known iron exporter (ferroportin) in lactating mammary glands by determining the expression of iron transporters in mRNA
from human milk fat globules (5). This suggested that the iron secretion mechanism in human lactating mammary glands is different from that of the rat - the only investigated rodent model. It also indicated that there is either another excretion routine for iron, or that there is no excretion pathway. This was the first study to investigate the expression of iron transporters in human lactating mammary tissue. However, these results were based only on mRNA from milk fat, and further studies are required to confirm these findings. Moreover, the expression of iron transporters in mRNA from human milk cells has not been studied, to the best of our knowledge, and it remains unknown as to whether mRNA from milk fat and milk cells reflects similar genomic information. In the present study, we investigated the expression of known iron transporters in RNA extracted from human milk fat and cells. We also aimed to determine which iron transporters are present in human milk cells, and conducted a comparative analysis of iron transporters expressed in the RNA extracted from human milk fat and cells.
5.3 Method

5.3.1 Human subjects

This study was approved by the University of Manitoba Bannatyne campus research ethics board. A written informed consent and a confidential questionnaire were obtained from all participants. Fresh human milk samples were obtained from fifteen eligible breastfeeding mothers. The inclusion criteria were that mothers must be successfully breastfeeding, the mothers and their infants were healthy (do not have any infection) at the time of the study, and the infants are no older than 6 month of the age. Exclusion criteria were not breastfeeding or receiving medication during breastfeeding or the mothers or infants have infection during the study.

5.3.2 Milk collection and RNA extraction

The breastmilk samples were collected with a sterile process. All the collection equipment was cleaned and heated before every collection. Alcohol swabs were used to clean the breasts before the milk collection. Approximately fifty milliliters of breast milk were collected simultaneously from both breasts via a standard breast pump (Medela AG, Baar, Switzerland) and immediately placed in a cooler surrounded by ice pads. The milk samples were transmitted to laboratory within 4 hours for the RNA extraction (Supplemental Digital Content -Figure S5-1). Breast milk was transferred into a sterile, RNasefree tube and then centrifuged at 1600 x g
for 20 minutes. The supernatant fat layer was transferred to a new tube, using a sterile cell scraper. Total mRNA of breast milk was prepared with a hybrid of the Trizol protocol and QIAGEN RNeasy Miniprep kit (Qiagen, Valencia, CA) as modified from Cai et. al, (2016) (5). Briefly, the supernatant fat layer was homogenized with seven and half milliliters of TRIzol reagent (Invitrogen Life Technologies, Burlington, ON). Then the homogenized samples were mixed with 1.5 mL of chloroform (Fisher scientific, Ottawa, ON) and centrifuged at 1600 x g for 15 minutes. The upper aqueous phase was transferred to a new tube and mixed with 1 volume of 70% ethanol. Total RNA was isolated from the mixture using a QIAGEN RNeasy Mini kit following the manufacturer’s suggested procedures. For the milk cell mRNA extraction, the lipid layer and skim milk were carefully removed, and the cell pellet was washed twice in PBS at 1600 x g for 5 minutes. Total RNA was extracted with the QIAGEN RNeasy Mini kit following the manufacturer’s suggested procedures (Qiagen, Valencia, CA). Total RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was measured by gel electrophoresis.

5.3.3 Gene expression analysis by qPCR

Samples were analyzed by Applied Biosystems StepOnePlusTM Real-Time PCR System as previously described (5). Briefly, total mRNA of each sample (1µg of
input RNA) was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The relative expressions of the iron transporters’ mRNA were determined by qPCR for *TFRC* (coding transferrin receptor 1), *TFR2* (coding transferrin receptor 2), *SLC11A1* (coding natural resistance-associated macrophage protein 1), *SLC11A2* (coding divalent metal transporter 1), *SLC40A1* (coding ferroportin), *TF* (transferrin) and *LTF* (lactoferrin). In order to confirm the source of mRNA in human milk, the expression of mRNAs encoding epithelial marker (*MUC1*, mucin 1, cell surface associated) and mRNAs encoding leukocyte marker (*CD45*, protein tyrosine phosphatase, receptor type, Cp) were examined by qPCR. Ten serial dilutions of standard cDNA (1:10 factor; from 10ng to 1x10⁻⁵ pg) were used to construct a standard curve for each gene. For the endogenous control, *18s rRNA* was used as the human housekeeping gene. Expression of the transcript of interest was normalized to the respective housekeeping gene.

5.3.4 Breastmilk cell isolation and cell culture

In addition to the mRNA extraction, the milk cells were subjected to cell culture for the observation study. After the cell isolation as described above, the cell pellets were resuspended in 7% Fetal Bovine Serum (PBS, Invitrogen, USA) in PBS. The total cell counts were determined with a Neubauer haemocytometer by Trypan Blue exclusion. The cell culture method was modified from Hassiotou et
The milk cells were grown in growth medium containing RPMI-1640 medium (Sigma, St. Louis, MO) with 20% Fetal Bovine Serum, 20 ng/mL Epidermal Growth Factor, 0.5 µg/mL hydrocortisone, 60 ng/mL cholera toxin, 4 µg/mL insulin, 10% antibiotic-antimycotic, and 2 µg/mL fungizone.

5.3.5 Statistical analysis

Data was expressed as mean ± SD and analyzed by Two-tailed unpaired t-test. Statistical analysis was conducted using GraphPad Prism 5.0. Significance is accepted at P < 0.05.
5.4 Results

5.4.1 Participant characteristics

Fifteen healthy lactating mothers were recruited (demographic characteristics are described in Supplemental Digital Content - Table S5-1). The women had an average age of 33 years, and an average body mass index of 22. All their babies were healthy, born full-term and younger than 6 months of age. No maternal or infant illness or infection was reported when the mothers provided the samples.

5.4.2 Total RNA concentration and purity of extracted RNA

Total RNA concentration and purity of RNA was different between human milk fat and milk cells (Table 5-1). Human milk fat contained a significantly higher concentration of extracted RNA compared to milk cells. The average concentration of RNA extracted from milk fat was 3999.8ng/µL, which was 5 times higher than that extracted from milk cells. The purity of the extracted RNA was also different. The mean 260/280 ratios of RNA extracted from milk fat and milk cells were both within the acceptable range. However, the mean 260/230 ratio of RNA extracted from milk cells was significantly lower than that of milk fat.

Table 5-1 Quantity and quality of RNA extracted from human milk fat and cells

<table>
<thead>
<tr>
<th>Milk Fraction</th>
<th>RNA concentration(ng/µL)</th>
<th>260/280 Ratio</th>
<th>260/230 Ratio</th>
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<tr>
<td>Fat</td>
<td>3999.8 ± 2006.7</td>
<td>1.96 ± 0.17</td>
<td>1.87 ± 0.31</td>
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<tr>
<td>Cell</td>
<td>653.6 ± 688.8</td>
<td>2.09 ± 0.06</td>
<td>1.20 ± 0.55</td>
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</table>
5.4.3 Cell morphology

The majority of cells lived for up to 2h after the milk collection, and most of them displayed an epithelial-like morphology. However, half of the cells were dead after 7 days’ culture (Supplemental Digital Content Figure S5-2).

5.4.4 Origin of the mRNA extracted from human milk fat globules and human milk cells

The relative expression of MUC1 and CD45, the gene markers for epithelial cells and leukocytes, respectively, were used to determine the origin of the mRNA extracted from the milk fat and milk cells. MUC1 was highly expressed in all of the milk fat samples (Figure 5-1A), while very low levels of CD45 were found in seven of the 15 samples. In the remaining eight samples, only MUC1 was observed. This indicates that the majority of the mRNA extracted from milk fat exclusively originated from epithelial cells.

MUC1 was also highly expressed in all of the milk cell samples (Figure 5-1B). Although expression of MUC1 was significantly higher than that of CD45 in milk cells, CD45 was expressed in all of the milk cell samples. This indicates that the mRNA extracted from the milk cells originated from both epithelial cells and leukocytes.
Figure 5-1 Gene markers gene expression in mRNA derived from human milk fat (A) and human milk cells (B)(N=15).

A, epithetical cell marker MUC1 is strongly expressed, while leucocyte marker CD45, is very low or absent in the human milk fat of fifteen individuals (S1-S15). B, both gene markers are expressed in the human milk cell of fifteen individuals (S1-S15)
5.4.5 Iron transporters transcripts in human milk fat globules and milk cells

The presence of iron transporter genes in milk fat and milk cells was consistent among the participants; however, the gene expression levels of these transporters varied (Figure 5-2).

TFRC, TFR2 and SLC11A2 were expressed in mRNA extracted from human milk fat globules, while SLC11A1 and SLC40A1 were not detected (Figure 5-2A). Iron-binding proteins, TF and LTF, were also detected. Of these protein genes, LTF had the highest expression level, followed by TFRC and TF.

All of the iron transporter genes were expressed in mRNA extracted from human milk cells (Figure 5-2B). This may have resulted from the combination of cells; however, a very low amount of SLC40A1 was expressed in four of the fifteen samples, and was not detected at all in the other eleven samples. When we divided the samples into two group by MUC1/CD45 expression ratio (<1.8 or ≥1.8), we found that SLC40A1 expression could only be detected when the ratio was lower than 1.8 (Figure 5-3). This means that when the expression levels of leukocytes was much lower than that of epithelial cells in the samples, either SLC40A1 expression was too low to detect, or SLC40A1 was absent.
Figure 5-2 Iron transporter gene expression in mRNA derived from human milk fat (A) and human milk cells (B) (N = 15).

A, transferrin receptors TFRC and TFR2, iron transporter SLC11A2 as well as transferrin (TF) and lactoferrin (LTF) are present in all the human milk fat samples (S1-S15), while natural resistance-associated macrophage protein 1 (SLC11A1) and the iron exporter SLC40A1 are absent. B, all of the transporters except SLC40A1 are present in all of the human milk cell samples (S1-S15). Very low of SLC40A1 is present in four of 15 human milk cell samples.
Figure 5-3 SLC40A1 expression levels with different MUC1/CD45 expression ratio.

* $P = 0.011$
5.5 Discussion

It has been suggested that breast milk allows non-invasive examination of the physiological functions of human lactating mammary glands. Two milk fractions, human milk fat globules and human milk cells, contain a rich source of maternal genetic information. In lactating epithelial cells, RNA is wrapped by fat globules and secreted in milk, while maternal cells are shed into breast milk during lactation. Some studies have showed that the human milk fat layer and milk cells are potential tools with which to examine lactating mammary epithelial cell gene expression during lactation, circumventing the need for an invasive tissue biopsy (7-9).

In our pilot study, we used only a column-based method, which could not extract RNA of good quality, and in sufficient quantity, from milk fat. However, a column-based method can effectively extract RNA from milk cells. Thus, we used TRIzol combined with a column method to extract RNA from the fat globules in the current study. With regard to the RNA extraction yield, the RNA obtained from milk fat was significantly higher than that obtained from milk cells. This could be due to the dissolute effect of TRIzol on the fat globules, largely ensuring cell lysis and thus liberation of RNA (9). In conclusion, TRIzol combined with a column
method effectively extracted a high concentration of RNA from milk fat, but it was possible to extract RNA from milk cells using column based method alone. The fact that human milk fat RNA primarily originated from the epithelial cells suggests its potential use as a tool that reflects the biological information of lactating epithelial cells. Although traces of leukocytes were found in some samples, the majority originated exclusively from epithelial cells. When running a gene expression study for individual participants, it was possible to select the pure candidate samples for further study. In contrast, RNA from human milk cells originated from both epithelial cells and leukocytes. Although the mothers and infants were healthy when the milk was collected, there still remained a negligible number of leukocytes in the total RNA. This suggests that RNA from milk cells might not be a good source with regard to the study of lactating epithelial cells. However, the RNA extraction method for milk cells is quick and convenient, as it uses only a single-column-based kit. Thus, it may be an effective tool in identifying the biological properties of lactating mammary glands, which include lactating epithelial cells and leukocytes. Real-time polymerase chain reaction confirmed the iron transporter profile in milk fat in the current study, and these transporters included TFRC, TFR2, DMT1, TF and LTF. However, FPN encoded by SLC40A1, the only known iron transmembrane release protein, was absent. This result is similar to those of
previous studies (5). In contrast, different levels of all of the iron transporters were found in milk cells, which may have been due to the combination of milk cells. Compared to other iron transporter genes, *LTF* was the most abundant, being expressed both in milk fat and milk cells. LTF is the second most common whey protein derived from mature humans (10), and this high level of expression may be associated with its great abundance in milk. Another two genes that were not found in milk fat, but were observed in milk cells, were *SLC11A1* and *SLC40A1*.

*SLC11A1* codes the protein NRAMP1, which is involved in iron metabolism and host resistance to certain pathogens. It is possible that this gene originated from leukocytes. Although a very low amount of *SLC40A1* was found in a few milk cell samples, it was absent from the majority. We also observed that *SLC40A1* could only be found in the samples with a lower epithelial cells/leukocytes ratio; when the ratio was higher than 1.8, *SLC40A1* was absent. This means that even the milk cells contained two cell types; if the epithelial cells were the domain cells (1.8 times higher than the leukocytes), then *SLC40A1* was absent or at too low a level to be detected. Moreover, moderate expression of *SLC40A1* in human leukocytes has been well-documented (11, 12). Combining this finding with that regarding the milk fat, we can suggest that SLC40A1 is not present in human lactating epithelial cells. The absence of the membrane release iron transporter indicates that there might be another iron transport pathway or non-secreting routine in human
lactating epithelial cells, which may explain why the iron content of human milk is extremely low. This study also had limitations. The mRNA of leukocyte was occasionally presented in the total mRNA extracted from milk fat globules in the present study. Further studies should improve the extraction method to avoid this limitation. More well-designed studies should be conducted to explore whether there is a new iron-release transporter or whether iron is not secreted in milk. Only then could we reveal the entire iron transport pathway in lactating human epithelial cells.
5.6 Conclusion

We showed the origins of RNA from milk fat and milk cells. Our data provided evidence that human milk fat RNA is primarily synthesised in lactating mammary epithelial cells, while human cell RNA is derived from both epithelial cells and leukocytes. Thus, RNA from milk fat and milk cells can be used as a novel tool to investigate the biological functions of lactating epithelial cells or lactating mammary glands via different purposes. Together with the results of previous studies, our data showed that $SLC40A1$ is not expressed in lactating epithelial cells, which suggests that an alternative iron transport pathway (or non-secreting routine) is used during the human milk synthesis process. Further research is required to identify the potential novel iron membrane exporter or to detect whether an iron-secreting routine is used during lactation.
5.7 References


Supplementary material

*Table S5-1 Characteristics of participants and their neonates.*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD/%</th>
</tr>
</thead>
<tbody>
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<td><strong>Maternal characteristics</strong></td>
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</tr>
<tr>
<td>N</td>
<td>15</td>
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<td>Age (years)</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>BMI before pregnancy</td>
<td>22 ± 3</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
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<tr>
<td>Age (days)</td>
<td>131 ± 47</td>
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<tr>
<td>Infant gestational age (weeks)</td>
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<td>Birth weight (g)</td>
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<td>Birth mode (natural birth %)</td>
<td>93%</td>
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<td>Gender (male %)</td>
<td>43%</td>
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</table>
Figure S5-1 RNA extraction flow chart
Figure S5-2 Milk cells under optical microscope in day 1(A) (40x), day 5 (100x) and day 7(40x)
Chapter VI. Overall Conclusion

6.1 General discussion and summary

Breast milk is broadly accepted as the gold standard in food for infants during the first months of life. Fe content in breast milk is low, and infants need to use their own Fe stores during the breast-feeding period (1). Therefore, Health Canada recommends that the first complementary food for infants should be Fe fortified (2). Whether there is enough Fe in breast milk for exclusively breast-fed infants is still controversial, as is the lack of agreement among scientists regarding how much Fe should be recommended for infants. Prior to 2001, the World Health Organization (WHO) recommended that infants be exclusively breast-fed for 4–6 months. In 2001, this advice was changed to exclusive breast-feeding for the first 6 months of life (3). Similarly, Health Canada supports exclusive breast-feeding until 6 months of age (4). These recommendations take into consideration that the Fe stored in the body is sufficient through 6 months of age and that excess Fe intake (rather than breast milk itself) may cause adverse effects in infants.

Conversely, the American Academy of Pediatrics has recommended that exclusively breast-fed full-term infants receive 1 mg/kg of Fe supplementation per day, beginning at 4 months of age. This policy supports the concept that the small amount of Fe in human milk may not be enough for the physical and neurological
development of exclusively breast-fed infants (5). Although some studies have evaluated the health benefits and risks of Fe-fortified food, it is important to determine whether these health effects and risks are associated with Fe intake alone or are due to other nutrients. Evidence of the benefits and risks of daily oral Fe supplementation with regard to hematologic, growth, and cognitive parameters and adverse effects in exclusively breast-fed infants is limited.

The meta-analysis of RCTs from current research (Chapter III) demonstrates that Fe interventions had no significant effect on Fe deficiency, Fe-deficiency anemia, serum ferritin levels, or hemoglobin levels. Fe interventions did result in a significant increase in Bayley psychomotor developmental indices in later life (MD= 7.00, 95% CI, 0.99-13.01) and mean corpuscular volume (MD=2.17 fL; 95% CI, 0.99-3.35 fL). Fe supplementation was associated with slower growth during the exclusive breast-feeding period, but the long-term effect is unclear. There was no evidence to suggest Fe supplementation could cause other adverse effects. This finding suggests that Fe supplementation may exclusively benefit breast-fed infants with respect to Fe status and mental development, and adverse effects are unlikely. This finding could assist policymakers and clinicians to better understand the benefits and safety of daily Fe supplementation in exclusively breast-fed infants.
Even though Fe is low in human milk, the underlying mechanism has not yet been identified. The expression of Fe transporters in human lactating epithelial cells (Chapter IV) was investigated in this research. The expression of the *TFRC*, *SLC11A2*, *TF*, and *LTF* was confirmed in RNA isolated from the human milk-fat globule. The expression patterns from the result strongly indicate the “transferrin cycle” as a mechanism of Fe uptake into the human mammary epithelial cell, as it is known for other cell types (6). FPN, the only known Fe exporter, is not expressed in human lactating epithelial cells. The lack of transmembrane release might explain the low Fe concentrations in human milk as compared to rat milk, where FPN is present in the lactating rat mammary gland (8). This suggests that if any Fe excretion into human milk occurs, it might be mediated through an LFT complex rather than direct transmembrane transport. LFT has been found that homogeneous distributing in the cytoplasm of secretory cells in the lactating mammary gland of sheep in a previous study (7). Hence, there is a potential route that free Fe in the cytoplasm might be taken up by the LFT and transport out of secretory cells as a complex. However, the data do not rule out a complete lack of Fe excretion capacity into human milk. As the suggested by Friel (2017), the low content of iron in human milk can be accounted for by the adventitious iron content of the cells that make up human milk (9). The total intracellular iron content of human milk is expected to be 0.58 to 0.65 mg/L [calculated by (N of epithelial cells in
human milk) X (average Fe content per epithelial cell)], which accounts for a large proportion of the total iron content in human milk. However, this hypothesis should be proved by the further studies.

The presented data also indicate that the mouse might be a more suitable model organism for human lactation because our data confirm a lack of Slc40a1 expression in a mouse model of mammary epithelial cells (HC11). Overall, current research suggests that there is a different Fe transporter pathway in the lactating human mammary gland different from what we have learned from the rat.

Because of technical and ethical difficulties, most studies use cell cultures and rodent models for lactating mammary gland biology research. Although breast tissue could be obtained via surgery, healthy lactating tissue is still difficult to obtain (10, 11). It should be noted that rodent models or cell cultures may not exactly reflect human physiology. Alternately, breast milk has been proposed as a non-invasive source of lactating epithelial cells for research. Current research provides evidence for the advance use of breast milk for maternal genetic research. We showed the origins of RNA from milk fat and milk cells. Our data provided evidence that human milk-fat RNA originates primarily in lactating mammary epithelial cells, whereas human cell RNA is derived from both epithelial cells and leukocytes. Thus, RNA from milk fat and milk cells can be used as a novel tool to
investigate the biological functions of lactating epithelial cells or lactating mammary glands via different research objectives. In addition, current research has modified the milk process and extraction method, which can be used as a standard method for future study.

6.2 Significance of the Research

This study is the first to summarise the effects of Fe supplementation on exclusively breast-fed infants. Based on our systematic review, we provided updated evidence on the beneficial and adverse effects of Fe, which would provide physicians and policymakers an overview of Fe nutrition in breast-fed infants.

In our current research, we standardised the isolation of RNA from human milk fat and milk cells and the appropriate sampling and analysis procedure for future studies. Moreover, we analysed the gene expression of gene markers and Fe transporters in individual participants and provided insights that relative gene expression varies greatly between individuals.

Most importantly, this study was the first to investigate Fe transporters in human lactating mammary cells. Remarkably, we revealed that human lactating mammary cells lack the only known membrane exporter, FPN. This finding suggests humans have different Fe transport mechanisms during lactation than have been seen in rats.
The evidence also supports the hypothesis that low Fe in human milk is due to the Fe transporters.

6.3 Limitations and Future Directions

Strict selection criteria were applied to the current meta-analysis. However, only four eligible studies were included. Although the outcome concluded the effects of Fe supplementation in exclusively breast-fed infants, which have not been summarised before, the conclusion was based on a small sample size. More well-designed RCTs with long-term follow-up should be done to provide further evidence on the effect of Fe supplementation on exclusively breast-fed infants with respect to Fe status, mental development, and adverse effects. For example, a long-term follow-up study of a double-blinded randomised controlled trial that recruit the exclusively breastfed infants from developed and developing countries should be done to address this issue. Iron supplementation will be given to the full-term infants from 4 months of age to 6 months of age in the treatment group. Fe status will be determined in the following time points: before the treatment (4-month-old), during the treatment (4 to 6-month-old, biweekly), after the treatment (9-month-old, 12-month-old, 24-month-old, 36-month-old, 9-year-old, 12-year-old, 15-year-old and 18-year-old). The mental development indexes and growth parameters will be determined at the 4-month-old, 6-month-old, 12-month-old, 36-month-old, 9-year-old, 12-year-old, 15-year-old and 18-year-old). The adverse effects related to
Fe supplementation (such as diarrhea, infection) should also be recorded during the treatment time (4 to 6-month-old). A study like this enables to analyze the effects of Fe supplementation in infants from developed and developing countries, also provide the evidence of the long-term beneficial and adverse effects of Fe supplementation.

Moreover, current study revealed the Fe transporters profile in the lactating human mammary gland. Although the Fe transport pathway was predicted based on the gene expression profile, the data was determined at the mRNA level, further studies to demonstrate expression at the protein level is needed to confirm this finding. Furthermore, the mRNA of leukocyte was occasionally presented in the total mRNA extracted from milk fat globules in the present study. Further studies should improve the extraction method to avoid this limitation. In addition, advanced culture technology could be used to culture the lactating epithelial cells from breast milk for localization and mechanistic studies. For example, flow cytometric analysis should be conducted in cells isolated from freshly expressed breastmilk first to extract the epithelial cells. Then monoclonal antibodies of iron transporter should be used for the localization study with immunohistochemistry. Ultimately, the Fe transport pathway in human lactating mammary gland could be confirmed.
6.4 Final Conclusion

This study revealed that the human fat RNA originated from epithelial cells, whereas the human cell RNA originated from both epithelial cells and leukocytes. The expression of \textit{TFRC}, \textit{TFR2}, \textit{SLC11A2}, \textit{TF}, and \textit{LTF} was confirmed in RNA isolated from both milk fat and milk cells. Two other transporters, \textit{SLC11A1} and \textit{SLC40A1}, were found only in RNA isolated from milk cells. No Fe export protein could be determined in the RNA isolated from fat globules in human breast milk and a human mammary epithelial cell line. From the current study, we conclude that RNA from milk fat and milk cells could be used as a novel tool to investigate the biological functions of lactating epithelial cells and lactating mammary glands, respectively, by different purposes. The lack of Fe exporter in lactating epithelial cells suggests that a novel Fe transport routine may be used during human lactation.
6.5 References


Appendices

Appendix 1: Copyright license for previously published materials

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Appendix 2: Recruitment poster

Are You Breastfeeding?

Join our research team!

Breastmilk samples (100-250ml) are needed for our nutrient (iron) in breastmilk study.

The whole process will be completed in 30 minutes.

- Contribute to knowledge that may help prevent iron-deficiency anemia in infants and improve their health.

- A gift card will be provided as honorarium.

For more information and to participate please contact:

Dr. James Friel,
Phone: (204) 474-8682,
Email: frielj@cc.umanitoba.ca

OR

Chenxi Cai,
Phone: (204) 230-7861,
Email: caic3@myumanitoba.ca
Appendix 3: REB approval letter

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF ANNUAL APPROVAL

PRINCIPAL INVESTIGATOR: Dr. J. Friel
INSTITUTION/DEPARTMENT: U of M/Human Nutritional Sciences
ETHICS #: HS 16261 (B2014:111)

BREB MEETING DATE (if applicable): APPROVAL DATE: October 14, 2015
EXPIRY DATE: October 27, 2016

STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (if applicable):

PROTOCOL NUMBER: N/A
PROJECT OR PROTOCOL TITLE: Iron Transporters in the Human Mammary Gland and their Relationship to Low Iron Concentrations in Human Milk

SPONSORING AGENCIES AND/OR COORDINATING GROUPS:

Submission Date of Investigator Documents: September 9, 2015
BREB Receipt Date of Documents: September 10, 2015

REVIEW CATEGORY OF ANNUAL REVIEW: Full Board Review ☐ Delegated Review ☒

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

<table>
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<th>Document Name(if applicable)</th>
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CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the annual study status report for the research study/project named on this Certificate of Annual Approval as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. Annual approval was granted by the Chair or Acting Chair, UM BREB, per the response to the conditions of approval outlined during the initial review (full board or delegated) of the annual study status report.

BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.
Appendix 4: Participant concern form

Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

203 RCFFN
Winnipeg, Manitoba
Canada R3T 2N2
Phone: (204) 474-8682
Fax: (204) 474-7552
frielj@cc.umanitoba.ca

Faculty of Human Ecology
Human Nutritional Sciences

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

Principal Investigator: Dr. James Friel, University of Manitoba, 196 Innovation Drive
Winnipeg, MB R3T 6C5 Canada, Phone: (204) 474-8692

You are being asked to participate in a research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your friends, family or (if applicable) your doctor before you make your decision. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Purpose of Study
Iron is important for physical growth and brain development of infants. Lack of adequate iron intake may lead to early iron deficiency, which is associated with immediate adverse health outcomes and implications later in life, such as lower IQs. In humans, iron concentrations are low in mother’s milk and the molecular background is not completely understood. In this original study, we will extract excreted epithelial cells (breast cells that produce milk) from mother’s milk in order to determine the iron transporting proteins present. We will examine which proteins are delivering iron into mother’s milk. We will determine whether lactating and non-lactating mothers have different iron transporting systems. We believe this proposal examining the mechanisms responsible for low iron concentration in human milk is important for breast-fed infants, since it will provide new knowledge that may help reduce anemia and improve their health. A total of 10 participants will participate in this study.

Study procedures
If you take part in this study, you will have the following procedures:
1. Schedule the collection time and place (your home or clinic).
Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

2. Breast milk collection: Breast milk (10 tsp.) will be collected simultaneously from both of your breasts via a standard breast pump (Playtex Embrace, Dover, DE) and immediately placed on ice.

Participation in the study will be for 1 day. The researcher may decide to take you off this study if you are not breastfeeding or are taking medicine during breastfeeding. You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the study staff first.

We will exam the iron concentration in your milk.

Genetic test: We will exam which iron transporter genes are in your milk with the method call ‘gene expression assay’. Your personal information will be confidential, the results will be recorded only as a sample number.

The samples will be marked by number, with the link maintained by PI. The breast milk will be stored in the research lab freezer in the Richardson Centre for Functional Foods and Nutraceuticals until the study is completed (2 years).

Risks and Discomforts
While on the study, your will have minimal physical risk, with possible bruising or discomfort from milk pumping.

Benefits
There may or may not be direct benefit to you from participating in this study. We hope the information learned from this study will benefit other breast-fed infants in the future.

Costs
All the procedures, which will be performed as part of this study, are provided at no cost to you.

Payment for participation
You will receive $20 gift card to taking part in this study.

Confidentiality
Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned patient number (or code) and /or initials.
Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

The University of Manitoba Bannatyne Campus Research Ethics Board may review research-related records for quality assurance purposes. All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your baby's medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the University of Manitoba.

Voluntary Participation/Withdrawal from the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time.

Medical Care for Injury Related to the Study

In the case of injury or illness resulting from this study, necessary medical treatment will be available at no additional cost to you.

If you become physically injured as a result of any research activity, the study research will provide any necessary treatment, at no charge, to help you recover from the injury.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff: Dr. James Friel, Phone: (204) 474-8682

For questions about your rights as a research participant, you may contact The University of Manitoba Bannatyne Campus Research Ethics Board at (204) 789-3389

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with Dr. James Friel and/or his study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statements or implied statements. Any relationship (such as employer, supervisor or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this research is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.
Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of any of my records that relate to this study by The University of Manitoba Research Ethics Board, for quality assurance purposes.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to being contacted in relation to this study. Yes ☐ No ☐

Participant signature ___________________________ Date ____________

Participant printed name: ____________________________ (day/month/year)

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name: ___________________________ Date ____________

Signature: ___________________________ (day/month/year)

Role in the study: ___________________________

Relationship to study team members: ___________________________
Appendix 5: Questionnaire

Iron transporters in the human mammary gland and their relationship to low iron concentrations in human milk

Date: 
Participant #: 

Human Nutritional Sciences
RESEARCH PARTICIPANT INFORMATION FORM

Mother's information:
Name: 
Age: 
Height: 
Weight: 
Any medication (or any supplementation):


Baby's information:
Birthday: 
Sex: Female Male 
Birth weight: 
Birth mode: Nature C-section 
Gestational weeks: 
Current feeding method:

Notes: