SUBCLINICAL MASTITIS IN GUATEMALAN WOMEN: ROLE OF MILK MINERALS AND INFLAMMATORY CYTOKINES

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ABSTRACT

Background: Subclinical mastitis (SCM) is an asymptomatic inflammatory condition of the mammary gland. It can progress to clinical mastitis, which is a painful and debilitating condition for lactating mothers and is recognized as one of the primary causes for early cessation of breastfeeding. A review by WHO suggests that a principal cause of SCM is infection. With infection, inflammatory cytokines are released transiently and act locally in mammary tissues to regulate host defense mechanisms. Previous bovine research has shown that mineral deficiencies can impact cows' ability to resist infection via altering mammary epithelial cells integrity and affecting the recruitment as well as the ability of immune cells to kill bacteria. Although the complex interactions between nutrition, infection and immunity have been recognized, little is known about the impact of SCM on the immunological and mineral concentrations in human breast milk.

Objectives: 1) Determine if milk minerals and cytokines during the 3 stages of lactation differed in mothers with and without SCM; 2) Explore the associations between minerals and cytokines in human breast milk in the context of SCM.

Methods: Transitional milk (TM: 5-17d, n=21), early mature milk (EMM: 18-46d, n=32) and mature milk (MM: 109-187d, n=59) samples were collected from *Mam*-Mayan women in Guatemala. Inductively Coupled Plasma Mass Spectrometry was used to analyze the concentration of 19 minerals (Na, K, Ca, Mg, Cu, Cr, Fe, Mn, Rb, Se, Sr, Zn, As, Ba, Cd, Co, Ni, Pb and Tl) and flow injection analysis with Lachat was used to measure the concentration of P in milk. Immunoassay with Luminex was used to determine the concentration of 6 cytokines (IL-1β, IL-6, IL-8, TNF-α, IL-10 and IL-13).

Results: By using Na/K ratio > 0.6 as an indicator, the prevalences of SCM were determined to be 26.3% in TM, 15.6% in EMM and 8.9% in MM. Na, K, P, Cu, Fe, Rb, Zn and IL-6 were higher in TM and EMM, whereas Mg was higher in MM, as was IL-8 SCM was associated with changes in P and Se and with the presence of 3 cytokines (IL-6, IL-8, and TNF-α), but in TM only. Regression analyses for each mineral showed that lactation stage was associated with Na, K, Mg, P, Cu, Cr, Fe, Mn, Rb and Zn. SCM was positively associated with Na, as expected, as well as with Se. Regression

analyses also showed that several cytokines were associated with higher milk mineral concentrations: IL-1β with P, Fe and Mn; IL-6 with K, Ca and Cu; IL-8 with Zn; and TNF-α with Mn.

Conclusion: Milk mineral and cytokine concentrations vary by lactation stages. Specific cytokines were associated with changes in milk mineral concentrations. Our finding suggests that SCM is associated with elevated Se in breast milk.

RESUME

Contexte de la recherche : La mammite sous-clinique (MSC) est une condition inflammatoire asymptomatique de la glande mammaire. Cette condition peut progresser au stade clinique, les effets duquel peuvent être très douloureux et débilitants et est reconnue comme étant la cause la plus importante de l'arrêt précoce de l'allaitement. Une étude par l'OSM suggère que la cause principale de la MSC est l'infection. Quand la glande mammaire combat une infection, elle libère des cytokines inflammatoires de façon transitoire qui agissent localement dans les tissues mammaires dans le but de règlementer les mécanismes de défenses de l'hôte. Des études chez les bovins ont démontrées que des déficiences minérales peuvent avoir un impact sur l'habileté de la vache à combattre des infections car ces déficiences peuvent altérées l'intégrité des cellules mammaires épithéliales et réduire le recrutement et l'efficacité des cellules immunitaires à combattre les bactéries. Malgré que le lien complexe entre la nutrition, l'infection et l'immunité est reconnu, peu est connu sur l'impact que la MSC à sur le profile immunologique et les concentrations de minéraux dans le lait maternel humain.

Objectif : 1) Déterminer si la composition de minéraux ou de cytokines diffère entre le lait maternel de mères avec et sans MSC par période de lactation; 2) Explorer les liens entre les minéraux et cytokines dans le lait maternel humain dans le contexte de MSC.

Méthodologie : Des échantillons de lait transitoire (LT : 5-17 jours (jrs), n=21), de lait mature précoce (LMP : 18-46jrs, n=32) et de lait mature (LM : 109-187jrs, n=59) furent collectionnés de femmes autochtones mayas, au sein du groupe ethnique Mam, du Guatemala. La concentration de 19 minéraux (Na, K, Ca, Mg, Cu, Cr, Fe, Mn, Rb, Se, Sr, Zn, As, Ba, Cd, Co, Ni, Pb et Tl) fut analysée par spectrométrie de masse avec plasma à couplage inductif (ICP-MS) et la concentration de P fut analysée par la technique d'analyse par injection en flux avec Lachat. La concentration de cytokines (IL-1β, IL-6, IL-8, TNF-α, IL-10 et IL-13) fut déterminée par immune-essai avec Luminex.

Résultats : La prévalence de la MSC, indiquée par un Na/K>0.6, était de 26.3% dans les échantillons de LT, 15.6% dans les échantillons de LMP, et 8.9% dans les échantillons de LM. Na, K, P, Cu, Fe, Rb, Zn et IL-6 étaient plus élevé dans le LT et le LMP tant disque Mg et IL-8 était plus élevé dans le LM. La MSC fut associée avec des changements de P et de Se ainsi qu'avec la présence de 3 cytokines (IL-6, IL-8, et TNF-α) dans les échantillons de LT seulement. Des analyses de régression ont

démontrées un lien entre la concentration de Na, K, Mg, P, Cu, Cr, Fe, Mn, Rb et Zn et la période de lactation. Comme prévue, la MSC fut associée de façon positive aux niveaux de Na. La MSC fut aussi associé de façon positive aux niveaux de Se. D'autres analyses de régression ont démontrées un lien entre certains cytokines et un niveaux plus élevé de certains minéraux: IL-1β avec P, Fe et Mn; IL-6 avec K, Ca et Cu; IL-8 avec Zn; et TNF-α avec Mn.

Conclusion : Les concentrations de minéraux et de cytokines dans le lait maternel varient par période de lactation. Certaines cytokines ont été liées avec des changements de concentrations de minéraux dans le lait maternel. Les résultats suggèrent que la MSC est liée avec des niveaux élevés de Se dans le lait maternel.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

ATP: Adenosine Triphosphate

CeSSIAM: Center for Studies of Sensory Impairment, Aging and Metabolism

CFM: Coliform Mastitis

CXCL8: Interleukin 8

DGGE-PCR: Denaturing Gradient Gel Electrophoresis PCR

DNA: Deoxyribonucleic acid

DTH Response: Delayed-Type Hypersensitivity Response

ELISA: Enzyme-Linked Immunosorbent Assay

EM: Early Milk

EMM: Early Mature Milk

GPx: Glutathione Peroxidase

HIV: Human Immunodeficiency Virus

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

IL: Interleukin

IFN-γ: Interferon gamma

LOD: Limit of Detection

LPS: Lipopolysaccharide

MHC-II: Major Histocompatibility II

MM: Mature Milk

Na/K: Sodium : potassium ratio

NFκB: Nuclear Factor kappa B

NK Cell: Natural Killer Cell

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

SCC: Somatic Cell Count

SCM: Subclinical Mastitis

SD: Standard Deviation

SEM: Standard Error of Mean

SOD: Superoxide Dismutase

SRM: Standard Reference Material

TLR-4: Toll Like Receptor-4

TM: Transitional Milk

TNF-α: Tumour Necrosis Factor alpha

WHO: World Health Organization

CHAPTER I. INTRODUCTION

1. BACKGROUND

Subclinical mastitis (SCM) is a common asymptomatic inflammatory condition of the lactating mammary gland (Lunney *et al.*, 2010; Semba, Kumwenda, Hoover*, et al.*, 1999; Willumsen *et al.*, 2002). In the past decade, this condition has been drawing increasing attention from researchers due to its association with elevated risk of mother-to-child transmission of HIV through breast milk (Dorosko, 2005; Gomo *et al.*, 2003; Kantarci, Koulinska, Aboud, Fawzi, & Villamor, 2007; Kasonka *et al.*, 2006; Lunney *et al.*, 2010; Semba & Neville, 1999; Willumsen *et al.*, 2002) and association with infant growth faltering (Aryeetey, Marquis, Brakohiapa, Timms, & Lartey, 2009; Filteau *et al.*, 1999; Gomo *et al.*, 2003; Morton, 1994). Hence, adequate diagnosis and treatment of SCM in breastfeeding mothers are both essential to prevent disease progression to clinical mastitis and breast abscess as well as recurrent mastitis and lactation failure (Sordillo, 2005; WHO, 2000).

SCM is believed to be much more prevalent than clinical mastitis in populations. Reported rates of SCM in lactating mothers range from 2% to 66% in African countries (Arsenault, Aboud, Manji, Fawzi, & Villamor, 2010; Nussenblatt *et al.*, 2005; Semba & Neville, 1999). Although, there is no standard definition of SCM proposed for humans, the Na/K ratio has often been used as a SCM diagnostic biomarker by researchers. A range of other different biomarkers such as cytokines and bacteria counts were also suggested (Osterman & Rahm, 2000; Pyörälä, 2003). Currently, studies indicate that SCM is most prevalent during early lactation and can occur for several reasons including milk stasis, infection, physical breast tissue trauma, micronutrient deficiency and poor lactation practices by the mother-infant dyad (Atakisi *et al.*, 2010; Kasonka *et al.*, 2006; Neville *et al.*, 1991).

Milk stasis and infection are the two main causes of mastitis. The resultant stagnation of milk from milk stasis can increase the severity of inflammation and the risk of bacterial infection in breast (Sordillo, 2011; WHO, 2000). The two most common infective agents associated with SCM and mastitis are *Staphylococcus aureus* and *Escherichia coli*. Other common bacterial contaminants include Staphylococcus spp. such as *Staphylococcus epidermidis* and *Staphylococcus pasteuri* (Collado, Delgado, Maldonado, & Rodriguez, 2009; Delgado, Arroyo, Martín, & Rodríguez, 2008; Hunt *et al.*, 2011; Jost, Lacroix, Braegger, & Chassard, 2013; Keane, Budd, Flynn, & McCoy, 2013; Martín *et al.*, 2007); Streptococcus spp. such as *Streptococcus mitis* and non-β-hemolytic streptococci and Corynebacterium. Normal skin and respiratory flora such as *Lactobacillus spp. and Enterococci spp.* have also been reported

in about half of the cases of clinically apparent mastitis (Delgado *et al.*, 2008; Delgado, Collado, Fernández, & Rodríguez, 2009; Keane *et al.*, 2013).

Human studies on SCM are scarce. The current available literature on breast milk has mainly focused on compositional changes during lactation (Krachler, Shi Li, Rossipal, & Irgolic, 1998; Matos, Moutinho, Almeida, Guerra, & Balcão, 2014). Researchers have found that concentrations of many macrominerals (chloride, sodium magnesium, phosphorus and potassium) and trace minerals (copper, iron, manganese, selenium and zinc) in breast milk decreased during lactation (Feeley, Eitenmiller, Jones, & Barnhart, 1983a, 1983b; Matos *et al.*, 2014; Silvestre *et al.*, 2001; Yamawaki *et al.*, 2005), whereas calcium remained relatively constant (Feeley *et al.*, 1983a; Yamawaki *et al.*, 2005). The serum concentrations of macrominerals are generally tightly regulated by homeostatic mechanism and hence maternal diet or mineral supplementation do not show an effect on their concentrations in milk. Conversely, the milk concentrations of trace minerals are influenced by a number of factors including maternal diet, infection and time of delivery etc. (Emmett & Rogers, 1997; Lönnerdal, 2000).

Most of our knowledge of SCM is extrapolated from animal studies, especially dairy cows. SCM has been extensively studied in bovine research, as it can disrupt the mammary gland functions in cows and lead to compromised quality and quantity of milk (Batavani, Asri, & Naebzadeh, 2007; Bruckmaier, Ontasouka, & Blum, 2004). As a result, this has tremendous economic impact on the dairy industry. As opposed to cows with SCM, healthy cows have a stable milk composition with the mammary epithelium tight junctions act as a barrier and all nutrient transportations tightly regulated by receptors (Lönnerdal *et al.*, 1996). Various mechanisms have been proposed to explain the adverse effects of SCM on milk. Firstly, the infecting pathogens in mammary gland can alter metabolic activity of the epithelial cells, which could result in a reduction of milk proteins. Secondly, the cytokines produced in response to the bacterial infection can initiate inflammation-mediated changes in the expression levels of nutrient transporter thereby altering nutrient absorption, distribution and elimination in the intra-mammary tissues. The inflammation can also trigger physical damage to the mammary epithelial layer, such as the opening of tight junctions, whereby an enhanced epithelial permeability distorts the overall milk composition with serum component such as sodium entering the milk and normal milk components such as lactose moving out of the alveolar lumen into the perivascular space (Batavani *et al.*, 2007; Bruckmaier *et al.*, 2004; Li, Zhou, Yuan, He, & Hu, 2009; Ling, 2011; Schukken *et al.*, 2011). The ionic environment of milk is therefore markedly changed by SCM. In SCM, sodium and chloride concentrations are increased in milk, while potassium, the predominant mineral normally found in milk declines (Batavani *et al.*, 2007).

Bovine SCM experimental studies have also helped to reveal the crucial roles of trace minerals in the host's antioxidant defense against infection (Spears & Weiss, 2008; Ustundag *et al.*, 2005). To date, the primary trace minerals of interest in dairy cattle diets are zinc, copper, manganese, and selenium (Oviedo-Boyso *et al.*, 2007). These are essential minerals, with classically defined roles as components in key antioxidant enzymes and proteins. A deficiency in any of these minerals may depress the immunity of cows by lowering its ability to resist pathogenic infection via two mechanisms. The first is due to the weakening of physical barriers such as tissue integrity, and the second is by affecting the recruitment as well as the ability of immune cells to kill bacteria during infection (Spears & Weiss, 2008; Weiss & Spears, 2006).

With infection, immune factors such as cytokines are released transiently by epithelial cells, tissue cells and immune cells. Cytokines, as intercellular mediators, play an important role in the nutrition-infection complex (Bannerman, 2009; Goldman, Chheda, Garofalo, & Schmalstieg, 1996; Mulokozi & Bilotta, 1999; Persson Waller, Colditz, Lun, & Östensson, 2003). On one hand, protein-calorie malnutrition, deficiency of fatty acids, vitamins and trace minerals can impair cytokine production. On the other hand, increased cytokine production during infection can interfere with the host's nutritional status by impairing metabolic activity and inducing anorexia (Mizuno *et al.*, 2012).

Cytokines have been detected in healthy and infected bovine mammary glands (Goldman *et al.*, 1996; Muñoz, Schlesinger, & Cavaillon, 1995; Wenz *et al.*, 2010). The pattern of cytokine expression by infected mammary cells differs depending on the mastitis-causing pathogen that elicits their response. Gram-negative bacteria such as *E. coli* are able to initiate a rapid and high magnitude of pro-inflammatory cytokine response (i.e., IL-1, IL-6, IL-8, and TNF-α), whereas Gram-positive bacteria such as *S. aureus* tend to induce a delayed or diminished cytokine response (Aitken, Corl, & Sordillo, 2011; Bannerman, 2009).

The interaction between nutrition, infection and immunity during lactation is highly complex. The impact of SCM on mothers' milk composition and subsequent infant growth and development remains to be established.

2. RATIONALE

SCM is an under-researched problem in public health. If left untreated, it can progress to mastitis, which is recognized as one of the primary causes for early cessation of breastfeeding (Aryeetey, 2007; Aryeetey, Marquis, Timms, Lartley, & Brakohiapa, 2008). In developing countries, including Guatemala, breastfeeding is of particular importance for optimum infant nutrition, health, and development.

One of the principal causes of SCM is infection. Nutrition status can affect the host ability to resist infection (Heinrichs, Costello, & Jones, 2009), but once an infection is established, it can elicit a cascade of immune responses, which in turn act to alter the host nutrient metabolism (Field, 2005; Latshaw, 1991; Muñoz *et al.*, 1995). The interaction between nutrition and immunity has long been a fascinating topic for researches. Localized inflammation in the mammary gland during SCM can cause an opening of the tight junction, which can result in enhanced permeability of the mammary epithelium and subsequent compositional changes in milk minerals (Li *et al.*, 2009; Ling, 2011). The released cytokines upon infection can also initiate inflammation-mediated changes in the expression levels of nutrient transporters, which may also affect the milk composition (Michie, Tantscher, Schall, & Rot, 1998). Evidence has also linked trace minerals to SCM and mastitis in the dairy cow. Animals with deficiency in minerals, such as selenium, copper and iron, are found to be more susceptible to infection with an increased risk of SCM (Filteau, 2009; Salman *et al.*, 2009; Weiss & Spears, 2006).

From literature search, it was observed that previous human studies have primarily focused on the impact of lactation stages on milk composition. In contrast, information or experimental data on the impact of SCM on milk composition was limited. As such, a comprehensive assessment concerning the influence of SCM, together with lactation stage, on milk mineral and cytokine composition will provide a better understanding of the pathophysiology of SCM in humans and is the primary goal of this thesis.

3. CONCEPTUAL FRAMEWORKS

Based on the literature review, two conceptual frameworks were constructed to demonstrate 1) the regulation process in non-SCM mammary gland (Fig 1.1); and 2) the mechanism of altered mineral concentrations in SCM milk (Fig 1.2). The elevated inflammatory cytokine production during SCM can promote the opening of tight junctions in mammary epithelial cells, which leads to an increase of mineral ion exchange between serum and breast milk.

FIG 1.1. A conceptual framework for conservation of milk minerals quantities in non-SCM mothers.

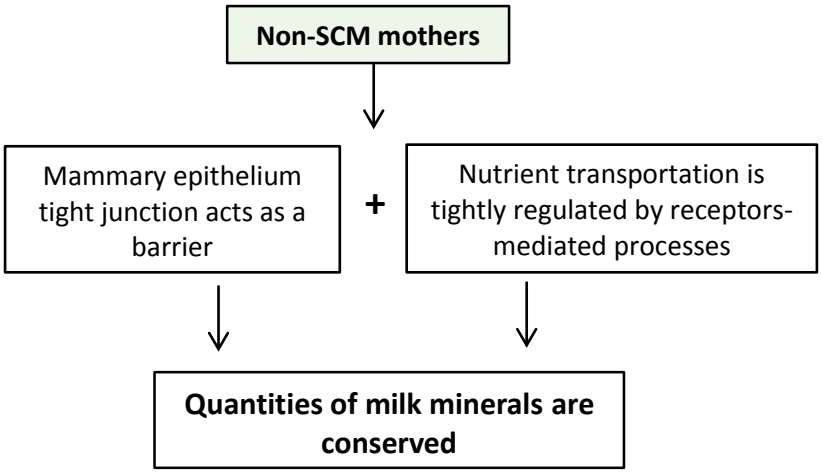
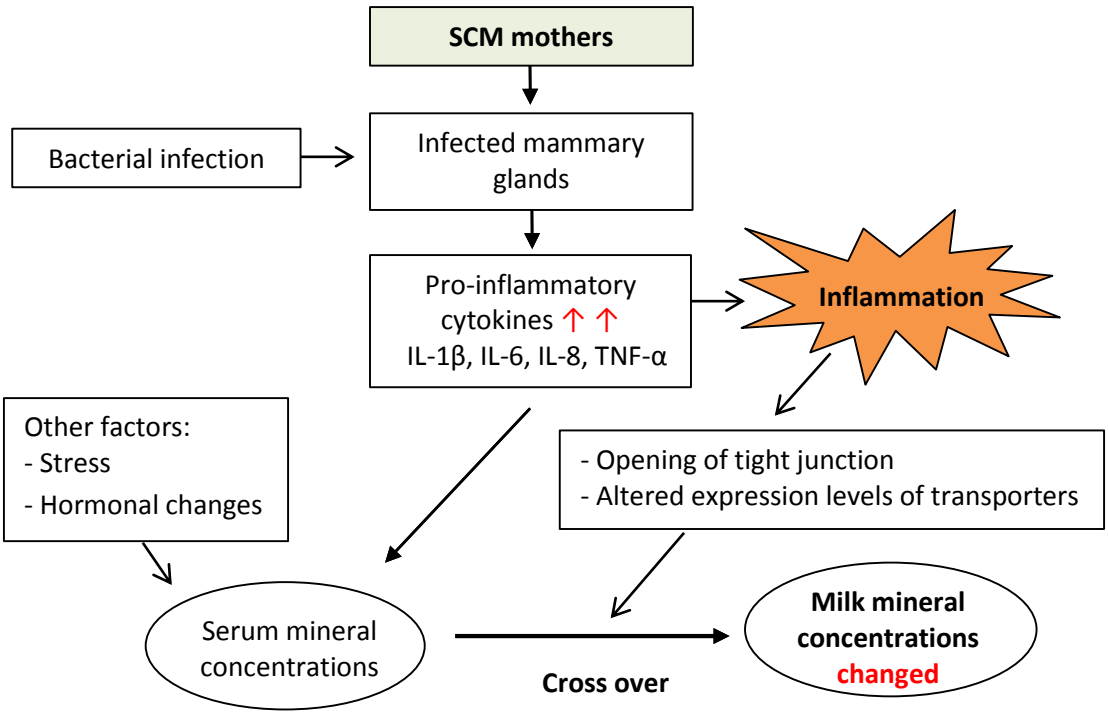


FIG 1.2. A conceptual framework for altered mineral concentrations in SCM milk.



4. HYPOTHESES

To our best knowledge, there is no systematic human study that measures and compares mineral and cytokine concentrations between SCM milk and non-SCM milk in lactating mothers. Our main hypothesis is that concentrations of milk minerals and cytokines will change during lactation and differ between SCM and non-SCM milk. Cytokines, being the mediators of inflammation, will be higher in milk from SCM mothers than from non-SCM mothers. Our secondary hypothesis is that a change in the cytokine concentrations will influence the concentrations of minerals in breast milk, especially the essential trace minerals such as selenium, copper and iron, which are required for the proper functioning of host immune system.

5. OBJECTIVES

1. To assess the differences in breast milk mineral and cytokines concentrations between SCM and non-SCM milk by stages of lactation.

2. To determine whether inflammatory cytokines, stage of lactation, and presence of SCM influence the macro- and trace minerals concentrations in breast milk.

CHAPTER II. LITERATURE REVIEW

1. INTRODUCTION

Breast milk is an extremely complex fluid containing more than 200 different components. It not only provides the infant with highly digestible and bioavailable forms of macro- and micronutrients, but also contains an array of essential anti-microbial and immunoactive factors which facilitate the neonate's transition from a relatively sterile environment of the mother's uterus to a postnatal environment with exposure to potential life-threatening pathogens. Breast milk is also a functional food providing many growth factors, hormones, and compounds that act as metabolic signals to the infant (Aryeetey, 2007; Aryeetey *et al.*, 2009; Foxman, D'Arcy, Gillespie, Bobo, & Schwartz, 2002; Martín *et al.*, 2007; Semba & Neville, 1999).

Breastfeeding, therefore, plays a crucial role in preventing the malnutrition–infection cycle, especially in developing countries. Epidemiological as well as experimental studies have demonstrated that infants derive nutritional, immunological, and psychosocial benefits from being breastfed. Exclusive breastfeeding has long been associated with lower infant morbidity and mortality, especially during the first year of life (Foxman, Schwartz, & Looman, 1994). Diseases of the mammary gland, such as mastitis, are associated with early cessation of breastfeeding i.e., lactation failure (WHO, 2000), altered milk composition, and reduced milk secretion (Contreras & Rodríguez, 2011). All of these may contribute to increased incidence and severity of infections, impaired infant growth and development.

Mastitis is broadly defined as "an inflammation of the mammary gland, including cellulitis of the interlobular connective tissue as well as related anatomical structures such as nipples, mammary areolas, milk ducts, etc. (Contreras & Rodríguez, 2011)." Mastitis is a painful and debilitating condition for breastfeeding mothers and a potentially serious illness that may result in breast abscess and septic fever (Osterman & Rahm, 2000). Early human studies have primarily focused on clinical mastitis, for it is recognized cause of milk reduction and association with early weaning. Approximately one quarter of mothers have cited clinical mastitis as their primary reason for cessation of breastfeeding (Michie, Lockie, & Lynn, 2003). In the past decade, there has been an influx of literature surrounding subclinical mastitis (SCM), as associations were found between SCM and higher HIV load in breast milk and elevated risk of vertical mother-to-child transmission of HIV (Dorosko, 2005; Semba, Kumwenda, Taha*, et al.*, 1999; Semba & Neville, 1999).

The purpose of the current literature review is to provide a comprehensive summary of SCM and its effects on mineral composition and cytokine levels in milk. The natural compositional changes

that take place in human milk during lactation is an important concept to understand and will be discussed prior to studying the SCM-related changes in milk. Present knowledge in regards to the clinical and experimental outcomes of SCM from both human and bovine research are later reviewed accordingly, followed by an in-depth discussion of findings mainly from veterinary studies on milk minerals and cytokines.

2. CHANGES OF MINERALS IN HUMAN MILK DURING LACTATION

Human milk, with unique composition characteristics, is regarded as the best source of nutrition and immune protection for the new born infant (Neville *et al.*, 1991). The nutritional components of human milk are derived from 3 primary sources: synthesis (in lactocyte), dietary intake, and maternal stores (Ballard & Morrow, 2013). In general, the quality and quantity of breast milk remains highly conserved as the maternal nutritional status has minimal effects on neither the macronutrient compositional profile (i.e., protein, carbohydrate, fat) nor the energy content of breast milk (Foxman *et al.*, 1994). Despite being unaffected by maternal nutritional status, the macronutrient composition in milk does vary between mothers as well as over the course of lactation (Ballard & Morrow, 2013; Salamon & Csapó, 2009). Studies of micronutrients (i.e., minerals and vitamins) and immunoactive factors in milk have also shown variations within and between individuals across lactation, in addition to influences by factors such as maternal diet, age, time, and method of delivery (Matos *et al.*, 2014). In particular, inter-individual variations and changes in milk composition are greatest and occur most rapidly during the first week post-partum in colostrum (Emmett & Rogers, 1997).

2.1. Macrominerals

In a comprehensive review article, published by Salamon and Csapó, where they analyzed and compared the different concentrations of macro- and trace minerals between lactating mother's colostrum and mother's milk(Salamon & Csapó, 2009),there appeared to be an general reduction in the overall mineral content in mature milk. Sodium concentration decreased from 400 mg/L in the colostrum to 150 mg/L in the mature milk, potassium from 600-700 mg/L to 400-550 mg/L, and chloride from 600-800 mg/L to 400-500 mg/L. In contrast, calcium concentrations in mother's milk varied in most studies between 84 and 462 mg/L, while phosphorus concentrations ranged from 17 to 278 mg/L.

Several other studies have also reported such changes of macromineral concentrations in milk through the course of lactation (Feeley *et al.*, 1983a, 1983b; Krachler *et al.*, 1998; Matos *et al.*, 2014; Salamon & Csapó, 2009). Table 2.1 below summarizes the findings from these studies.

TABLE 2.1. Summary of changes in milk macromineral concentrations during lactation.

Macromineral	Study Population	N	Lactation Stages / Milk Concentrations	Significance
Sodium	Gambia - Rural mothers	 192 183 193	(mmol/L) 30d / 5.20 90d / 4.11 180d / 3.78	(Richards *et al.*, 2010) No statistical analysis performed between lactation stages
	USA - Preterm	 20 19 16 13	(mEq/L) 7d / 17.23±1.88# 14d / 12.36±1.42# 28d / 10.51±0.96# 42d / 9.56±0.86#	(Lemons, Moye, Hall, & Simmons, 1982) #Significant difference between preterm and term mothers
	USA - Term	 7 7 7	(mEq/L) 7d / 9.54±1.30# 14d / 9.37±1.95# 28d / 7.04±0.96#	No statistical analysis performed between lactation stages
	Japan	 21 38 541 56	(mg/100 mL) 1-5d / 32.7±17.0* 6-10d / 24.1±11.1* 21-89d / 13.9±7.2* 181-365d / 11.6±6.1*	(Yamawaki *et al.*, 2005) *Significant difference between all lactation stages
Calcium	USA - Preterm	 20 19 16 13	(mEq/L) 7d / 293.11±16.35 14d / 266.32±15.03 28d / 282.45±11.73 42d / 309.79±16.02	(Lemons *et al.*, 1982) NS between preterm and term No statistical analysis performed between lactation stages
	USA - Term	 7 7 7	(mEq/L) 7d / 293.14±7.61 14d / 274.44±12.76 28d / 267.46±12.58	
	USA - Caucasian	 28 23 30	(µg/mL) 1-3mo / 257±29$ 7-9mo / 175±28$ 19-31mo / 150±38$	(Vaughan, Weber, & Kemberling, 1979) $Significant differences in log concentrations between consecutive months
	USA	 93 163 159	(mg/100g) 4-7d / 26.3±0.6 10-14d / 25.0±0.5* 30-45d / 26.2±0.5	(Feeley *et al.*, 1983a) *Significant difference between lactation stages
	Japan	 21 38	(mg/100 mL) 1-5d / 29.3±7.2 6-10d / 31.0±9.7	(Yamawaki *et al.*, 2005)

		550 56	21-89d / 25.7±6.3* 181-365d / 26.0±5.4	*Significant difference between lactation stages
Magnesium			(mg/L)	
	USA - Preterm	20 19 16 13	7d / 37.07±1.39# 14d / 31.75±1.44# 28d / 31.03±1.79# 42d / 35.19±1.28	(Lemons *et al.*, 1982) #Significant difference between preterm and term mothers No statistical analysis performed between lactation stages
	USA - Term	7 7 7	(mg/L) 7d / 30.97±2.51# 14d / 29.17±3.06# 28d / 28.21±2.44#	
	USA - Caucasian	28 23 30	(µg/mL) 1-3mo / 31±1.7 7-9mo / 26±3.3 19-31mo / 26±5.1	(Vaughan *et al.*, 1979) NS between lactation stages
	USA	93 163 159	(mg/100g) 4-7d / 5.3±0.1 10-14d / 4.9±0.1* 30-45d / 4.9±0.1	(Feeley *et al.*, 1983a) *Significant difference between lactation stages
	Japan	21 38 550 56	(mg/100 mL) 1-5d / 3.2±0.5* 6-10d / 3.0±0.9* 21-89d / 2.5±0.7* 181-365d / 3.3±0.7*	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages
Phosphorus			(mg/L)	
	USA - Preterm	20 19 16 13	7d / 134.48±6.45# 14d / 138.84±6.97# 28d / 131.63±5.69# 42d / 129.47±15.96#	(Lemons *et al.*, 1982) #Significant difference between preterm and term mothers No statistical analysis performed between lactation stages
	USA - Term	7 7 7	(mg/L) 7d / 169.30±8.55# 14d / 151.99±7.98# 28d / 138.53±9.09#	
	USA	93 163 159	(mg/100g) 4-7d /14.6±0.4 10-14d / 14.4±0.1 30-45d / 13.3±0.3*	(Feeley *et al.*, 1983a) *Significant difference between lactation stages
	Japan	21 38 550 56	(mg/100 mL) 1-5d / 15.9±4.0 6-10d /19.0±6.1* 21-89d / 15.6±3.4 181-365d / 13.0±2.5*	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages
Potassium			(mEq/L)	
	USA - Preterm	20 19 16 13	7d / 17.28±0.67 14d / 15.59±0.48 28d / 13.75±0.39 42d / 14.43±0.81	(Lemons *et al.*, 1982) NS between preterm and term No statistical analysis performed between lactation stages
	USA - Term	7 7	(mEq/L) 7d / 16.86±1.22 14d / 14.57±0.51	

		7	28d / 13.00±0.51	
Potassium	Japan	21 38 550 56	(mg/100 mL) 1-5d / 72.3±13 6-10d / 70.9±22.8 21-89d / 46.6±8.3* 181-365d / 43.2±7.0*	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages
Chloride	USA -Preterm	20 19 16 13	(mEq/L) 7d / 18.60±1.42 14d / 14.35±1.23 28d / 12.65±0.68 42d / 11.98±0.61	(Lemons *et al.*, 1982) NS between preterm and term No statistical analysis performed between lactation stages
	USA -Term	7 7 7	(mEq/L) 7d / 13.90±1.57 14d / 12.11±1.82 28d / 10.46±0.91	
	Japan	21 39 556 56	(mg/100 mL) 1-5d / 34.1±12 6-10d /33.8±14.8 21-89d / 33.4±16.3 181-365d / 28.6±14*	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages

Among the macrominerals, sodium represents the most important mineral for the assessment of lactogenesis (Morton, 1994) or SCM status (Aryeetey, 2007; Aryeetey, Marquis, Timms, Lartley*, et al.*, 2008). Breast milk sodium concentration has been shown to be elevated in antenatal and postnatal colostrum, falling dramatically by day 3 and declining at a steady rate for at least 6 months. Morton and colleagues have previously suggested that, after measuring the sodium concentrations of 130 nursing mothers, a normal drop in sodium concentration is highly predicative of a successful lactation, whereas a prolonged elevation of sodium signifies impaired lactogenesis with a high risk of failure. This was consistent with other reports where unusually high concentrations of sodium found in breast milk were associated with mothers whose infants developed malnutrition, dehydration, and hypernatremia between day 10 and 15 (Morton, 1994).

Based on the above summary table (Table 2.1), milk concentrations of sodium, magnesium, phosphorus, potassium and chloride are notably decreased during lactation, while calcium in milk remains relatively constant.

Under normal conditions, concentrations of macrominerals such as sodium, calcium, phosphorus and magnesium in the maternal serum are tightly controlled by homeostatic mechanisms, therefore their concentrations in human milk remain relatively constant independent of the mother's dietary or supplemental intake (Emmett & Rogers, 1997; Lönnerdal, 2000). Also, no correlations were

observed between maternal age, parity, or previous history of lactation and the elemental content of milk (Feeley *et al.*, 1983b).

2.2. Trace minerals

In comparison to the tightly regulated macrominerals, the concentrations of trace minerals in milk are highly variable and are influenced by a number of factors including maternal diet, infection, and time of delivery. The findings compiled by Salamon and Csapó's review indicated that iron concentrations in milk were between 0.04-1.92 mg/L with an average of 0.40 mg/L, while concentrations of copper varied between 0.03-219 mg/L with an average of 0.350 mg/L. Zinc and manganese contents in breast milk ranged between 0.15-5.41 mg/L and 0.07-3.8 μg/L, respectively (Salamon & Csapó, 2009). In addition to the substantial inter-individual variations observed, the concentrations of trace minerals also change during the course of lactation. Table 2.2 below summarizes findings from studies that examined the compositional changes of trace minerals during lactation.

TABLE 2.2. Summary of changes in milk trace mineral concentrations during lactation.

Trace Minerals	Study Population	N	Lactation Stages / Milk Concentrations	Significance
Copper	USA		(μg/100g)	
		88	4-7d / 104.4±5.4*	(Feeley *et al.*, 1983b)
		151	10-14d / 93.9±3.6*	*Significant difference between all lactation stages
		151	30-45d / 84.7±3.8*	
	USA - Caucasian		(μg/mL)	(Vaughan *et al.*, 1979)
		28	1-3mo / 0.43±0.05$	$Significant differences in log concentrations between consecutive months
		23	7-9mo / 0.30±0.03$	
		30	19-31mo / 0.28±0.06	
	Spain		(mg/L)	
		22	2-4d / 0.38±0.20*	(Silvestre *et al.*, 2001)
		22	30d / 0.34±0.07*	*Significant difference between all lactation stages
		22	90d / 0.19±0.10*	
	Austria		(μg/L)	
		44	1-3d / 570±336	(Rossipal & Krachler, 1998)
		44	4-7d / 228±114*	*Significant difference between lactation stages
		44	42-293d / 148±52*	
	Vietnam		(mg/L)	
		37	6-8mo / 0.19±0.05	(Nakamori *et al.*, 2009)
		22	9-12mo / 0.18±0.05	NS between lactation stages
	Portugal		(μg/kg)	
		31	7d / 529±149.6	(Matos *et al.*, 2009)
		31	28d / 379.6±93.7*	

Copper		31 31	56d / 292.4±77.0* 112d / 240.7±98.0	*Significant difference between lactation stages
	Libya	25 25 25	(mg/L) 0-4d / 0.84±0.06 5-9d / 0.66±0.06* 10-20d / 0.39±0.05*	(Hannan, Dogadkin, Ashur, & Markus, 2005) *Significant difference between lactation stages
	Turkey - Preterm	20 20 20 20	(µg/dL) 0-7d / 91.0±8.6# 7-14d / 87.0±7.8 21d / 93.0±8.7 60d / 84.0±5.3	(Ustundag *et al.*, 2005) #Significant difference between preterm and term mothers No statistical analysis performed between lactation stages
	Turkey - Term	20 20 20 20	(µg/dL) 0-7d / 112±13.8# 7-14d / 103±9.8 21d / 109±10.7 60d / 97±8.8	
	Japan	20 38 555 476 39	(mg/100 mL) 1-5d / 37±15 6-10d / 48±10* 21-89d / 34±19 90-180d / 36±25 181-365d / 16±5*	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages
Iron	USA	92 163 157	(µg/100g) 4-7d / 96.5±6.5* 10-14d / 85.4±4.5* 30-45d / 76.1±3.8*	(Feeley *et al.*, 1983b) *Significant difference between all lactation stages
	USA - Preterm	20 19 14 11	(mg/L) 7d / 1.50±0.11# 14d / 1.52±0.21# 28d / 1.31±0.16# 42d / 1.39±0.42#	(Lemons *et al.*, 1982) #Significant difference between preterm and term mothers No statistical analysis performed between lactation stages
	USA - Term	7 7 7	(mg/L) 7d / 0.77±0.10# 14d / 0.98±0.20# 28d / 0.81±0.13#	
	USA - Caucasian	28 23 30	(µg/mL) 1-3mo / 049±0.05 7-9mo / 0.42±0.06 19-31mo / 0.42±0.08	(Vaughan *et al.*, 1979) NS between lactation stages
	Spain	22 22 22	(mg/L) 2-4d / 0.56±0.22* 30d / 0.39±0.19* 90d / 0.40±0.17*	(Silvestre *et al.*, 2001) *Significant difference between all lactation stages
	Vietnam	37 22	(mg/L) 6-8mo / 0.40±0.14 9-12mo / 0.46±0.15	(Nakamori *et al.*, 2009) NS between lactation stages
	Japan	20 38	(µg/100mL) 1-5d / 110±54 6-10d / 96±70*	(Yamawaki *et al.*, 2005)

		542 476 39	21-89d / 180±327* 90-180d / 52±143* 181-365d / 85±66*	*Significant difference between lactation stages
Manganese	USA - Caucasian	 28 23 30	(µg/mL) 1-3mo / 1.98±0.21 7-9mo / 2.53±0.39 19-31mo / 1.99±0.86	(Vaughan *et al.*, 1979) NS between lactation stages
	Austria	 44 44 44	µg/g (median, mean) 1-3d / 9.4±6.0 4-7d / 4.2±1.6* 42-293d / 4.5±1.7*	(Rossipal & Krachler, 1998) *Significant difference between lactation stages
	Portugal	 31 31 31 31	(µg/kg) 7d / 3.73±2.64 28d / 3.65±1.99 56d / 2.29±1.13* 112d / 2.44±1.49	(Matos *et al.*, 2009) *Significant difference between lactation stages
	Japan	 18 38 555 476 39	(µg/100 mL) 1-5d / 1.2±0.8* 6-10d /1.8±5.3* 21-89d 0.8±2.2* 21-89d / 1.1±1.1* 181-365d / 0.9±1.1*	(Yamawaki *et al.*, 2005) *Significant difference between all lactation stages
	USA	 93 163 159	(mg/100 g) 4-7d /5.3±0.1 10-14d / 4.9±0.1* 30-45d / 4.9±0.1*	(Feeley *et al.*, 1983b) *Significant difference between lactation stages
Selenium	Austria	 13 18 8 8	µg/kg (median, mean) 1-3d / 32.7, 33.4* 4-7d / 16.0, 16.1* 42-60d / 12.5, 11.8* 97-293d / 8.9, 10.2*	(Krachler *et al.*, 1998) *Significant difference between all lactation stages
	Portugal	 31 31 31 31	(µg/kg) 7d / 30.69±9.21 28d / 25.47±7.10* 56d / 19.95±6.76* 112d / 19.49±3.58	(Matos *et al.*, 2009) *Significant difference between lactation stages
	Lybia	 25 25 25	(µg/L) 0-4d / 104±9.46 5-9d / 69±7.86* 10-20d / 41.8±6.66*	(Hannan *et al.*, 2005) *Significant difference between lactation stages
	Japan	 10 10 129 134 10	(µg/100mL) 1-5d / 2.5±0.7 6-10d /2.4±0.6 21-89d / 1.8±0.4* 21-89d / 1.5±0.6* 181-365d / 1.3±0.4	(Yamawaki *et al.*, 2005) *Significant difference between lactation stages
	USA	 91 163	(mg/100g) 4-7d / 0.52±0.02* 10-14d / 0.41±0.01*	(Feeley *et al.*, 1983b)

Zinc		158	30-45d / 0.29±0.01*	*Significant difference between all lactation stages
	USA - Caucasian	 28 23 30	(µg/mL) 1-3mo / 1.60±0.23 7-9mo / 0.75±0.11$ 19-31mo / 0.60±0.19$	(Vaughan *et al.*, 1979) $Significant differences in log concentrations between consecutive months
	Spain	 22 22 22	(mg/L) 2-4d / 7.99±3.23* 30d / 2.41±0.90* 90d / 1.05±0.71*	(Silvestre *et al.*, 2001) *Significant difference between all lactation stages
	Austria	 44 44 44	(µg/L) 1-3d / 6040±3590 4-7d / 760±600* 42-293d / 470±310*	(Rossipal & Krachler, 1998) *Significant difference between lactation stages
	Vietnam	 37 22	(mg/L) Median (25%,75%) 6-8mo / 0.59(0.47, 0.85)* 9-12mo / 0.38(0.20, 0.73)*	(Nakamori *et al.*, 2009) *Significant difference between lactation stages
	Portugal	 31 31 31 31	(µg/kg) 7d / 4010.6±1177.7 28d / 2160.6±589.4* 56d / 1491.4±619.5* 112d / 1014.1±461.5*	(Matos *et al.*, 2009) *Significant difference between lactation stages
	Lybia	 25 25 25	(mg/L) 0-4d / 16.1±2.67 5-9d / 7.07±1.04* 10-20d / 4.95±1.3*	(Hannan *et al.*, 2005) *Significant difference between lactation stages
	Turkey - Preterm	 20 20 20 20	(µg/dL) 0-7d / 241±28.1# 7-14d / 228±18.9# 21d / 239±20.0# 60d / 201±17.5#	(Ustundag *et al.*, 2005) #Significant difference between preterm and term mothers No statistical analysis performed between lactation stages
	Turkey - Term	 20 20 20 20	(µg/dL) 0-7d / 308±30.4# 7-14d / 272±19.8# 21d / 265±20.3# 60d / 281±18.0#	
	Japan	 20 38 551 476 39	(µg/100mL) 1-5d / 475±248 6-10d / 384±139 21-89d / 177±108* 21-89d / 67±80* 181-365d / 65±43	(Yamawaki *et al.*, 2005) *Significant difference Between lactation stages

In addition to the trace minerals listed in Table 2.2, previous studies have also measured the concentrations of other trace minerals present in milk. Bjorklund's group determined the breast milk concentration of 32 minerals in Swedish mothers in early lactation (14-21 day) by using inductively coupled plasma mass spectrometry (ICP-MS) (Björklund *et al.*, 2012). They observed large inter-individual differences in the levels of some trace elements in human milk such as cobalt, chromium, manganese, and molybdenum, as well as in the levels of toxic elements including arsenic, cadmium, lead, vanadium, and antimony. The arsenic concentration in breast milk was significantly correlated with fish consumption of Swedish women, suggesting the influence of maternal diet on the concentrations of toxic elements in breast milk (Björklund *et al.*, 2012). Furthermore, Matos group studied changes in concentrations of 13 oligoelements present in human milk during the first four months of lactation (Matos *et al.*, 2014). Milk samples were collected from 31 lactating women at 1, 4, 8, 12, and 16 weeks postpartum, respectively, and measured by ICP-MS. A significant reduction in the milk levels of rubidium, vanadium, and thallium was observed: from 891.4±284.8, 2.56±1.20, and 0.062±0.19 μg/kg in the first week to 579.5±229.4, 1.51±0.48, and 0.014±0.13 μg/kg by week 16, respectively. In contrast, there were no significant changes in the milk levels of cobalt, nickel, molybdenum, lead, and cadmium throughout the 16 weeks of lactation (Matos *et al.*, 2014). A decrease in the concentration of trace elements in human milk during the course of lactation has been proposed to be linked to the decreasing binding capacity of milk due to a culminating reduction in the overall concentrations of protein and fat (Leotsinidis, Alexopoulos, & Kostopoulou-Farri, 2005; Matos *et al.*, 2014).

In comparison to the compositional changes observed in trace minerals during lactation, the maternal dietary intake of chromium, copper, iron, and zinc does not appear to influence their concentrations in milk. This suggests the involvement of regulatory mechanisms present in the mammary gland in governing the uptake and export of these minerals in milk (Lönnerdal, 2000). Lonnerdal has proposed that this regulation may occur at two different sites, either at the mammary epithelial cells where the uptake of trace minerals from serum occurs or at the site where synthesis and secretion of milk takes place.

Lastly, the maternal diet has a direct effect on milk selenium concentrations (i.e., circulating serum selenium is closely correlated with milk selenium). Mothers living in areas where low selenium intake is common have lower than normal concentrations of both serum and milk selenium. For example, plasma and milk selenium levels in Nepalese mothers were lower than American mothers

due to the lack of selenium-rich food sources in their diet (Emmett & Rogers, 1997). It appears that the uptake and export of selenium in milk is unregulated.

3. CHANGES OF CYTOKINES IN HUMAN MILK DURING LACTATION

The mammary gland immune system comprises of a diverse array of physical, cellular, and molecular components that function within innate and adaptive (acquired) immune responses (Rainard & Riollet, 2006). The innate immune system constitutes the primary line of defense during the initial stages of infection and is a key determinant of mastitis outcome. Following exposure to invading pathogens, cytokines are released transiently in the teat and udder cisterns by epithelial cells, other resident tissue cells, recruited leukocytes, or by a combination of these sources (Persson Waller *et al.*, 2003). Although cytokines do not confer any direct antibacterial functions, they regulate host defense indirectly by exerting their diverse effects on the local mammary gland effector cell populations to perform antimicrobial activities and to maintain immunological homeostasis (Aitken *et al.*, 2011; Oviedo-Boyso *et al.*, 2007).

In addition to their roles in the maternal immune system, cytokines are equally important to newborns' host defense by preventing autoimmunity and aiding in their development of the digestive system (Srivastava *et al.*, 1996). Physiological changes of the cytokine profile during the course of lactation reflects the changes in the mammary immune system as well as any alteration in the needs of the recipient newborn for their mucosal defense development and immune protection (Ustundag *et al.*, 2005). For example, in mature milk, the levels of IL-6 and IL-8 were significantly lower, while the levels of IL-10 and TNF-α were significantly higher than their levels in transitional milk, indicating a shift in pro- and anti-inflammatory cytokine production at different periods of lactation (Meki, Saleem, Al-Ghazali, & Sayed, 2003).

In general, a considerable variability exists between individuals in terms of the types and concentrations of cytokines present in milk. Cytokine levels were higher in colostrum and transient milk, but reduced during lactation (Ustundag *et al.*, 2005); and women delivering preterm have lower levels of several cytokines than women delivering at term (Srivastava *et al.*, 1996).

3.1. Interleukin-8

CXCL8 (or IL-8) is the most studied chemokine in the bovine mastitis immune response. Chemokine is a specific class of cytokines mediating the recruitment of innate immune effector cells,

such as myeloid and NK cells, to the site of inflammation to initiate pathogen clearance and the migration of T- and B-cells to induce a humoral immune response (Schukken *et al.*, 2011; Sordillo & Streicher, 2002). This chemokine interacts with CXC chemokine receptor 1 (CXCR1) and CXCR2 expressed on the surface of effector immune cells, mediating the recruitment of granulocytes and accumulation of macrophages from the maternal circulation to the breast milk and possibly later across the neonatal bowel wall (Michie *et al.*, 1998). This is an important characteristic of IL-8 and is thought to contribute to the newborn's mucosal defense and immune system development.

A few studies have reported changes in IL-8 concentrations during lactation (Meki *et al.*, 2003; Michie *et al.*, 2003; Srivastava *et al.*, 1996; Ustundag *et al.*, 2005). Meki *et al.* discovered that IL-8 concentration was found to be at the highest in colostrum, followed by in transitional milk, and in mature milk. The changes in IL-8 concentrations appear to be parallel to changes in immunoglobulin A (IgA) levels (Meki *et al.*, 2003). Similar observations were made by Michie *et al.* as well (Michie *et al.*, 1998). Srivastava *et al.* also demonstrated that among the many cytokines measured, IL-8 was the only one present in milk samples. Moreover, IL-8 was significantly elevated in mature milk of mothers with allergies (Ustundag *et al.*, 2005).

3.2. Tumour necrosis factor alpha and interleukin-6

Tumour necrosis factor-alpha (TNF-α) is the main pro-inflammatory cytokine produced by macrophages, neutrophils and epithelial cells during the early stage of infection, and it participates in the chemotactic activity of neutrophil such as inducing migration of leukocytes into the udder (Fitzgerald *et al.*, 2007). Its physiological role also extends to the development of the mammary gland by stimulating the growth and branching morphogenesis of mammary epithelial cells, and modulating their functional differentiation. In the context of human milk, TNF-α stimulates interleukin-6 (IL-6) production via mononuclear leucocytes, and its concentration is increased especially within the 1st week postpartum (Ustundag *et al.*, 2005). Correlation analyses have shown a positive correlation between TNF-α and IL-6 cytokines in milk, independent of the newborn's gestation age, as well as a positive association between IL-6 and IgA levels (Meki *et al.*, 2003). The latter association was previously seen in Garofalo *et al.*'s study, where the addition of neutralizing antibody to IL-6 resulted in the inhibition of IgA production by stimulated colostrum mononuclear cells (Garofalo *et al.*, 1995).

IL-6 is often used as a marker for systemic activation of pro-inflammatory cytokines since it is responsible for regulating acute phase protein synthesis and promoting the movement of monocytes to the mammary gland (Oviedo-Boyso *et al.*, 2007). Large amounts of IL-6 are secreted into whey with

the corporation of TNF-α and IL-1β (Rudloff, Schmalstieg, Palkowetz, Paszkiewicz, & Goldman, 1993; Saito, Maruyama, Kato, Moriyama, & Ichijo, 1991). A study by Meki *et al.* showed that TNF-α and IL-6 were detected in all milk samples throughout lactation, whereas Srivastava *et al.* reported no significant TNF-α and IL-6 presence in any of the milk samples (Srivastava *et al.*, 1996). Several studies reported that both cytokines were at the highest concentration in colostrum and decreased markedly in transitional milk and mature milk (Rudloff *et al.*, 1992; Rudloff *et al.*, 1993; Saito *et al.*, 1991; Ustundag *et al.*, 2005). Ustundag *et al.* further discovered that the lowered levels of TNF-α in transitional milk were later found to be elevated again in mature milk. Conversely, Munoz *et al.* noted low levels of TNF-α in the first two weeks after birth (Muñoz *et al.*, 1995).

3.3. Interleukin-1 beta

Interleukin-1 (IL-1) is subdivided into two types, the cytoplasmic IL-1α and the secreted IL-1β. IL-1β is a key mediator of local and systemic immune response (Schukken *et al.*, 2011). It initiates the recruitment of neutrophils to the mammary gland (Oviedo-Boyso *et al.*, 2007) and regulates the expression of a complex series of immune response genes that encode for other cytokines, enzymes and acute phase proteins which are involved in cellular proliferation and apoptosis (Schukken *et al.*, 2011).

IL-lβ, but not IL-1α, was found in the colostrum and early (day 7) milk samples from healthy lactating mothers. During early lactation, the milk cells were activated and spontaneously produced IL-1 (Garofalo *et al.*, 1995). Hawkes *et al.* reported that IL-lβ levels declined gradually during lactation, and were higher in milk from mothers of mature infants than mothers of premature ones during the first two months. Conversely, no significant IL-lβ was present in milk samples from study by Srivastava *et al.* (Srivastava *et al.*, 1996).

3.4. Interleukin-10

Interleukin-10 (IL-10) is one of the best examined anti-inflammatory interleukins in udder host response (Bannerman, 2009; Castellote *et al.*, 2011; Meki *et al.*, 2003; Schukken *et al.*, 2011; Silvestre *et al.*, 2001; Wenz *et al.*, 2010). It is an 18-kD protein homodimer and a member of the α-helix family of hematopoietic cytokines. IL-10 has two major functions, the inhibition of cytokine synthesis and the reduction of factors mediated by the major histocompatibility II (MHC-II) complex. It acts to inhibit the production of IL-1, IL-2, 1L-6, IL-8, TNF-α, granulocyte-macrophage colony-

stimulating (GM-CS) factor, granulocyte colony-stimulating factor, macrophage migration inhibitory factor, and interferon-γ (IFN-γ), many of which are pro-inflammatory cytokines (Garofalo *et al.*, 1995).

IL-10 was measured at very low concentrations in human milk and was undetectable in some milk whey samples. During lactation, IL-10 concentrations undergo a gradual decrease in milk from both mothers of mature and premature infants (Meki *et al.*, 2003).

4. CLINICAL AND SUBCLINICAL MASTITIS

4.1. Prevalence

Clinical mastitis and SCM occur in all populations worldwide. Estimates for the prevalence of mastitis vary among literature, from 1.1% to 16.5% (Table 2.3) in lactating women depending on the case definition used. Incidence and prevalence of human mastitis are difficult to determine because of substantial differences in case definition and reporting. This condition most frequently develops in the early stages of lactation, with highest incidence during the first 4 weeks after parturition. The majority of cases (74–95%) are observed in the first 3 months (WHO, 2000). However, mastitis may occur at any stage of lactation, including in the second year (Contreras & Rodríguez, 2011). Some mothers may suffer from recurrent mastitis, whether with one infant or those with successive pregnancies (Michie *et al.*, 2003). The reported percentages of SCM in populations are likely an underestimate of the actual prevalence of the condition as most epidemiology studies only record cases based on signs of clinical symptoms.

Human studies regarding SCM are scarce in medical literature in comparison to clinical mastitis, perhaps due to its lack of physical symptoms. However, there has been an increasing interest in recent years as studies have demonstrated that the prevalence of SCM among lactating mothers, ranging from 2% to 66% (Table 2.3), is much higher than clinical mastitis. Its ability to enhance vertical mother to infant transmission of infection (Semba, 2000) and the emerging threat of uprising multi-resistant bacterial strains all suggest the potential serious health consequences pose by SCM (Oviedo-Boyso *et al.*, 2007).

TABLE 2.3. Mastitis and SCM: Prevalence and incidence timeline.

Postpartum Time	Prevalence		Incidence	Reference
	Mastitis	SCM	Mastitis	
2 weeks	0%	12.2%, 66%		(Arsenault *et al.*, 2010; Nussenblatt *et al.*, 2005)
4 weeks	0%	7.8%		(Nussenblatt *et al.*, 2005)
6 weeks	1.4%, 16.4%	6.8%, 26%		(Arsenault *et al.*, 2010; Nussenblatt *et al.*, 2005; Semba, 2000)
9 weeks		20%		(Arsenault *et al.*, 2010)
10 weeks	0%	3.7%		(Nussenblatt *et al.*, 2005)
14 weeks	1.4%	10.6%		(Nussenblatt *et al.*, 2005)
< 3 months			27.1%, 74-95%	(Contreras & Rodríguez, 2011; Semba & Neville, 1999)
3 months		33%		(Arsenault *et al.*, 2010)
< 6 months	1.1%, 2.8%	5.1%	20%	(Nussenblatt *et al.*, 2005; Semba, 2000)
6 months		26%		(Arsenault *et al.*, 2010)
9 months	1.4%	4.9%, 20%		(Nussenblatt *et al.*, 2005)
12 months	2.0%	1.9%, 20%		(Arsenault *et al.*, 2010; Nussenblatt *et al.*, 2005)
>12 months		25%		(Arsenault *et al.*, 2010)

4.2. Etiology

Prompt diagnosis and treatment of mastitis in women is required to prevent lactation failure, recurrent mastitis, and breast abscess (Sordillo, 2011). A review by WHO in 2000 suggests that the two principal causes of SCM/mastitis are milk stasis and infection. Milk stasis occurs when milk is not removed from the breast efficiently, resulting in the stagnation of milk within the breast which provides a favourable condition for bacterial growth (WHO, 2000). Milk stasis can also lead to a halt of milk flow from the alveolus and increase the severity of inflammation. Without effective removal of milk, non-infectious mastitis is likely to progress to infectious mastitis, and infectious mastitis to the formation of an abscess (Barbosa-Cesnik, Schwartz, & Foxman, 2003). The problem can usually be treated by encouraging women to continue breast-feeding or to express milk from the affected

breast (Thomsen, Espersen, & Maigaard, 1984). Causes of milk stasis include poor infant feeding practices such as suboptimal attachment of the infant to the breast, ineffective suckling, restriction of the frequency or duration of feeds, overabundant milk supply and blockage of milk ducts (WHO, 2000). All of these are predisposing factors for mastitis in addition to fatigue, stress, mother's age, parity and breast trauma (Foxman *et al.*, 2002; Foxman *et al.*, 1994; Kaufmann & Foxman, 1991)

The source of entry of pathogenic bacteria to the mammary gland remains uncertain. Several routes have been suggested: via the lactiferous ducts into a lobe, by haematogenous spread, and/or through a nipple fissure into periductal lymphatic system (WHO, 2000). Localized infections by *Staphylococcus aureus* is thought to be responsible for up to 50% of clinically apparent mastitis but other common skin and respiratory flora, such as *Escherichia coli* and *Klebsiella pneumonia*, have also been implicated (Kudi, Bray, Niba, & Kalla, 2009). Moreover, systemic inflammation has been proposed, although unconfirmed, as another cause of SCM/mastitis. Although, SCM lacks the clinical symptoms, it exhibits similar pathology as clinical mastitis as both are associated with raised sodium, pH, and inflammatory cytokines (Filteau *et al.*, 1999; Willumsen *et al.*, 2002). It is not known whether women with SCM have sterile inflammation in the breast or are harbouring microbial pathogens that may be causing the non-symptomatic infectious mastitis (Nussenblatt *et al.*, 2005).

In addition to milk stasis and infection, nutritional factors have often been thought to play a role in the predisposition to mastitis, including high salt and fat intake and anaemia but the evidence is inconclusive (WHO, 2000). However, veterinary research in cows has suggested that micronutrient deficiencies, particularly in copper, selenium, vitamin E and vitamin A, are known to increase the progression of SCM (Smith, Hogan, & Weiss, 1997; Weiss & Spears, 2006; Wintergerst, Maggini, & Hornig, 2007).

4.3. Bacteria associated with mastitis

The two most common infective agents responsible for mastitis are *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). Infection induced by *E. coli* is usually acute and tends to resolve within a few days, whereas mastitis caused by *S. aureus* often results in a chronic low-grade infection in the mother (Ling, 2010). *S. aureus* has been isolated from the milk of approximately 40-50% of women with clinically apparent mastitis (Delgado *et al.*, 2009). Other bacterial species and genera that have been frequently isolated in milk from mothers with mastitis or SCM include non-β-hemolytic streptococci, *Staphylococcus epidermidis, Klebsiella oxytoca, corynebacterium*, *Enterococcus faecalis,* and *Pseudomonas fluorescens* (Delgado *et al.*, 2008; Delgado *et al.*, 2009; Osterman & Rahm, 2000). Normal

skin and respiratory flora, such as *Lactobacillus spp., Enterococci spp., E. coli, Klebsiella pneumoniae*, and *Bacteroides fiagilis*, have been reported in about half of the cases of clinically apparent mastitis (Osterman & Rahm, 2000).

Preliminary results have indicated that human mastitis may derive from mammary bacterial dysbiosis, a process in which the population of mastitis agents increases while the population of normal or commensal mammary microbiota decreases. As pathogenic bacteria concentration increases, it induces a series of detrimental biochemical and immunological changes in the mammary gland and milk, which are later manifested as clinical symptoms (Contreras & Rodríguez, 2011).

4.4. Diagnosis of SCM: Traditional approaches

Clinical mastitis is characterized and diagnosed by signs of inflammation in the mammary gland including hyperemia, pain, erythema, oedematous and increased gland size and density. These symptoms may or may not be accompanied by systemic signs, such as fever or malaise. (Michie *et al.*, 2003; Pyörälä, 2003; WHO, 2000; Wöckel, Abou-Dakn, Beggel, & Arck, 2008). SCM, on the other hand, is asymptomatic and lacks a clear definition and standard diagnosis.

In humans, there is no routine quantitative approach to diagnose SCM. Current literature show that SCM can be defined as cases characterized by the absence of external inflammatory symptoms along with one or more of the following indicators: reduced milk secretion, increased sodium/potassium (Na+/K+) ratio, elevated IL-8 count, elevated milk leukocytes or somatic cell count (SCC), and/or high bacterial count in milk sample (Aryeetey, 2007; Aryeetey *et al.*, 2009; Aryeetey, Marquis, Timms, Lartley*, et al.*, 2008; Gomo *et al.*, 2003; Nussenblatt *et al.*, 2005; Rasmussen *et al.*, 2008; Willumsen *et al.*, 2002).

Although no standard definition of SCM has been proposed, sodium levels and/or Na/K ratio are the two most commonly used diagnostic biomarkers for SCM (Aryeetey, Marquis, Timms, Lartey, & Brakohiapa, 2008; Kantarci *et al.*, 2007; Morton, 1994; Richards *et al.*, 2010). Elevated sodium concentrations in human milk are considered to be a sensitive indicator of mastitis on the basis that sodium concentrations do not differ between fore-milk and hind-milk, and that it is not under the influence of maternal sodium intake. Moreover, milk sodium concentrations, expressed as a ratio with potassium permit the use of spot milk samples regardless of the time of sampling or the time since the infant was last fed (Filteau *et al.*, 1999). A number of reports have indicated that Na/K ratios in the milk of healthy women at one month postpartum generally averaged 0.6 or less (Aryeetey, Marquis, Timms, Lartey*, et al.*, 2008), which is in correspondence to the average breast milk sodium and

potassium concentrations ranging between 5 to 6 mmol/L and 13 to 14 mmol/L, respectively. Conversely, the mean sodium concentrations in mastitic milk are >16 mmol/L (Semba & Neville, 1999). Therefore, a Na/K ratio ≤ 0.6 is considered to be normal, > 0.6 to 1.0 is considered to be moderately raised, and > 1.0 is greatly raised (Kantarci *et al.*, 2007; Willumsen *et al.*, 2002). The Na/K ratio of breast milk has been considered as an indirect measure of epithelial damage, leakiness, and subsequently inflammation status.

Results from a recent study by Hunt *et al.* indicated that SCC counts and IL-8 concentrations in milk may be better and more informative indicators of mammary inflammation than sodium contents alone due to their higher sensitivity to changes in breast milk (Hunt et al., 2012). However, despite this recent discovery, previous studies have pointed out that high leukocyte counts or SCC cannot be used as the sole indicator for SCM because women from areas where high prevalence of infections is common (i.e., poor hygiene practices) may naturally have elevated baseline leukocyte counts (Abou-Dakn, Richardt, Schaefer-Graf, & Wöckel, 2010; Pyörälä, 2003). Although cytokines such as IL-8 are direct indicators of inflammation in milk and can be determined using rapid and reliable routine techniques (Pyorala, 2003), their high variability between individuals makes them a less effective tool for the early detection of SCM.

There are also several ancillary tests that are used to detect both clinical and SCM, these include: N-acetyl-beta-D-glucosaminidase activity, pH, lactose content, electrical conductivity, flow measurements, and quantification of acute phase proteins (Contreras & Rodríguez, 2011).

4.5. Etiological diagnosis of mastitis: Microbiological analysis

Microbiological analysis was described as the only method that allows for an etiological diagnosis of mastitis (Contreras & Rodríguez, 2011). Earlier identification of bacterial diversity in breast milk has been based almost exclusively on the use of selective and differential culture media, however, majority of the bacteria failed to grow on conventional culture, especially anaerobic bacteria. It was reported that at least 20%-30% of milk samples taken from bovine with clinical mastitis show no bacterial growth on culture (Taponen, Salmikivi, Simojoki, Koskinen, & Pyörälä, 2009). The lack of identification in culture may have multiple explanations, including inability to culture certain bacteria species, presence of bacteria below current detection thresholds, and absence of bacteria at the time culture is initiated (Jost *et al.*, 2013; Keane *et al.*, 2013; Kuehn *et al.*, 2013). The limitations of classical bacterial cultures have spurred the interests of adopting culture-independent, molecular

techniques as a better alternative method for the assessment of a complete biodiversity profile in breast milk.

Methods such as real-time PCR, multiplex PCR, denaturing gradient gel electrophoresis (DGGE) PCR, and 16S RNA pyro-sequencing are now being used to identify bacterial DNA in milk samples (Collado *et al.*, 2009; Delgado *et al.*, 2008; Keane *et al.*, 2013; Martineau *et al.*, 2001; Rinttilä, Kassinen, Malinen, Krogius, & Palva, 2004; Sorg *et al.*, 2012). In addition to addressing the above limitations imposed by classical culture-dependent techniques, molecular techniques have the potential to offer some insight on the microbial communities present in milk and assess the changes in bacterial populations throughout disease progression. Several studies have reported the use of microbial identification techniques based on pyrosequencing of the hypervariable regions within the 16S ribosomal RNA gene extracted directly from milk to obtain a description of the healthy human milk microbiome. They showed that such technique identifies a much greater diversity of bacteria in milk than what has previously been reported. The milk microbiome is more complex than expected, with several bacterial genera representing greater than 5% of the relative community abundance (Collado *et al.*, 2009; Delgado *et al.*, 2008; Hunt *et al.*, 2011).

These culture-independent, molecular techniques have provided an in-depth evaluation of DNA-based characterization of mastitis-causing pathogens at the subspecies level. They have also contributed to the understanding of how the identification of allelic profiles for housekeeping genes or virulence genes is a useful diagnostic tool to assess pathogen persistence (Sordillo, 2011). Hence, future developments of molecular biology analyses should target the complex interaction between immunological defenses of the mammary gland and the diversity of microorganisms that cause infection but not necessarily lead to the manifestation of clinical symptoms.

5. MASTITIS AND MILK MACROMINERALS

Clinical and SCM are known to have adverse effects on the quality and quantity of bovine milk produced. This causes substantial economic damages to the global dairy industry and hence, veterinary scientists have a particular interest in this disease and extensive studies were conducted in cows (Heinrichs *et al.*, 2009; Wöckel *et al.*, 2008). Therefore, much of our knowledge on the pathophysiology of mastitis and its effects on milk composition is derived from experimental studies of cows. However, even within the realm of bovine research, there are limited data on SCM in comparison to clinical mastitis.

Bovine studies have found that clinical mastitis disrupts mammary gland function via a number of mechanisms. The infecting pathogens can alter the metabolic activity of mammary epithelial cells, which could result in the reduction of α-lactalbumin and β-lactoglobulin in milk. Bacteria are also capable of producing proteolytic enzymes such as proteinase that have the capacity to degrade milk proteins, such as casein. The inflammation of intra-mammary tissues during mastitis can trigger physical damages to the epithelial layer, decreasing its synthetic and secretory capacity as well as enhancing mammary epithelium permeability via the opening of tight junctions between cells. Leaky tight junctions are responsible for the lowered lactose and elevated sodium levels in breast milk (Li *et al.*, 2009).

5.1. Sodium, potassium and chloride

The ionic environment of milk is noticeably altered by mastitis. The sodium and chloride concentrations are increased, while potassium, the normally predominant mineral in milk, declines. The disturbances seen in sodium, chloride, and potassium levels have been confirmed by a number of studies. Compared to the milk from healthy quarters, milk from quarters with SCM showed elevated sodium (91.97 vs. 52.93 mg/dL), chloride (>0.14 vs. <0.14 g/dL), pH (6.69 vs. 6.59), albumin (5.62 vs. 2.65 g/dL) levels as reported by Batavani and coworkers. These changes in pH, mineral concentrations and protein fractions in milk of quarters suggest the presence of tissue damage or cellular apoptosis provoked by SCM (Batavani *et al.*, 2007). In another study by Bruckmaier *et al.*, sodium concentrations were positively correlated with chloride concentrations ($r = 0.94$, $P < 0.001$), but negatively with potassium concentrations in the infected quarters ($r = -0.72$, $P < 0.05$) (Bruckmaier *et al.*, 2004). Wegner and Stull have noted a 20% decline in potassium concentrations with increasing infection (Wegner & Stull, 1978). In addition, the elevation in sodium concentrations was also noted in a few human studies, whereby higher sodium levels were detected in milk samples from both SCM mothers (Aryeetey, Marquis, Timms, Lartley*, et al.*, 2008) and mothers with clinical mastitis (Hunt *et al.*, 2013).

The high sodium and chloride concentrations in clinical and subclinical mastitic milk are the result of blood transudation that is complemented osmotically by the reduction in lactose and potassium levels. During mastitis, the intra-mammary infection and resultant inflammation result in significant damages to the ductal and secretory epithelium in the mammary gland. Consequently, this causes an opening of the "tight junctions" between secretory cells, and increased permeability of the blood capillaries. As such, high levels of sodium and chloride in the extracellular fluid pour into the

lumen of the alveolus in response to the changes in the concentration gradient, and potassium level is decreased proportionately in order to maintain osmolarity (Wegner & Stull, 1978).

5.2. Other macrominerals

In the milk obtained from cows with evidence of udder infections, Batavani's group has reported a reduction in both calcium and phosphorus levels, whereas Leiner, Merin and Silanikove did not observe any change in the calcium levels, but instead indicated significantly lowered calcium ion activity in infected glands (Leitner, Merin, & Silanikove, 2004). In Wegner and Stull's study, the milk calcium concentrations dropped by 11%, while the magnesium content of mastitic milk remained relatively constant. Either magnesium did not contribute to the osmotic adjustment which accompanied udder inflammation, or its association with casein altered the milk-blood permeability of this mineral (Wegner & Stull, 1978).

In general, plasma calcium is low in periparturient dairy cows due to the massive secretion of this mineral into colostrum and milk. Clinical or subclinical hypocalcemia impairs systemic and mammary gland immune functions, thus increasing the risk for mastitis (Contreras & Rodríguez, 2011).

6. MASTITIS AND MILK TRACE MINERALS

Trace minerals have critical roles in the interrelated system of host defense, oxidative and energy metabolism in cows as well as in their susceptibility to mastitis. Technological advances in immunological research have increased our ability to explore the immunity of the bovine mammary gland during mastitis. A pathology of SCM is increased oxidative stress, as indicated by lipid peroxides and glutathione peroxidase in bovine blood (Semba, Kumwenda, Taha*, et al.*, 1999). Following oxidation-dependent bacterial killing, oxygen radicals enter the cytosol and cause oxidative damage to the host cells (Sordillo, 2013). To prevent cell damage, an array of antioxidant defenses derived from trace minerals is initiated. Cytosolic enzymes such as selenium-dependent glutathione peroxidase, copper- and zinc-dependent superoxide dismutases protect the leukocytes from oxidation by degrading the potential substrates for oxygen radical production (Overton & Yasui, 2014; Spears & Weiss, 2008). Therefore, trace minerals are important in the promotion of efficient leukocyte function and their supplementation is a well-recognized means of decreasing the inflammatory response during SCM in the dairy industry (Spears & Weiss, 2008). The modulation of oxidative mechanisms and

immune function by trace minerals are particularly significant for cows during the transition period and early lactation. It has been understood that for some trace minerals, the amount required by the animal for optimal immune function is greater than the amount required for growth and reproduction (Heinrichs *et al.*, 2009). On the other hand, existing literature on trace minerals reveal substantial heterogeneity of responses in the outcome variables measured (Overton & Yasui, 2014).

6.1. Selenium

Function of selenium in immune system as well as bovine clinical mastitis has been well documented. Selenium is essential for optimum immune response. It influences both innate and acquired immune responses of the mammary gland through its role in redox regulation and antioxidant function and contributes to membrane integrity and protection against DNA damage (Salman *et al.*, 2009; Wintergerst *et al.*, 2007). Selenium is an integral component of the enzyme glutathione peroxidase (GPx) (Spears & Weiss, 2008). During the metabolism of oxygen within cells and microbe killing by neutrophils, large quantities of superoxide anions and hydrogen peroxide are generated and these reactive oxygen species (ROS) can severely damage membrane lipids, DNA, cellular proteins, and enzymes if not neutralized (Heinrichs *et al.*, 2009; Smith *et al.*, 1997). The specific function of GPx is to convert hydrogen peroxide to water and lipid hydroperoxides to the corresponding alcohols. When the concentration of hydrogen peroxide is low, there is less chance that the hydroxyl radical will be formed. The hydroxyl radical is an ROS that is extremely damaging to cells (Smith *et al.*, 1997).

The soil in many parts of the world is poor in selenium, and foods grown from these soils do not provide adequate dietary selenium (WHO, 2000). This inadequate intake of selenium is considered an important risk factor of mastitis. Compromised neutrophil function and increased milk somatic cell count (SCC) in milk were reported in selenium deficient cattle along with increased incidence of infectious disease such mastitis (Sordillo, 2013). The neutrophils from selenium deficient cows accumulate hydrogen peroxide, have decreased viability and reduced ability for intracellular killing of mastitis pathogens. However, selenium status does not influence the ability of neutrophils to phagocytize bacteria (Grasso, Scholz, Erskine, & Eberhart, 1990). Furthermore, bovine mammary endothelial cells grown in selenium deficient cell culture media were found to exhibit enhanced neutrophil adherence when stimulated with TNF-α, IL-1 or hydrogen peroxide. This tight adhesion between neutrophils and endothelial cells could hinder neutrophil migration to the infection site (Spears & Weiss, 2008).

It is clear that low selenium status is linked to increased susceptibility of dairy cows to mastitis and adequate intake of selenium is directly related to disease resistance. High serum selenium concentrations (i.e. supplemented heifers), were associated with fewer infected quarters, reduced prevalence of infection, lowered incidence of mastitis, shorter infection duration and lowered milk somatic cell count compared with unsupplemented heifers. Selenium supplementation appears to strengthen antibacterial activity in milk by enhancing bacterial killing by neutrophils (Semba & Neville, 1999). The influx of polymorphonuclear neutrophils into bacteria challenged quarters was more rapid in the selenium-supplemented cows, restricting the *E. coli* load in milk to a low level. As a result, infections were less severe and were eliminated more rapidly, and milk loss was reduced in the selenium-supplemented cows whereas selenium-deficient cows appear to be much less resistant to experimental clinical mastitis with *E. coli* (Smith *et al.*, 1997).

Administration of selenium before expected calving has been shown to reduce the duration of clinical mastitis by 46% compared to controls (Smith *et al.*, 1997). Weiss *et al.* have also reported that higher dietary supplementation with selenium resulted in less severity and shorter duration for experimental *E. coli* mastitis. The influx of neutrophils into challenged quarters was more rapid in supplemented cows, where milk loss was reduced and infections were eliminated more rapidly (Weiss & Spears, 2006). Both phagocytosis ($P<0.05$) and killing ($P<0.01$) of *S. aureus* by blood leukocytes were higher when the dairy cows received between 10 and 17 mg of selenium per day (Salman *et al.*, 2009). Many clinical studies have revealed the positive association between selenium supplementation, either alone or in combination with vitamin E, and udder health (Spears & Weiss, 2008).

6.2. Copper

Copper plays an important biological role as a cofactor for several copper dependent enzymes, such as superoxide dismutase (SOD) and ceruloplasmin (Spears & Weiss, 2008). Copper not only affects the oxidant-antioxidant balance in dairy cow, but also the circulating concentrations of thyroid hormones, suggesting further links with energy metabolism (Overton & Yasui, 2014). SOD is responsible for conversion of superoxide radicals to hydrogen peroxide in the cytosol, thereby protecting cells from the toxic effects of oxygen radicals generated during phagocytosis. Ceruloplasmin is an acute phase protein that acts to reduce *in vitro* adhesion of activated neutrophils to endothelial cells and facilitates iron absorption and transportation. Both functions of copper help to reduce the incidence of periparturient intra-mammary infection (Heinrichs *et al.*, 2009; McClellan, Miller, & Hartmann, 2008; Meglia, Johannisson, Petersson, & Persson Waller, 2001).

Copper status alters functions of neutrophils, monocytes, and T cells production (Wintergerst *et al.*, 2007). Cows with clinical mastitis exhibit higher concentrations of plasma ceruloplasmin than non-mastitic cows, and neutrophils from copper-deficient cows exhibit reduced ability to kill *S. aureus* during infection i.e. reduced bactericidal activities (Heinrichs *et al.*, 2009). However, the phagocytic activity of neutrophils and responses of mononuclear cells to mitogen stimulation were not affected (Spears & Weiss, 2008).

Overall, copper-supplemented dairy cows had lower bacterial counts, lower SCC, lower clinical udder scores, and lower peak rectal temperatures than responses in control animals after intra-mammary challenge with *E. coli*. The decreased clinical severity could be due to increased capability of neutrophils in supplemented animals to kill the invading *E. coli*. Copper supplementation appear to reduce the severity of clinical response during experimental *E. coli* mastitis but had no effect on duration (Scaletti, Trammell, Smith, & Harmon, 2003).

6.3. Chromium

The primary function of chromium appears to be its ability to enhance insulin actions. However, it has also been shown to influence both immune function and energy metabolism of cattle; dairy cows fed chromium during the transition period and early lactation have evidence of improved immune function, increased milk production, and decreased cytological endometritis (Spears & Weiss, 2008).

A study showed that chromium supplementation with a Cr-amino acid chelate prepartum and during the first 16 weeks of lactation had no beneficial effect on health status and mastitis-related parameters in dairy cows (Overton & Yasui, 2014). However, in periparturient dairy cows supplementing chromium in diets may affect cell-mediated and humoral immune responses (Spears & Weiss, 2008).

6.4. Iron

Iron is essential for electron transfer reactions, gene regulation, binding and transport of oxygen. Iron-dependent enzymes, such as catalase and peroxidase, are critical in controlling ROS. In addition, iron is involved in the regulation of cytokine production, their mechanism of action, and in the activation of protein kinase C, which is essential for phosphorylation of factors regulating cell proliferation (Overton & Yasui, 2014; Spears & Weiss, 2008). Therefore, a deficiency in iron can affect multiple systems in cows such as immune function, oxygen and energy metabolism.

A review by Wintergerst *et al.* has summarized that iron deficiency results in impaired secretion of cytokines (IFN-γ, TNF-α, IL-2), reduced NK cell activity, reduced T-cell proliferation, lowered delayed-type hypersensitivity (DTH) response, impaired bactericidal macrophage activity and causes unfavorable functional consequences on the immune system (Wintergerst *et al.*, 2007). On the other hand, an excess of iron in serum is reactive and pro-oxidant. It interferes with the absorption of other trace minerals and generate ROS (Overton & Yasui, 2014).

6.5. Zinc

Zinc is involved with cell proliferation, differentiation and apoptosis i.e. cell turnover, as it is an essential component of numerous enzymes, including enzymes involved in the synthesis of DNA and RNA (Weiss & Spears, 2006). In the antioxidant system, zinc is a component of SOD, which scavenges for superoxide in immune cells. Zinc also induces synthesis of metallothionein, a metal binding protein that can capture hydroxyl radicals. Therefore, zinc may affect immunity via its important role in autoxidation, cell replication and proliferation (Overton & Yasui, 2014; Spears & Weiss, 2008). Severe zinc deficiency in calves and in lambs has been shown to weaken immune responses and increase rate of infection (Wintergerst *et al.*, 2007).

In cows, zinc is essential for production of keratin, which has an important role in the mechanism of defense against pathogens in mammary gland (Spears & Weiss, 2008). Dietary supplementation of cows with zinc proteinate produced a lower rate of new intra-mammary infections than those supplemented with inorganic zinc oxide. It was suggested that the organic form of zinc i.e. zinc proteinate may enhance resistance to mammary infections by increasing keratin synthesis in the teat canal (Spain, 1993). The keratin acts as a physical barrier, which blocks the entry of pathogens into the udder. Studies have also shown that dairy cows supplemented with zinc methionine tended to produce more milk with a lower SCC compared with cows without supplemental zinc (Overton & Yasui, 2014).

7. MASTITIS AND MILK CYTOKINES

Current knowledge of cytokine function in mastitis is predominantly derived from animal studies, where the researchers induced clinical mastitis in animals via bacterial infection and observed the subsequent host immune response (Blum *et al.*, 2000; Grönlund, Johannisson, & Persson Waller, 2006; Persson Waller *et al.*, 2003; Rambeaud, Almeida, Pighetti, & Oliver, 2003; Vels, Røntved,

Bjerring, & Ingvartsen, 2009; Yang *et al.*, 2008). From these studies, cytokines, especially pro-inflammatory cytokines, were found to play a crucial role in the pathophysiology of bovine mastitis. They regulate a number of the immune cell activities, such as increase in phagocytic bactericidal capacity of macrophages and neutrophils, production of prostaglandins and leukotrienes, recruitment of neutrophils towards the site of infection, maturation of dendritic cells, and control of the acquired immune response(Oviedo-Boyso *et al.*, 2007). However, very little work has been conducted to specifically study these cytokines during SCM.

In cows, mastitis is the inflammatory reaction of the udder to invading pathogens. The adhesion of bacteria to mammary epithelial cells and the interaction of bacterial toxins with mammary tissues induce the expression of several acute phase cytokines (IL-1β, IL-6, IL-8, and TNF-α). A strong rise in the abundance of mRNAs encoding these cytokines in the udder tissue as well as elevated cytokine abundance in milk are observed (Bannerman, 2009; Günther, Liu, Esch, Schuberth, & Seyfert, 2010; Yang *et al.*, 2008; Zhu, Berg, Fossum, & Magnusson, 2007). These cytokines are responsible for the subsequent development of an unlimited local inflammatory reaction in mammary gland during mastitis (Oviedo-Boyso *et al.*, 2007).

7.1. Differential cytokine responses to pathogens

A variety of cytokines has been detected in healthy and infected bovine mammary glands. The pattern of cytokine expression by mammary cells differs depending on the mastitis-causing pathogen that elicits their response. In general, Gram-negative bacteria such as *E. coli* initiate a greater magnitude of pro-inflammatory cytokine responses (i.e., IL-1β, IL-6, IL-8, and TNF-α) when compared to Gram-positive bacteria such as *S. aureus* that tend to express a weaker and slower cytokine response during the early stage of infection (Aitken *et al.*, 2011). The endotoxin lipopolysaccharide (LPS) expressed on multiplying and dying Gram-negative bacteria tends to provoke a much stronger increase in TNF-α and IL-1β expression and secretion than Gram-positive pathogens (Georgeson & Filteau, 2000; Sordillo & Streicher, 2002). The LPS binds to the toll like receptor (TLR)-4 complex on numerous mammary gland cells. Activation by TLR-4 via the NF-κB pathway results in cellular production and release of several pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) that mediate the localized response to Gram-negative mastitis (Aitken *et al.*, 2011). Conversely, an immune response elicited by *S. aureus* is dominated by the cytokine IL-6 and generally exhibit a relatively low and short duration of expression in cells. Plotted on graphs, *S. aureus* infection demonstrates a cytokine response pattern that has an initial delayed response. However, ultimately there is a strong expression of the

cytokines since the positive feedback loop of cytokine production (induced by IL-1 and TNF-α) is diminished in the *S. aureus* triggered immune response (Bannerman, 2009; Schukken *et al.*, 2011).

TABLE 2.4. Cytokine response of bovine mammary gland to different bacterial species. [1]

Bacteria	Type of mastitis	Cytokine production
Staphylococcus aureus	Subclinical	Increase in IL-1β, IL-8, IL-6
	Clinical	Transit Increase in TNF-α, IL-12
Escherichia coli	Clinical	Increase in TNF-α, IL-1β, IL-6 IL-8, IL-12, IFN- γ
Streptococcus uberis	Clinical	Increase in TNF-α, IL-1β, IL-8, IL-12, IFN- γ
Serratia macrcescens	Clinical	Low concentration of IL-12, IFN- γ
Klebsiella pneumonia	Clinical	Increase in TNF-α, IL-1β, IL-8 and IL-12
Pseudomonas aeruginosa	Clinical	Increase in TNF-α, IL-8, IL-12, IL-10

[1] Table adapted from Bannerman (2009).

7.2. Chemokine: Interleukin-8

IL-8 is a chemokine produced by monocytes, T lymphocytes, and macrophages, as well as epithelial and endothelial cells. In a study on lactating mothers, milk collected from the symptomatic breast of women with mastitis (n = 14) was compared with that collected from the contralateral asymptomatic breast. The IL-8 level was parallel to the increase in milk sodium level and was elevated nearly 10-fold in the milk from mastitis breasts (Hunt, 2012). In another study, the median IL-8 levels were about 13 times higher among women who had elevated breast milk sodium levels than in women with normal sodium levels, and IL-8 levels were significantly correlated with levels of sodium and immunological factors measured in breast milk (Semba & Neville, 1999). Plasma acute phase protein concentrations were also found to correlate with milk Na/K ratio and IL-8, suggesting these biomarkers all measure the same phenomenon of subclinical breast inflammation (Mulokozi & Bilotta, 1999).

In cows, the Bannerman's group has detected a significant increase in the CXCL8 mRNA abundance in udder tissue and CXCL8 protein in milk after infection with various mastitis pathogens (Bannerman, 2009). IL-8 is actively produced in clinical mastitis caused by *E. coli*, whereas in mastitis caused by *S. aureus*, it is present in low concentrations (Oviedo-Boyso *et al.*, 2007).

7.3. Tumour necrosis factor alpha and interleukin 6

Expression of cytokines such as TNF-α and IL-6 have been studied considerably during various bacteria and endotoxin-induced mastitis experiments in cows (Oviedo-Boyso *et al.*, 2007). TNF-α is the main cytokine produced during the early stage of infection and is responsible for endotoxic shock in acute mastitis caused by *E. coli*. Elevated sera and milk concentrations of TNF-α were found in cows that had died from acute *E. coli* mastitis during the periparturient period. Upon LPS stimulation, the monocytes isolated from periparturient dairy cows can produce more TNF-α than cells isolated from cows in mid to late lactation (Sordillo, Pighetti, & Davis, 1995). This suggest that the enhanced ability of localized cell populations to produce TNF-α around calving may contribute to the greater frequency and severity of acute clinical coliform mastitis during the periparturient period. Therefore, limiting TNF-α production by monocytes, particularly within the mammary gland, may reduce the severity of clinical coliform mastitis in periparturient dairy cattle (Sordillo *et al.*, 1995).

IL-6 is involved in acute septic shock during mastitis caused by coliforms or *S. aureus*. This cytokine facilitates the exchange of neutrophils for monocytes in the mammary gland and regulate acute phase protein synthesis in hepatocytes. In a human study, Mizuno and coworkers observed that IL-6 level in the milk obtained from mothers affected with mastitic inflammation is significantly higher ($P < 0.01$) than that in milk from healthy mothers (Mizuno *et al.*, 2012). This difference becomes larger when the inflammation was accompanied by systemic symptoms of mastitis (fever/malaise). With treatments, the difference between healthy and mastitic milk decreased over time (Mizuno *et al.*, 2012), indicating that early initiation of appropriate treatment may be useful in limiting the production of IL-6 in breast milk. The elevated cytokine concentrations in milk can have potential adverse effects on the maternal mammary gland via induction of apoptosis in a variety of cell types (Bannerman, Paape, Hare, & Hope, 2004). Moreover, the IL-6 concentration in the subclinical mastitic animals was significantly higher (30.8 ng/mL) than both the clinically manifested (18.0 ng/mL) and healthy animals (5.2 ng/mL) (Osman *et al.*, 2010).

A study by Nakajima *et al.* measured the presence of TNF-α and IL-6 activities in milk and serum of cows with naturally occurring coliform mastitis (CFM). TNF-α had a higher concentration in the serum than in the milk. IL-6 was high in the sera of surviving CFM animals, but was low in animals that died and in healthy controls. The mean IL-6 level was 20-fold higher in the milk than in the sera of mastitic cows and a correlation was observed between the serum and milk levels of IL-6 (Nakajima *et al.*, 1997). On the other hand, in Slebodzinski's study, the TNF-α levels in infected milk

was found to be 2-fold higher than that in control, while the concentration of IL-6 was unchanged (Slebodziński, Malinowski, & Lipczak, 2002).

7.4. Interleukin-1 beta

During inflammatory response, IL-1β regulates the expression of adhesins by mammary endothelial cells and neutrophil chemotaxis in infections caused by *E. coli*, whereas, it's role in infections caused by *S. aureus* is important only in the early stages (Oviedo-Boyso *et al.*, 2007).

7.5. Interleukin-10

IL-10 is an anti-inflammatory cytokine. It suppresses the production of inflammatory cytokines mentioned above. It is also involved in the uptake of antigens and differentiation and function of T and B lymphocytes. Cows infected with Gram-negative bacteria had a higher IL-10 concentration in milk than cows infected with Gram-positive pathogens (Wenz *et al.*, 2010). Studies show that IL-10 was significantly elevated in milk following *E. coli* infection (Rinaldi *et al.*, 2010), whereas *S. aureus* was only able to slightly induce IL-10 (Sorg *et al.*, 2012). Furthermore, the concentration of IL-10 was higher in cows with moderate to severe inflammation than mild systemic inflammation (Schukken *et al.*, 2011).

7.6. Cytokines and nutrient transportation

Apart from their roles in host immune response, cytokines also regulate nutrient transportation in the mammary epithelium. Both *in vitro* and *in vivo* studies have identified the ability of inflammatory cytokines such as TNF-α and IL-6 to initiate inflammation-mediated changes in the expression levels of nutrient transporter, affecting the substrate availability to lactating mammary epithelial cells. Changes in nutrient transporter expression can alter nutrient absorption, distribution and elimination in the intra-mammary tissues. During lactation, enhanced ATP generation is necessary to satisfy the energy requirements for milk synthesis and secretion in the mammary gland. Glucose and, to a certain extent, fatty acids are the major energy substrates for ATP production. In the lactating mammary gland, transporters play a decisive role in making the substrates that support mammary epithelial cell energy metabolism, synthesis functions, and various milk components (i.e. lactose, fat, micronutrient) available to the lactating epithelial cells (Ling, 2011).

8. SIGNIFICANCE

SCM is an under-researched problem. This review has identified our severe lack of knowledge on the impact of SCM on breastfeeding mothers. The sparse emerging literature on SCM presents a more serious outlook than previously believed. It is a disease capable of high economic, social, and public health impact, which may be more apparent in developing countries with poor socioeconomic status and poor hygiene. SCM plays a role in the negative spiral of infection and malnutrition in the context of maternal and infant health. Elevated milk sodium in SCM mothers were found to be associated with poor weight gain in American and Bangladeshi infants (Aryeetey *et al.*, 2009; Filteau *et al.*, 1999). This disease deserves more research focus. Results from the present study will further scientific understanding of the pathophysiology of SCM in human as this is the first comprehensive study to evaluate multiple minerals and cytokines in breast milk in relation to SCM.

CHAPTER III. MATERIALS AND METHODS

1. ETHICS STATEMENT

This study is part of a larger cross-sectional study, which is a collaboration between McGill University and CeSSIAM (Center for Studies of Sensory Impairment, Aging and Metabolism) in Guatemala. Ethical approval was obtained from Institutional Review Boards of both institutions. All mothers provided written informed consent for participation in this study. The breast milk sample collection procedures were designed to be culturally-sensitive within an Indigenous population using non-invasive breast milk collection and anthropometric techniques.

2. STUDY SETTING AND POPULATION

The study population has been described in detail in Chomat *et al.*'s work (Chomat *et al.*, 2014). Briefly, the population of interest for this cross-sectional study is pregnant women from 8 rural *Mam*-speaking communities (Buena Vista, Los Alonzo, La Esperanza, Los Perez, Espumpuja, Los Romero, La Union, Los Lopze) in the San Juan Ostuncalco region located approximately 30 km outside of the city of Quetzaltenango in Guatemala. Community selection was based on size (2000 to 4000 population), intra-community similarity in SES, interest in participation in the study and feasibility.

3. STUDY RECRUITMENT AND ELIGIBILITY

All eligible lactating women in the 8 selected communities were informed of the study and invited to participate. Study visits occurred at a local health centre or at the participants' house. Milk samples were collected from May to August 2012.

Inclusion criteria: Indigenous lactating women with infants 1) <46 days, and 2) from 3 to 6 months.

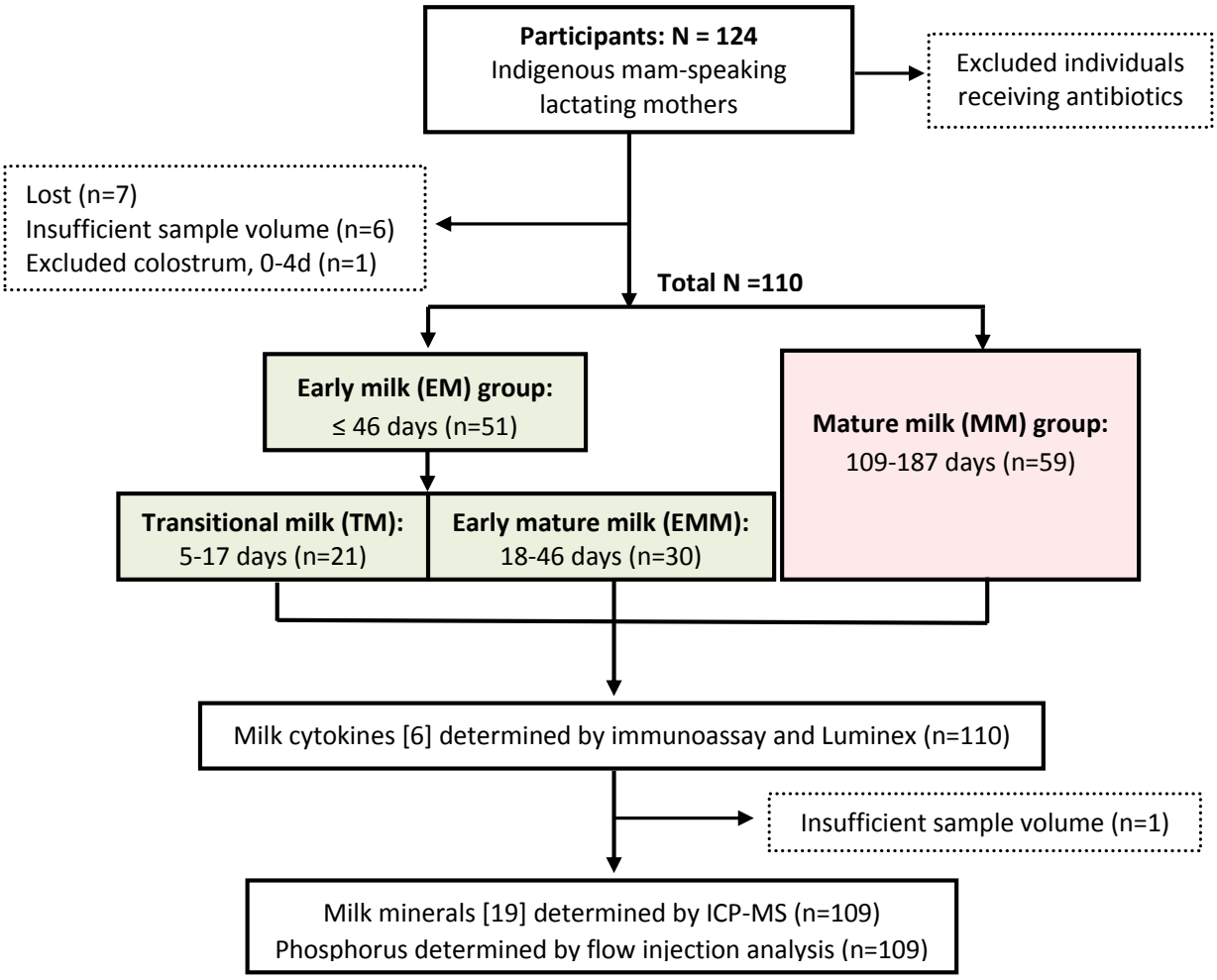
Exclusion criteria: 1) Mothers with colostrum (milk < 4 days postpartum) and; 2) mothers known to be treated with antibiotics by a licensed physician during postpartum period. The use of antibiotics has been demonstrated to decrease rate of SCM (WHO, 2000).

4. DATA COLLECTION

A single unilateral human milk sample was collected via manual expression by a trained midwife on site. Before expressing milk, the nipple and areola of breast was cleaned with cotton soaked in 70% ethyl alcohol to reduce microbial contamination (Groer & Beckstead, 2011). As milk composition can vary between foremilk and hindmilk, women were asked to provide full milk expression from the breast not recently used for feeding the infant. Milk, collected in 60 ml plastic vials, was immediately stored in a cold chest and subsequently aliquotted under sterile conditions in the field laboratory before being stored at -30°C prior to transfer on dry ice to McGill University for storage at -80°C until analysis.

5. STUDY DESIGN: CROSS-SECTIONAL STUDY

FIG. 3.1. Study design, identifying study population, study exclusions and sample sizes at various stage of analysis.



The breast milk samples (n = 110) were initially categorized into two lactation stages: early milk, EM (≤ 46 days) and mature milk, MM (109-187d) based on days postpartum. However, from previous studies of breast milk, we observed that samples are usually categorized as colostrum, transitional milk and mature milk. Colostrum has been defined as 0-4d, 1-3d and 1-5d postpartum. Early transitional milk was defined as 4-7d postpartum and transitional milk has been defined as 5-9d, 4-17d, 6-15d and 10-14d postpartum (Aydin, Aydin, Ozkan, & Kumru, 2006; Boersma, Offringa, Muskiet, Chase, & Simmons, 1991; Krachler *et al.*, 1998; Rossipal & Krachler, 1998). The definition for mature milk was even more diverse, it ranged from 10-30d to 30-45d to anything beyond 15d and 40-293d postpartum (Aydin *et al.*, 2006; Boersma *et al.*, 1991; Feeley *et al.*, 1983b; Krachler *et al.*, 1998; Rossipal & Krachler, 1998). In addition, these studies have showed compositional difference in milk between the different lactation stages.

In this study, we recognize the importance of accurate categorization of lactation stages, hence we have taken all the previous definitions into consideration and defined our lactation stages as the following: colostrum, 1-4d postpartum; transitional milk (TM), 5-17d postpartum; early mature milk (EMM), 18-46d postpartum and mature milk (MM), 109-187d postpartum. Using this definition, our initial EM group was further subdivided into TM and EMM, leaving us with 3 lactation stages. The sample size for colostrum (n=1) was deemed too small and was excluded from subsequent analysis.

6. INDICATOR FOR SCM

SCM, the condition of interest, was defined using the sodium/potassium (Na/K) ratio. A Na/K ratio ≤ 0.6 is considered to be non-SCM, whereas a Na/K ratio > 0.6 indicated SCM. This cut-off was established using the Na/K ratio distribution according to previously published categories in human breast milk: Na/K ratio ≤ 0.6 is considered to be normal; > 0.6 to 1.0 is considered to be moderately raised with subclinical inflammation; and > 1.0 is considered to be highly raised and is indicative of severe subclinical mastitis (Kantarci *et al.*, 2007; Willumsen *et al.*, 2002).

7. MILK CYTOKINE ANALYSIS

Immunoassays with Luminex®

7.1. Instrumentation and Multiplex kit

The EMD Millipore's MILLIPLEX® MAP Human High Sensitivity Cytokine panel was used to measure 6 cytokines: IL-1ß, IL-6, IL-8, IL-10, IL-13 and tumor necrosis factor alpha (TNF-α) in breast milk samples according to manufacturer's specifications (EMD Millipore, 2012). Samples were analysed in duplicates.

The immunoassay was conducted with Luminex® 200™ using xMAP® platform (Luminex Corp., Austin, TX) in a magnetic bead format featuring high speed and sensitivity. Data was processed using the MasterPlex® CT software version 1.2 (MiraiBio, Inc., Alameda, CA). Minimum detectable concentrations for the various cytokines were based on calculated values by StatLIA® Immunoassay Analysis Software from Brendan Technologies. It ranged from 0.3 and 10.3 pg/mL, depending on the analyte (Luminex Corporation, 2010).

7.2. Principle

The Luminex® xMAP® technology is based on polystyrene or paramagnetic microspheres, or beads, which are physically and thermally stable polystyrene particles that are internally dyed with red and infrared fluorophores of differing intensities. Each dyed bead is given a unique number, or bead region, allowing the differentiation of one bead from another. For multiplex immunoassay kits, individual bead sets are then coated with a capture antibody qualified for one specific analyte of interest such as different cytokines. Multiple analyte-specific beads can then be combined in a single well of a 96-well microplate-format assay to detect and quantify multiple targets simultaneously from one sample, using one of the Luminex® instruments for analysis.

The Luminex analyzer contains a fluidics system that controls sample uptake and sorts the particles. The sample is injected into a central channel with an outer sheath that contains faster moving fluid relative to the center. The faster flowing sheath fluid alters the velocity of the sample flow in the gradually narrowing central channel and forces the particles to proceed in a single file. This is called hydrodynamic focusing.

The Luminex analyzer also uses an excitation system, which contains two lasers, a classification laser that excites the fluorochromes contained within the microspheres themselves and a reporter laser that excites the reporter molecule that is bound to biological reactants at the surface. The fluorescent emissions from the classification and reporter lasers represent a distinct assay within a multiplex of

assays and quantitate each distinct assay, respectively. The fluorescent signals can be specified using optical filters, which block certain wavelengths and transmit others. The Luminex analyzer employs photodiodes and a photo multiplier tube to receive filtered fluorescent signals, which are then converted to intensity units via the digital signal processor.

7.3. Milk sample preparation

Breast milk samples (n=110, excluding colostrum) were thawed on ice. 200 µl of each sample were aliquotted and centrifuged at 14000 rpm for 15 minutes at 4°C (Groer & Beckstead, 2011). The top fat layer was removed and 50 µL of supernatant in duplicates were carefully loaded onto the 96-well plate.

After addition of samples, 25 µL of antibody-immobilized beads were added to each well on the 96-well plate. The plate and beads are then washed twice with 200 µL wash buffer according to instructions. After washing, 50 µL of high sensitivity human cytokine standards and controls were transferred to appropriate wells according to pre-designed well map followed by addition of 50 µL of Assay Buffer to the 0 pg/mL standard (Background) and sample wells and 50 µL of Serum Matrix to the background, standards, and control wells. Finally, 50 µL of sample was transferred into appropriate sample wells. Samples were sealed, covered with aluminum foil, and incubated with agitation on a plate shaker (Sanofi Diagnostics Pasteur, Chaska, MN, Model # 2202) overnight at 4°C (EMD Millipore, 2012).

The well contents were gently removed using a Hand Held Magnetic Separation Block (EMD MILLIPORE catalog # 40-285) and the plate was washed according to instructions. After washing, 50 µL of Detection Antibodies was added to each well and the sealed, foil-covered plate was incubated with agitation on a plate shaker for 1 hour at room temperature. Thereafter, 50 µL of Streptavidin-Phycoerythrin was added to each well and the sealed, foil-covered plate was incubated with agitation for 30 minutes at room temperature. After each incubation, the well contents were gently removed and plate was washed. Following the final wash, 150 µL of Sheath Fluid was added to all wells and the beads were re-suspended on a plate shaker for 5 minutes (EMD Millipore, 2012).

7.4. Sample analysis

Samples were analysed on the Luminex using MasterPlex® CT software. A minimum of 50 events (beads) was collected for each cytokine and median fluorescence intensities (MFI) were obtained. Cytokine concentrations were automatically calculated based on standard curve data using

MasterPlex® QT analysis software version 3.0 (MiraiBio, Inc., Alameda, CA), and were expressed as pg/ml±S.E.M.

In 96.3% of samples, the concentrations of IL-10 and IL-13 were below the LOD and these two cytokines were not included in further statistical analysis.

TABLE 3.1. Limit of Detection (LOD) and % of samples below LOD for each cytokine analysed.

Cytokines	IL-1ß	IL-6	IL-8	IL-10	IL-13	TNF-α
LOD (pg/mL)	0.06	0.20	0.05	0.48	0.18	0.07
% < LOD	27.3%	0	1.8%	96.3%	96.3%	0

8. MILK MINERALS ANALYSIS

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

8.1. Instrumentation

All minerals (with the exception of P) have been quantified on a Bruker (formerly Varian) ICP-820MS (Billerica, MA, USA) equipped with a Collision Reaction Interface to allow fast, flexible, and interference-free analysis. The 820-MS system features patented 90 degree reflecting ion optics system for gigahertz sensitivity (1000 Mc/s/mg/L).

8.2. Principle

An ICP-MS combines a high-temperature ICP source with a mass spectrometer. The sample is introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer, where it is converted into a fine aerosol with argon gas at about 1 L/min. The fine aerosol then emerges from the exit tube of the spray chamber and is transported into the plasma torch via a sample injector.

The plasma is produced by the interaction of an intense magnetic field (generated by radio frequency [rf] passing through a copper coil) on a tangential flow of argon gas through a concentric quartz tube (torch). This ionizes the gas and, when seeded with a source of electrons from a high-voltage spark, forms a very high temperature plasma discharge (~10,000 K) at the open end of the tube. Positively charged ions are generated by the plasma torch. The produced ions are then directed into the mass spectrometer via the interface region, which is maintained at a vacuum of 1 - 2 torr with

a mechanical roughing pump. This interface region consists of two metallic cones, called the sampler and a skimmer cone. Each cone features a small (0.6 - 1.2 mm) orifice to allow the ions beam containing all the analyte and matrix ions through the ion optics, where they are guided into the mass spectrometer.

The mass spectrometer allow analyte ions of a particular mass-to-charge ratio (m/z) through to the detector and filter out all the non-analyte, interfering, and matrix ions. Depending on the design of the mass spectrometer, this is either a scanning process where the ions arrive at the detector sequentially, or a simultaneous process where the ions are sampled at the same time. In the final process, an ion detector converts the ions into an electrical signal. This electronic signal is then processed by the data handling system and converted into analyte concentration using ICP-MS calibration standards.

8.3. Standards

Five standard solutions were prepared from 10 mg/L multi-element PlasmaCAL Calibration Standards (SCP Science, Ref #141-110-015) by dilution with 4% HNO_3 v/v in ultrapure deionised water (the same background matrix as the samples). The Limits of Detection (LOD) were established from three times the standard deviation (SD) of the mean for each mineral, measured on 8 replicates of the lowest calibration standard.

8.4. Quality assurance

Analytical quality controls comprised of reference materials and reagent blanks. No suitable reference material for human milk was available. Instead, a commercial brand of milk powder was included in each analytical run as the internal quality control along with a Standard Reference Material (SRM) peach leaves 1547 (National Institute of Standards and Technology, Gaithersburg, MD) as the external quality controls for all minerals analysed. Six reagent blanks were also included in each ICP-MS run to monitor potential contamination from the Teflon tubes or the reagents. The reagent blanks held <0.1% of the average concentrations of the analysed samples for all elements.

8.5. Sample preparation and digestion

Breast milk samples (n=109, excluding colostrum) were thawed on ice and thoroughly homogenized by vortexing. After thorough mixing, 0.5 ml of each sample was digested in 2 mL concentrated nitric acid (69.0-70.0%, M.W. 63.01, J.T.Baker 9598-34) for 5 hours at 125°C in

triplicates. Quality controls were also prepared in triplicates; 0.16 g of commercial milk powder and 0.12 g of SRM peach leaves 1547 (NIST, Gaithersburg, MD) were digested in 2 mL concentrated nitric acid. 6 reference materials and 6 reagent blanks were included in each analytical run. Digested samples (completely clear, colorless, and homogenous solutions), reference materials and reagents blanks were transferred to acid-washed polyethylene tubes and diluted with deionized water (from Milli-RO / Milli-Q system (Millipore) to a nitric acid concentration of 20%.

The obtained solutions were measured for 19 elements: Arsenic (75 As), barium (135 Ba), cadmium (110 Cd), calcium (43 Ca), chromium (53 Cr), cobalt (59 Co), copper (65 Cu), iron (56 Fe), lead (208 Pb), magnesium (26 Mg), manganese (55 Mn), nickel (60 Ni), potassium (39 K), rubidium (85 Rb), selenium (78 Se), sodium (23 Na), strontium (86 Sr), thallium (205 Tl) and zinc (68 Zn) in breast milk samples collected from the indigenous Mam-Mayan women using ICP-MS.

Concentrations of the toxic trace minerals measured: As, Ba, Cd, Co, Ni Pb and Tl were 100% below the LOD and hence were not included in further statistical analysis. In the cases of Cr and Mn, where 0.9% and 11.9% of the samples, respectively, were below LOD. The <LOD values were replaced by half of the LOD values of the instrument for statistical analysis (Croghan & Egeghy, 2003).

TABLE 3.2. Limit of Detection (LOD) and % of samples below LOD for each mineral analysed.

Macrominerals	LOD (µg/L)	% <LOD	**Trace minerals**	LOD (µg/L)	% <LOD	**Toxic trace minerals**	LOD (µg/L)	% <LOD
Ca	1.505	0	**Cr**	0.031	0.9%	**As**	0.007	100%
K	4.887	0	**Cu**	0.396	0	**Ba**	0.031	100%
Mg	0.232	0	**Fe**	1.34	0	**Cd**	0.020	100%
Na	1.816	0	**Mn**	0.005	11.9%	**Co**	0.002	100%
P	0.008	0	**Rb**	0.032	0	**Ni**	0.032	100%
			Se	0.007	0	**Pb**	0.038	100%
			Sr	0.026	0	**Tl**	0.002	100%
			Zn	0.116	0			

Flow Injection Analysis with Lachat

Total phosphorus was measured colorimetrically on a flow injection analyzer Lachat (Milwaukee, WI, USA). Standard solutions ranging from 0.05 to 2.00 mg P/L were prepared in the same background matrix as the samples. Phosphorus were measured following the standard protocol QuikChem Method 10-115-01-1-A on Lachat at 880nm. The orthophosphate ions in the digested samples (see above for preparation) reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex. This complex is then reduced with ascorbic acid to form a blue complex, which absorbs light at 880nm. The absorbance is proportional to the concentration of orthophosphate in the sample.

9. STATISTICAL ANALYSIS

Data analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) and SPSS version 20.0 (SPSS, Inc, Chicago, IL, USA, 2007). *P* values < 0.05 were considered statistically significant. Prior to analyses, all data were checked for i) normality of residuals using Shapiro-Wilk Test; ii) homogeneity of variances using Levene's Test and transformed when necessary. Concentrations of Mg, Na, Fe, Rb, Se Sr, Zn, IL-1ß, IL-6, IL-8 and TNF-α were natural log transformed [Ln(y)] to achieve normality and Cr and Mn were square-root transformed [Sq(y)]. Unless otherwise indicated, non-transformed means and standard error of mean (SEM) are reported in tables.

Using Proc GLM model in SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA), one-way analysis of variance (ANOVA) test was performed to compare milk mineral and cytokine concentrations between lactation stages (EM vs. MM and TM vs. EMM vs. MM). Two-way ANOVA (2x3 design) was then used to study the main effects of SCM status (SCM vs. non-SCM) and lactation stages (TM vs. EMM vs. MM), as well as their interaction on the concentrations of milk minerals and cytokines. When appropriate, post-hoc analyses were carried out using Bonferroni correction for multiple comparisons between groups. The strict Bonferroni correction was also applied to control type-1 error at $P = 0.05$ during multiple testing. In one-way ANOVA, where 18 minerals and cytokines were tested, the corrected probability = 0.05/18 = 0.0028. In two-way ANOVA, where 17 minerals and cytokines were tested, the corrected probability = 0.05/17 = 0.0029.

Spearman's rank correlations were performed with SPSS (SPSS, Inc, Chicago, IL, USA, 2007) between all variables of interest to determine associations. The correlation matrices of cytokines were examined in particular to determine which cytokine could be included together within the same

regression models. Although some of the correlations among cytokines are significant, the correlation coefficients only range from 0.1 to 0.4, indicating very weak correlations. A series of multiple regression models were then utilized to assess the association between milk minerals (dependent variables) and cytokines and SCM (explanatory variables). Each model was controlled for lactation stage. Therefore, a total of 6 potential predictors were entered into each model and using Bonferroni correction, the adjusted probability to test for significance = 0.05/6 = 0.0083.

CHAPTER IV. RESULTS

PART A

1. IMPACT OF LACTATION STAGES

1.1. Macrominerals

To evaluate the impact of lactation stages on macrominerals, we first examined the frequency distributions of milk macromineral between EM and MM groups (Fig. 4.1). For sodium (Fig. 4.1A), the distribution of EM group overlapped with the MM group between 4 and 8 mmol/L, but only the EM group had sodium concentrations > 8 mmol/L. Potassium (Fig 4.1B), magnesium (Fig 4.1D) and phosphorus (Fig 4.1E) showed two distinct concentration ranges, one for EM and another for MM with a minimal overlap. In contrast, the distributions of calcium (Fig. 4.1C) were relatively similar between EMM and MM groups.

The effect of lactation stages on milk macrominerals was supported by one-way ANOVA (Table 4.1 and 4.2). A comparison between EM and MM demonstrated that, except for calcium, the mean concentrations of all macrominerals differed between lactation stages. Sodium ($P < 0.0001$), potassium ($P < 0.0001$) and phosphorus ($P < 0.0001$) had higher mean concentrations in EM than in MM, whereas magnesium ($P < 0.0001$) was lower (Table 4.1). Similar results were observed when 3 lactation stages (TM, EMM and MM) were compared (Table 4.2), with the additional finding that potassium was higher in TM than EMM.

1.2. Trace minerals

The inter-individual variations in concentrations of trace minerals, especially iron and zinc (Fig 4.2C and 4.2H), were large compared to the macrominerals. As with the macrominerals, different patterns in frequency distributions were again observed. The distribution of copper clearly depicted two distinct concentration ranges between EM and MM groups with separate peaks for each group (Fig. 4.2B). In contrast, the distribution of other trace minerals did not demonstrate any noticeable difference between the two lactation groups.

The ANOVA results (Table 4.1 and 4.2) showed that selenium and strontium were the only two trace minerals that were not affected by lactation stages. Consistent with the change of macrominerals over lactation stages, the concentrations of chromium ($P < 0.0001$), copper ($P < 0.019$), iron ($P < 0.0001$), manganese ($P < 0.006$), rubidium ($P < 0.0001$) and zinc ($P < 0.0001$) were higher in EM than in MM (Table 4.1). ANOVA between 3 lactation groups (Table 4.2) further revealed

that chromium concentration was higher in TM than in EMM and lowest in MM (0.45±0.05, 0.34±0.04 and 0.31±0.02 μmol/L respectively, $P < 0.0001$).

Concentrations of manganese in milk were lower than the rest of trace minerals, with 11.9% of the measurements below the detection limit of ICP-MS. None of the toxic trace minerals, arsenic, barium, cadmium, cobalt, lead, nickel and thallium were detected in the milk samples.

1.3. Inflammatory cytokines

The histograms of milk cytokines showed that, except for IL-8 (Fig. 4.3C), the other cytokines all had similar frequency profiles for EM and MM groups (Fig. 4.3A, 4.3B and 4.3D). In the case of IL-8, the EM group depicted a strong positively skewed distribution where 68.6% of the samples had concentrations at 10 pg/mL or less, while in the MM group, the measured concentrations ranged from 5 to over 100 pg/mL (Fig. 4.3C).

Using one-way ANOVA (Table 4.1), the differences by lactation stages were observed for IL-1β, IL-6 and IL-8. IL-8 had a higher concentration in MM ($P < 0.001$), whereas IL-1β ($P = 0.015$) and IL-6 ($P < 0.0001$) were higher in EM. Similar results were found when 3 lactation stages were compared (Table 4.2). Among the cytokines measured, IL-8 had the highest inter-individual variations and also displayed the most drastic change over the course of lactation. Its mean concentration in breast milk quadrupled from 7.80±2.33 pg/mL in EMM to 38.27±7.41 pg/mL in MM.

In the majority of cases (96.3%), IL-10 and IL-13 were not detected. IL-1β and IL-8 were also undetectable in 27.3% and 1.8% of the samples respectively, whereas IL-6 and TNF-α were detected in all samples.

1.4. Bonferroni correction

In one-way ANOVA (Table 4.1 and 4.2), the stages of lactation effects on sodium, potassium, magnesium, phosphorus, chromium, iron, rubidium, zinc, IL-6 and IL-8 remained significant after Bonferroni correction ($P = 0.0028$).

2. CONNECTING STATEMENT

The results from the one-way ANOVAs (Table 4.1 and 4.2) demonstrated that multiple minerals and cytokines in milk differed by stages of lactation, making it a crucial variable to consider while examining milk composition under SCM. Therefore, the main effect of lactation stages, along with SCM status, was included in the subsequent two-way ANOVA.

Furthermore, categorization of milk samples into 3 (TM, EMM and MM) rather than 2 stages (EM and MM) have enabled us to capture the difference in potassium and chromium concentrations between TM and EMM. Therefore, the categorization of TM, EMM and MM was used in the following analyses (Part B), which focuses on how stages of lactation as well as presence or absence of SCM alters the minerals and cytokines in human breast milk samples.

FIG 4.1. Frequency distributions of macromineral concentrations: Sodium (A), Potassium (B), Calcium (C), Magnesium (D) and Phosphorus (E) in EM (5-46d) and MM (109-187d).

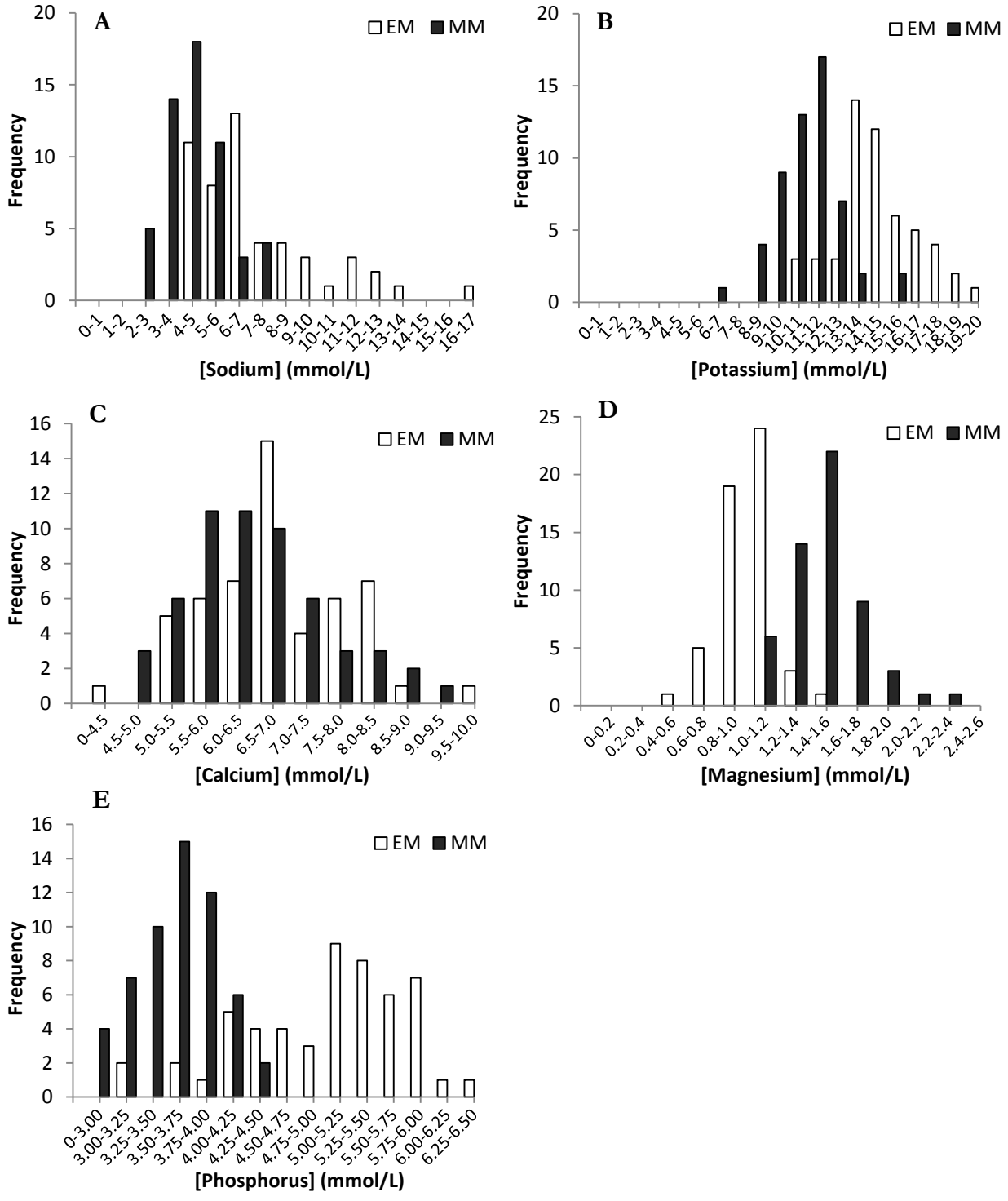
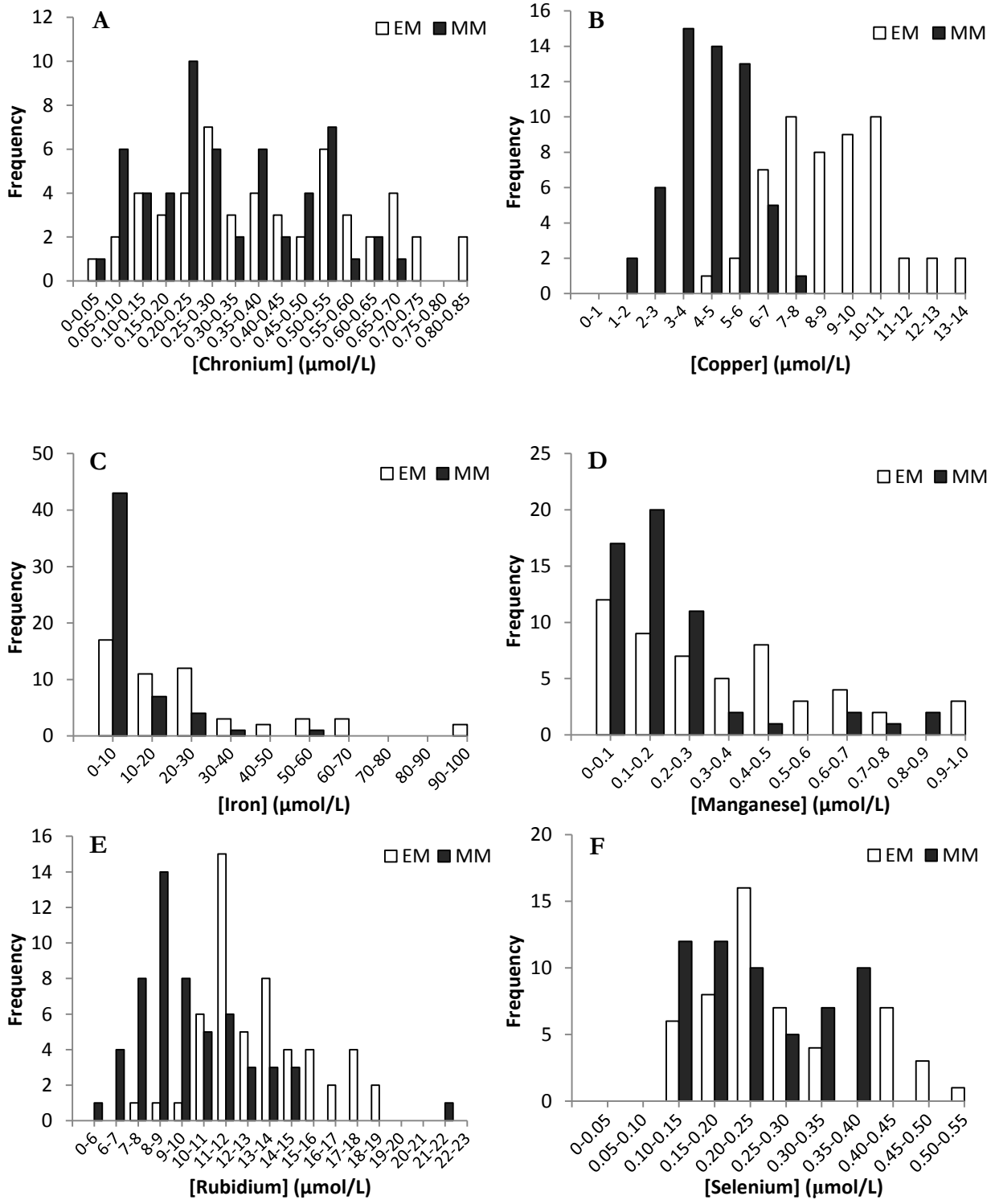


FIG 4.2. Frequency distributions of trace mineral concetnrations: Chromium (A), Copper (B), Iron (C), Manganese (D), Rubidium (E), Selenium (F), Strontium (G) and Zinc (H) in EM (5-46d) and MM (109-187d).



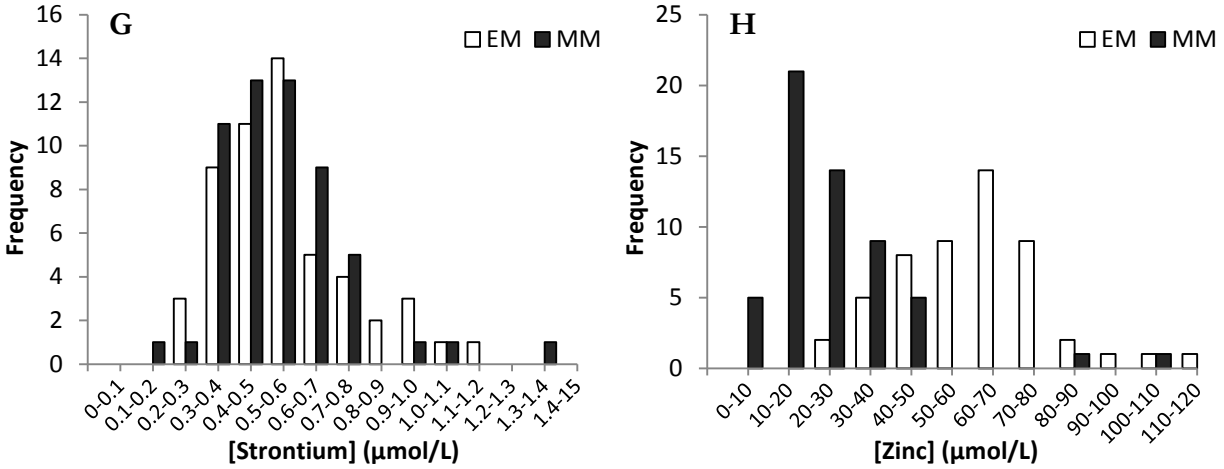

G
EM
MM
Frequency
16
14
12
10
8
6
4
2
0
0-0.1
0.1-0.2
0.2-0.3
0.3-0.4
0.4-0.5
0.5-0.6
0.6-0.7
0.7-0.8
0.8-0.9
0.9-1.0
1.0-1.1
1.1-1.2
1.2-1.3
1.3-1.4
1.4-15
[Strontium] (µmol/L)
H
EM
MM
Frequency
25
20
15
10
5
0
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100-110
110-120
[Zinc] (µmol/L)

FIG 4.3. Frequency distributions of inflammatory cytokine concentrations: IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D) in EM (5-46d) and MM (109-187d).

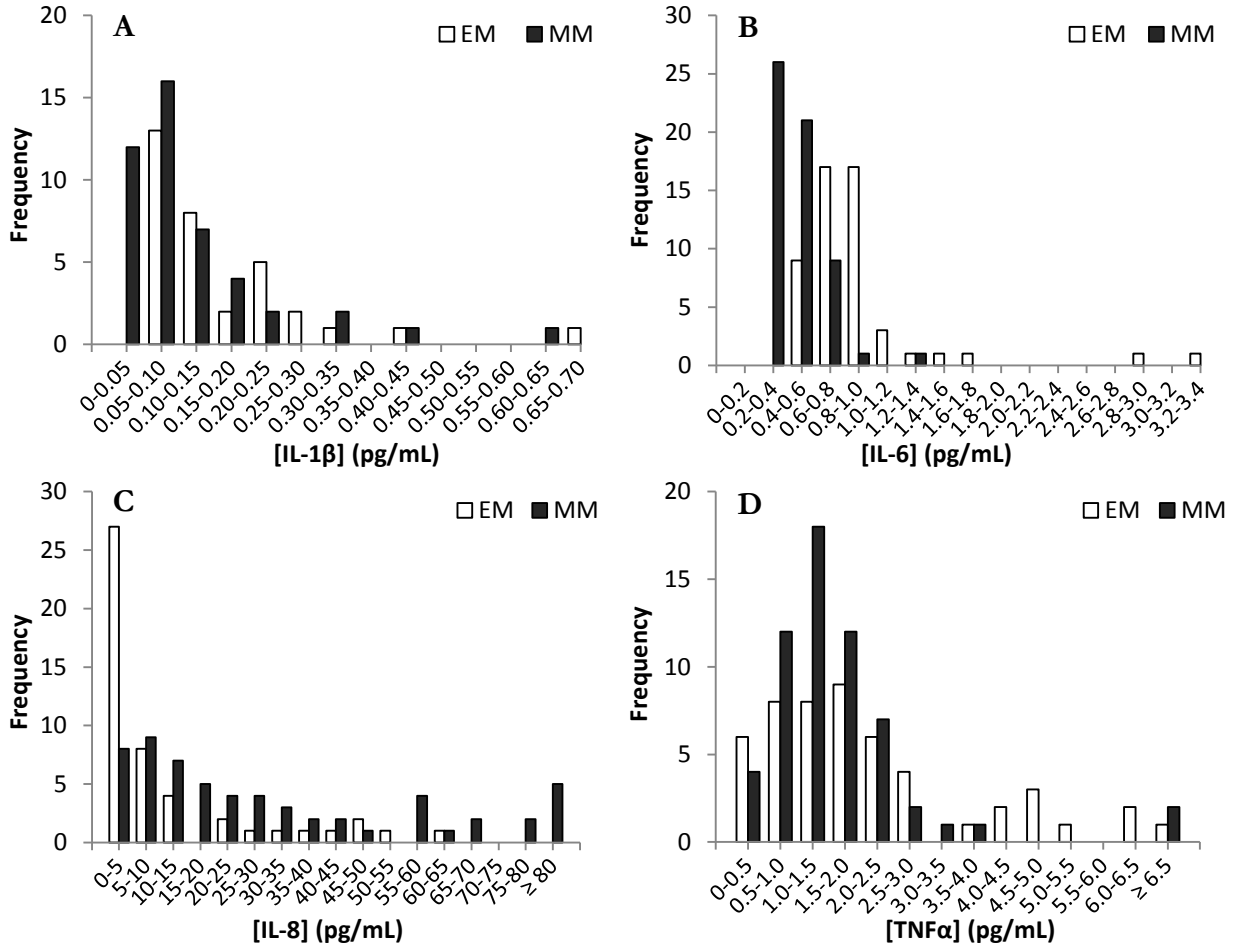


TABLE 4.1. Effect of lactation stages (EM and MM) on concentration of minerals and inflammatory cytokines in breast milk of Guatemalan women.[1]

Milk Biomarkers [2, 3]	Lactation Stages		*P* values
	Early Milk, 5-46 d	Mature Milk, 109-187 d	
Macrominerals (mmol/L)	**(n = 53)**	**(n = 56)**	
Na/K ratio	0.49 ± 0.02	0.42 ± 0.02	**0.015**
Sodium	7.24 ± 0.39	4.61 ± 0.16	**<0.0001***
Potassium	14.56 ± 0.27	11.01 ± 0.22	**<0.0001***
Calcium	6.81 ± 0.15	6.45 ± 0.14	0.088
Magnesium	1.00 ± 0.02	1.47 ± 0.03	**<0.0001***
Phosphorus	5.00 ± 0.10	3.59 ± 0.05	**<0.0001***
Trace Minerals (µmol/L)			
Chromium	0.40 ± 0.03	0.31 ± 0.02	**<0.0001***
Copper	8.79 ± 0.26	4.39 ± 0.18	**0.019**
Iron	25.64 ± 2.99	9.85 ± 1.20	**<0.0001***
Manganese [4]	0.33 ± 0.04	0.19 ± 0.03	**0.006**
Rubidium	13.10 ± 0.34	9.87 ± 0.36	**<0.0001***
Selenium	0.26 ± 0.02	0.24 ± 0.01	0.115
Strontium	0.55 ± 0.03	0.53 ± 0.03	0.664
Zinc	59.57 ± 2.81	25.19 ± 2.42	**<0.0001***
Cytokines (pg/mL)	**(n = 51)**	**(n = 59)**	
IL-1β	0.16 ± 0.02	0.12 ± 0.02	**0.015**
IL-6	0.91 ± 0.07	0.48 ± 0.02	**<0.0001***
IL-8	10.82 ± 2.30	38.27 ± 7.41	**<0.0001***
TNF-α	2.15 ± 0.25	1.75 ± 0.24	0.449

[1] Values are Mean ± SEM.
[2] Mg, Na, Fe, Rb, Se, Sr, Zn, IL-1β, IL-6, IL-8 and TNF-α were log transformed [Ln(y)] to achieve normality. Cr and Mn was square-root transformed [Sq(y)] to achieve normality.
[3] Based on the outlier labelling rule, 1 outlier was identified and excluded from the Na, Se, Zn and IL-6 data set and 2 outliers from the IL-1β data set.
[4] IL-1β had a smaller sample size. Early milk n = 33 and mature milk n = 46.
* Indicates statistical significance after Bonferroni correction ($P = 0.0028$).

TABLE 4.2. Effect of lactation stages (TM, EMM and MM) on concentration of minerals and inflammatory cytokines in breast milk of Guatemalan women.[1]

Milk Biomarkers [2, 3]	Lactation Stages			*P* values
	Transitional Milk, 5-17d	Early Mature Milk, 18-46d	Mature Milk, 109-187d	
Macrominerals (mmol/L)	**(n = 21)**	**(n = 32)**	**(n = 56)**	
Na/K ratio	0.52 ± 0.05	0.48 ± 0.03	0.42 ± 0.02	**0.036**
Sodium	8.11 ± 0.71 [b]	6.72 ± 0.43 [b]	4.61 ± 0.16 [a]	**<0.0001***
Potassium	15.37 ± 0.38 [c]	14.03 ± 0.35 [b]	11.01 ± 0.22 [a]	**<0.0001***
Calcium	6.78 ± 0.25	6.82 ± 0.20	6.45 ± 0.14	0.232
Magnesium	1.01 ± 0.03 [a]	0.99 ± 0.03 [a]	1.47 ± 0.03 [b]	**<0.0001***
Phosphorus	5.21 ± 0.17 [b]	4.86 ± 0.13 [b]	3.59 ± 0.05 [a]	**<0.0001***
Trace Minerals (µmol/L)				
Chromium	0.45 ± 0.05 [c]	0.37 ± 0.04 [b]	0.31 ± 0.02 [a]	**<0.0001***
Copper	9.67 ± 0.32 [b]	8.21 ± 0.35 [ab]	4.39 ± 0.18 [a]	**0.021**
Iron	27.96 ± 4.29 [b]	24.12 ± 4.10 [b]	9.85 ± 1.20 [a]	**<0.0001***
Manganese	0.35 ± 0.06 [b]	0.31 ± 0.05 [ab]	0.19 ± 0.03 [a]	**0.016**
Rubidium	13.49 ± 0.55 [b]	12.85 ± 0.44 [b]	9.87 ± 0.36 [a]	**<0.0001***
Selenium	0.27 ± 0.02	0.26 ± 0.02	0.24 ± 0.01	0.291
Strontium	0.59 ± 0.06	0.52 ± 0.03	0.53 ± 0.03	0.657
Zinc	66.57 ± 4.60 [b]	54.98 ± 3.35 [b]	25.19 ± 2.42 [a]	**<0.0001***
Cytokines (pg/mL)	**(n = 21)**	**(n = 30)**	**(n = 59)**	
IL-1β [4]	0.11 ± 0.02 [ab]	0.18 ± 0.03 [b]	0.12 ± 0.02 [a]	**0.027**
IL-6	0.98 ± 0.11 [b]	0.85 ± 0.09 [b]	0.48 ± 0.02 [a]	**<0.0001***
IL-8	15.21 ± 4.42 [a]	7.80 ± 2.33 [a]	38.27 ± 7.41 [b]	**<0.0001***
TNF-α	2.63 ± 0.49	1.81 ± 0.25	1.75 ± 0.24	0.224

[1] Values are Mean ± SEM. Means with differing superscripts within rows are significantly different at the $p < 0.05$ based on Bonferroni post hoc paired comparison.

[2] Mg, Na, Fe, Rb, Se, Sr, Zn, IL-1β, IL-6, IL-8 and TNF-α were natural log transformed [Ln(y)] to achieve normality. Cr and Mn was square-root transformed [Sq(y)] to achieve normality.

[3] Based on the outlier labelling rule, 1 outlier was identified and excluded from the Na, Se, Zn and IL-6 data set and 2 outliers from the IL-1β data set.

[4] IL-1β has a smaller sample size. TM n = 11, EMM n = 22 and MM n = 46.

* Indicates statistical significance after Bonferroni correction ($P = 0.0028$).

PART B

1. NA/K AND SCM PREVALENCE

In this study, SCM was defined as having a Na/K ratio > 0.6. Using this definition, a total of 15 participating Guatemalan mothers had SCM (14%). The prevalence was highest in TM group (26.3%), and followed by EMM group (15.6%). The MM group, with only 8.9%, had the lowest prevalence of SCM. Fig 4.4 shows the frequency distributions of the TM, EMM and MM.

As expected, milk sodium concentration was influenced by stages of lactation ($P < 0.0001$) as well as SCM status ($P < 0.0001$). Sodium was higher in TM and EMM than in MM, and all SCM mothers had higher sodium than the non-SCM mothers. In other words, sodium concentrations were higher in SCM TM and SCM EMM compared with other experimental groups (Table 4.3). In the case of potassium, there was a significant interaction between lactation stages and SCM status ($P = 0.026$). Potassium in non-SCM TM was higher than non-SCM EMM, while the SCM TM and EMM had similar potassium concentrations (Table 4.3).

2. IMPACT OF LACTATION STAGES, SCM STATUS AND THEIR INTERACTION

The results of the two-way ANOVA between stages of lactation (TM, EM and MM) and SCM status are presented in Table 4.3.

2.1. Macrominerals

Magnesium, with higher concentrations in MM than in TM and EMM, was only affected by stages of lactation ($P < 0.0001$) and not by SCM status. In contrast, phosphorus was influenced by both stages of lactation ($P < 0.0001$) and SCM status ($P = 0.003$). Phosphorus had a higher concentration in TM and EMM than in MM. Although post-hoc multiple comparisons did not indicate a difference in phosphorus concentrations between non-SCM and SCM groups, a trend toward lower phosphorus in SCM milk was observed. No differences were noted in calcium concentrations.

2.2. Trace minerals

Both rubidium ($P < 0.0001$) and zinc ($P < 0.0001$) were only affected by stages of lactation and had higher concentrations in TM and EMM than in MM. SCM status had no effect on these trace minerals. Among the trace minerals, SCM status was only associated with selenium ($P = 0.01$).

Although post-hoc pairwise comparisons did not indicate any difference between experimental groups, a trend towards higher selenium concentrations in SCM milk was observed.

Significant interactions were identified for copper ($P = 0.004$), iron ($P = 0.007$) and strontium ($P = 0.023$). The copper concentrations in non-SCM mother were higher in TM than in EMM, which in turn was higher than in MM. Both SCM and non-SCM mothers had the lowest copper concentration in MM. SCM status had no effect on copper in TM and MM, but in EMM, SCM mothers had higher copper than non-SCM mothers. Similar to copper, iron was also lower in MM than in TM and EMM. A trend of higher iron concentrations in EMM and MM from SCM mothers than non-SCM mothers was seen. However, since iron has the highest inter-individual variability amongst the trace minerals, the difference between SCM and non-SCM milk was not significant. Despite showing a significant interaction, post-hoc multiple comparison were unable to detect any difference in strontium concentrations between all experimental groups. Moreover, no differences were noted in chromium and manganese concentrations throughout lactation and with SCM.

2.3. Inflammatory cytokines

IL-8 was influenced by both lactation stages ($P < 0.0001$) and SCM status ($P = 0.001$). In TM group, SCM mothers had a higher IL-8 concentration than non-SCM mothers. The IL-8 concentration in SCM mothers was similar throughout lactation, whereas IL-8 in non-SCM mothers was higher in MM than in earlier milk (Fig 4.5C). Conversely, IL-1β was only affected by stages of lactation and post hoc multiple comparisons failed to detect any difference in its concentrations between the groups.

Interactions between lactation stages and SCM status were identified for IL-6 ($P = 0.049$) and for TNF-α ($P = 0.02$). Fig 4.5B and 4.5D depict the impact of SCM on these cytokines during lactation. IL-6 concentrations were higher in TM and EMM than in MM. In addition, TM of SCM mothers had a higher IL-6 concentration than TM of non-SCM mothers. In the case of TNF-α, its concentrations in non-SCM mothers did not fluctuate much during lactation, whereas its concentrations in SCM mothers differed significantly between TM and EMM. Similar to IL-8 and IL-6, higher TNF-α concentration in TM of SCM mothers was also observed.

It is interesting to note that the change in IL-8 concentrations of non-SCM mothers during lactation was in the opposite direction to changes observed in IL-6. IL-8 was higher in MM than in TM and EMM, whereas IL-6 was higher in the earlier milk (Fig 4.5B and 4.5C).

2.4. Bonferroni correction

The effects of lactation stages on IL-1β and the effects of SCM status on phosphorus, selenium and IL-6 did not reach significance ($P = 0.0028$) when the conservative Bonferroni correction was applied to avoid type 1 error at $P = 0.05$. No interaction was also detected after the correction.

3. ASSOCIATION BETWEEN MINERALS, CYTOKINES AND SCM

To examine the association between minerals, cytokines and SCM, multiple regression models were constructed using milk minerals as the dependent variables. Cytokines and SCM status were entered as the independent or explanatory variables, while controlling for lactation stages (Table 4.4).

The regression analyses showed that lactation stage was negatively associated with sodium, potassium, phosphorus, chromium, copper, iron, manganese, rubidium and zinc and positively associated with magnesium. The cytokines were found to be associated with changes in specific milk minerals (Table 4.4). IL-6 ($P = 0.003$) alone explained approximately 11.4% of the variability in calcium and IL-6 ($P = 0.015$) with lactation stages explained 54.6% of the total variance for potassium. In addition, about 68.2% of the variance in copper concentrations could be accounted for by the combination of IL-6 ($P = 0.003$) and lactation stages. IL-1β was associated with higher phosphorus ($P = 0.016$), iron ($P = 0.001$) and manganese ($P = 0.001$) and IL-8 ($P = 0.034$, $r^2 = 0.530$) with higher zinc. TNF-α ($P = 0.044$) only had a weak negative association with manganese. Approximately 59% and 37.3% of the variances in milk phosphorus and iron concentrations respectively, were accounted by IL-1β and lactation stages.

Regression analyses of minerals (Table 4.4) also showed that SCM was positively associated with sodium. This was expected as sodium was part of the Na/K ratio indicator for SCM. Together, SCM and lactation stages, explained up to 64.4% of the variability in sodium. Last, but most important finding from these analyses was that SCM was associated with higher milk selenium, but only explained 7% of its variability ($P = 0.021$, $r^2 = 0.071$). To our knowledge, this was the first report of such association in human studies. This observation suggests that selenium might play a crucial role in the pathophysiology of SCM.

3.1. Bonferroni correction

The association between stages of lactation and sodium, potassium, magnesium, phosphorus, copper, iron, manganese, rubidium and zinc remained significant after the Bonferroni correction (P = 0.0042). Moreover, IL-6 was still positively associated with calcium and copper and IL-1β with iron and manganese after correction.

FIG. 4.4. Frequency distribution of Na/K ratio in TM (5-17d), EMM (18-46d) and MM (109-187d).

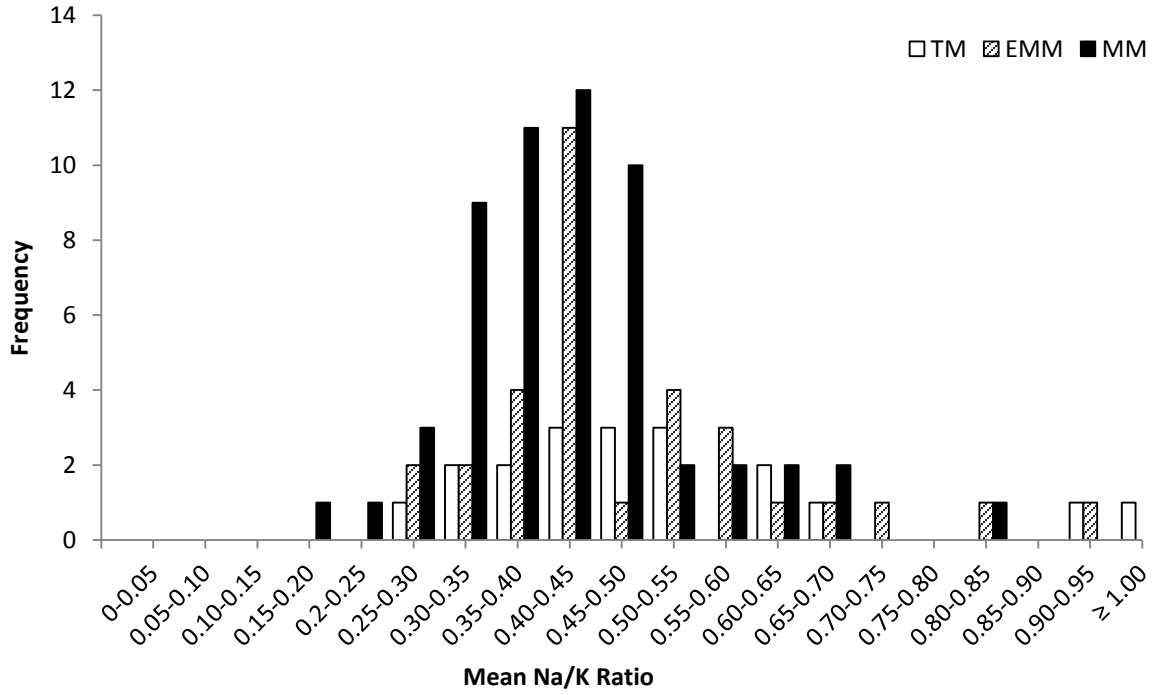


TABLE 4.3. Effects of lactation stages (TM, EMM and MM) and SCM status (SCM confirmed by Na/K > 0.6) on concentration of minerals and inflammatory cytokines in breast milk of Guatemalan women[1]

Milk Biomarkers [3, 4]	Transitional Milk, 5 – 17 d		Early Mature Milk, 18 – 46 d		Mature Milk, 109 – 187 d		Two-way ANOVA [2], *P* values		
	Non-SCM	SCM	Non-SCM	SCM	Non-SCM	SCM	Lactation Stages (A)	SCM Status (B)	A x B Interaction
Macrominerals (mmol/L)	**(n = 14)**	**(n = 5)**	**(n = 27)**	**(n = 5)**	**(n = 51)**	**(n = 5)**			
Na	6.66 ± 0.40 ^{b}	12.17 ± 1.24 ^{c}	5.82 ± 0.23 ^{b}	11.56 ± 0.62 ^{c}	4.39 ± 0.15 ^{a}	6.86 ± 0.12 ^{b}	**< 0.0001***	**< 0.0001***	0.315
K	15.69 ± 0.34 ^{c}	15.30 ± 0.94 ^{bc}	13.73 ± 0.35 ^{b}	15.67 ± 0.99 ^{bc}	11.11 ± 0.23 ^{a}	10.07 ± 0.53 ^{a}	**< 0.0001***	0.675	**0.026**
Ca	6.46 ± 0.25	8.17 ± 0.28	6.86 ± 0.16	6.64 ± 0.99	6.44 ± 0.14	6.60 ± 0.58	0.276	0.172	0.109
Mg	0.95 ± 0.03 ^{a}	1.14 ± 0.06 ^{ab}	0.98 ± 0.03 ^{a}	1.06 ± 0.10 ^{a}	1.47 ± 0.03 ^{c}	1.47 ± 0.15 ^{bc}	**< 0.0001***	0.074	0.215
P	5.48 ± 0.10 ^{c}	5.32 ± 0.19 ^{bc}	4.92 ± 0.14 ^{b}	4.52 ± 0.29 ^{b}	3.63 ± 0.05 ^{a}	3.20 ± 0.18 ^{a}	**< 0.0001***	**0.003**	0.944
Trace Minerals (µmol/L)									
Cr	0.47 ± 0.05	0.29 ± 0.07	0.37 ± 0.04	0.36 ± 0.11	0.31 ± 0.02	0.31 ± 0.09	0.285	0.596	0.737
Cu	9.97 ± 0.34 ^{c}	9.63 ± 0.63 ^{bc}	7.82 ± 0.30 ^{b}	10.31 ± 1.31 ^{c}	4.40 ± 0.19 ^{a}	4.22 ± 0.61 ^{a}	**< 0.0001***	0.271	**0.004**
Fe	34.50 ± 5.51 ^{c}	12.63 ± 3.87 ^{abc}	20.24 ± 2.93 ^{bc}	45.09 ± 20.04 ^{bc}	9.29 ± 1.00 ^{a}	15.65 ± 9.24 ^{ab}	**0.0004***	0.811	**0.007**
Mn	0.42 ± 0.07 ^{b}	0.14 ± 0.06 ^{ab}	0.29 ± 0.04 ^{ab}	0.45 ± 0.21 ^{ab}	0.19 ± 0.03 ^{a}	0.21 ± 0.14 ^{ab}	0.062	0.334	0.098
Rb	13.63 ± 0.55 ^{b}	14.68 ± 1.23 ^{b}	12.63 ± 0.46 ^{b}	14.02 ± 1.24 ^{b}	9.87 ± 0.37 ^{a}	9.90 ± 1.23 ^{ab}	**< 0.0001***	0.465	0.768
Se	0.24 ± 0.02	0.33 ± 0.06	0.27 ± 0.02	0.22 ± 0.06	0.23 ± 0.01	0.34 ± 0.02	0.960	**0.010**	0.394
Sr	0.51 ± 0.05	0.88 ± 0.14	0.54 ± 0.03	0.42 ± 0.05	0.53 ± 0.03	0.52 ± 0.10	0.126	0.787	**0.023**
Zn	70.75 ± 4.20 ^{c}	49.57 ± 12.71 ^{bc}	54.36 ± 3.71 ^{bc}	58.34 ± 8.32 ^{bc}	24.41 ± 2.59 ^{a}	33.14 ± 5.75 ^{ab}	**< 0.0001***	0.315	0.276
Cytokines (pg/mL)	**(n = 14)**	**(n = 5)**	**(n = 26)**	**(n = 4)**	**(n = 51)**	**(n = 5)**			
IL-1β [5]	0.10 ± 0.03	0.12 ± 0.02	0.18 ± 0.04	0.19 ± 0.03	0.12 ± 0.02	0.08 ± 0.03	**0.018**	0.718	0.501
IL-6	0.78 ± 0.03 ^{b}	1.59 ± 0.36 ^{c}	0.77 ± 0.04 ^{b}	1.40 ± 0.62 ^{bc}	0.47 ± 0.03 ^{a}	0.44 ± 0.05 ^{a}	**<0.0001***	**0.004**	**0.049**
IL-8	7.04 ± 3.86 ^{a}	37.23 ± 9.20 ^{b}	5.38 ± 1.86 ^{a}	22.88 ± 10.24 ^{ab}	31.17 ± 7.52 ^{b}	71.43 ± 32.94 ^{b}	**0.001***	**<0.0001***	0.229
TNF-α	1.50 ± 0.21 ^{a}	5.28 ± 1.11 ^{b}	1.72 ± 0.24 ^{a}	2.40 ± 1.11 ^{a}	1.41 ± 0.09 ^{a}	3.29 ± 1.14 ^{ab}	**0.042**	**0.0001***	**0.020**

[1] Values are Means ± SEM. Means with differing superscripts within rows are significantly different at the $p < 0.05$ based on Bonferroni post hoc paired comparison.
[2] Main effects included Lactation Stages: TM, EMM, MM (A) and SCM Status: non-SCM and SCM (B) and Lactation Stages x SCM Status interaction (A x B).
[3] Mg, Na, Fe, Rb, Se, Sr, Zn, IL-1β, IL-6, IL-8 and TNF-α were natural log transformed [Ln(y)] to achieve normality. Cr and Mn was square-root transformed [Sq(y)] to achieve normality.
[4] Based on the outlier labelling rule, 1 outlier was identified and excluded from the Na, Se, Zn and IL-6 data set and 2 outliers from the IL-1β data set.
[5] IL-1β has a smaller sample size for non-SCM milks. TM: n = 5, EMM: n = 18 and MM: n = 38.
* Indicates statistical significance after Bonferroni correction ($P = 0.0029$).

FIG 4.5. Effect of lactation stages and SCM status on cytokine concentrations: IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D). All values represent Means ± SEM from each group. Means with differing superscripts are significantly different at the $p < 0.05$

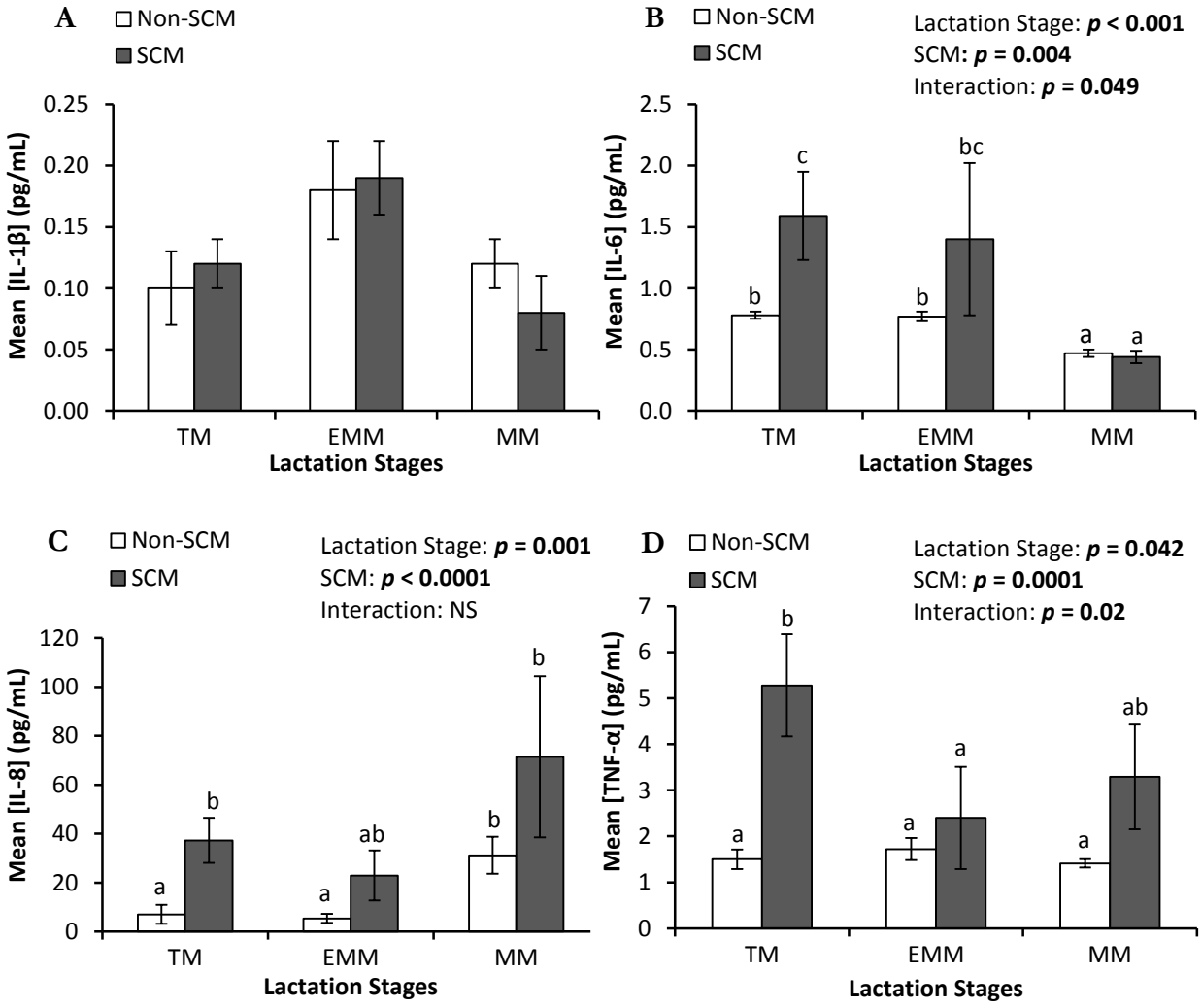


TABLE 4.4. Multiple regression models of milk minerals as dependent variables.

Dependent Variable [1]	Explanatory variables	Coefficient (β)	*P* - value	R^2
Macrominerals				
Sodium	SCM (1, 2) [2]	0.553	<0.0001*	0.644
	Lactation Stages (1, 2, 3) [3]	-0.488	<0.0001*	
Potassium	Lactation Stages (1, 2, 3)	-0.599	<0.0001*	0.546
	IL-6	0.227	0.015	
Calcium	IL-6	0.338	0.003*	0.114
Magnesium	Lactation Stages (1, 2, 3)	0.675	<0.0001*	0.455
Phosphorus	Lactation Stages (1, 2, 3)	-0.737	<0.0001*	0.590
	IL-1β	0.186	0.016	
Trace Minerals				
Chromium	Lactation Stages (1, 2, 3)	-0.264	0.022	0.070
Copper	Lactation Stages (1, 2, 3)	-0.685	<0.0001*	0.682
	IL-6	0.233	0.003*	
Iron	Lactation Stages (1, 2, 3)	-0.507	<0.0001*	0.373
	IL-1β	0.318	0.001*	
Manganese	IL-1β	0.346	0.001*	0.243
	Lactation Stages (1, 2, 3)	-0.288	0.008*	
	TNF-α	-0.215	0.044	
Rubidium	Lactation Stages (1, 2, 3)	-0.534	<0.0001*	0.286
Selenium	SCM (1, 2)	0.266	0.021	0.071
Zinc	Lactation Stages (1, 2, 3)	-0.752	<0.0001*	0.530
	IL-8	0.180	0.034	

[1] Na, Mg, Fe, Rb, Se, Sr and Zn were natural log transformed [Ln(y)] to achieve normality. Cr and Mn was square-root transformed [Sq(y)] to achieve normality

[2] SCM status: 1 = Non-SCM and 2 = SCM.

[3] Lactation stages: 1 = TM, 2 = EMM and 3 = MM.

* Indicates statistical significance after Bonferroni correction ($P = 0.0083$).

CHAPTER V. DISCUSSION

In the present study, we measured various macro- and trace minerals and cytokines in breast milk samples of non-SCM and SCM affected Guatemalan mothers at different lactation stages. Our results (Results – Table 4.3) showed that sodium, potassium, phosphorus, manganese, rubidium, and zinc as well as IL-6 had higher concentrations in TM and EMM than in MM, whereas magnesium and IL-8 were lower. In contrast to the widespread effect of lactation stages, the impact of SCM status was only observed in selective minerals and cytokines, namely phosphorus, selenium and IL-8. However, this effect did not reach significance for phosphorus and selenium after the application of conservative Bonferroni correction for multiple comparisons. Moreover, although no interaction between lactation stages and SCM status was detected after correction, the effect of lactation stages on copper, iron and IL-6 remained significant, as was the effect of SCM on TNF-α. Copper, iron and IL-6 had higher concentrations in TM and EMM than in MM, while TNF-α concentrations in TM were higher in SCM mothers than in non-SCM mothers.

The multiple regression model of milk minerals as the dependent variables and cytokines and SCM as the explanatory variables (controlling for lactation stages), further revealed the association between several minerals and cytokines (Results – Table 4.4). IL-6 was associated with higher potassium, calcium and copper; IL-1β with higher phosphorus, iron and manganese; and IL-8 with higher zinc and TNF-α with lower manganese. The most interesting finding was that SCM was shown to be positively associated with selenium. However, only the association between IL-6 and calcium and copper, IL-1β with iron and manganese remained significant after Bonferroni correction.

SCM, the condition of interest, was defined as having a Na/K ratio > 0.6 according to previously published categories: a Na/K ratio ≤ 0.6 is considered to be normal, > 0.6 to 1.0 is considered to be moderately raised, and > 1.0 is greatly raised (Kantarci *et al.*, 2007; Willumsen *et al.*, 2002). Na/K ratio of breast milk is recognized as an indirect measure of epithelial damage, leakiness, and subsequently inflammation status. An elevation in Na/K ratio is indicative of increased severity of inflammation. It is reported that Na/K ratios in milk of healthy women at one month postpartum generally averaged 0.6 or less (Aryeetey, Marquis, Timms, Lartey*, et al.*, 2008), and that the average breast milk sodium and potassium concentration ranges between 5 to 6 mmol/L and 13 to 14 mmol/L, respectively. The mean sodium and potassium concentrations in non-SCM milk seen from our results

(Results – Table 4.3) were in line with these earlier values. In addition, our ANOVA showed that sodium concentrations are significantly higher in SCM milk than in non-SCM milk across all lactation stages. The elevated sodium concentrations in mastitic breast milk are believed to be the result of serum sodium transudating into milk via "leaky" tight junctions in order to offset the osmotic pressure caused by reduced levels of lactose (Wegner & Stull, 1978). Potassium is also believed to be part of the osmotic adjustment in milk, associated with casein altered milk-blood permeability during udder inflammation (Wegner & Stull, 1978).

Earlier studies have reported that SCM and mastitis may develop at any stage of lactation ranging from 2 weeks to one year after parturition, with higher incidence during the second and third week postpartum (WHO, 2000). In our study, the prevalence of SCM was indeed found to be the highest in TM (26.3%) and dropped to 8.9% in MM. These values agree with several other reports on SCM prevalence, which ranged from 2% to 66% in lactating women. (Barbosa-Cesnik *et al.*, 2003; Foxman *et al.*, 2002; Nussenblatt *et al.*, 2005; Vogel, Hutchison, & Mitchell, 1999). The elevated SCM prevalence during early lactation was also seen in cows, and researchers have suggested that this is due to depressed host resistance mechanism observed from approximately 3 weeks before calving until 3 weeks after calving. However, the underlying mechanisms and factors have not been fully explained. It could be related to the many metabolic and hormonal changes that take place during this period (Meglia *et al.*, 2001). Perhaps, the maternal immune system also undergoes a similar depression before and after childbirth, making the mother susceptible to pathogenic infections, hence leading to higher chances of developing diseases, such as SCM.

Paralleling to the compromised host resistance mechanism around calving is the drop in serum levels of calcium, magnesium, phosphorus, potassium, selenium, and zinc among periparturient cows, suggesting possible early mineral deficiencies (Meglia *et al.*, 2001). A similar mineral shift might also occur in lactating mothers, however, there are no exiting studies on the maternal serum mineral concentrations around childbirth. On the other hand, studies have revealed that the concentrations of macrominerals such as sodium, calcium, phosphorus, and magnesium in the maternal serum are tightly controlled by homeostatic mechanisms, independent of maternal diet and nutritional status, as such no effects are carried over in breast milk as well (Emmett & Rogers, 1997; Lönnerdal, 2000). Similarly, the maternal dietary intake also do not show an influence on chromium, copper, iron and zinc concentrations in milk. It appears that even though trace minerals exhibit larger inter-individual differences, under normal conditions, the influx of trace elements into the mammary gland and their

secretion into milk remains tightly regulated, possibly by up- and down-regulation of receptor-mediated processes (Lönnerdal *et al.*, 1996). More studies are needed to investigate the relationship between blood electrolytes, maternal mineral deficiencies, and milk mineral concentrations in the context of SCM.

Summary table 5.1 and 5.2 provide an overview of previous human studies that have measured the concentrations of macro and trace minerals in breast milk. The method of detection, postpartum time, sample size, and mean concentrations are described accordingly. Most of the milk mineral concentrations measured by ICP-MS in the present study were very similar to the data reported by Björklund *et al.* and Deng *et al.* (Discussion – Table 5.1 and 5.2), with the exception of chromium concentrations being lower (Björklund *et al.*, 2012), while iron and rubidium concentrations being higher than the literature values (Björklund *et al.*, 2012; Deng, Zhang, Yan, & Zhang, 2009). The discrepancy could be attributed to the differences in the time when milk was sampled postpartum. The milk samples in Björklund *et al.*'s study were collected during the third week after delivery (14–21 days postpartum), whereas our TM and EMM groups ranged from 5 to 17 days and 18 to 46 days postpartum, respectively.

Our measurements of breast milk also indicated that sodium, potassium, phosphorus, copper, iron, manganese, rubidium, and zinc were present at higher concentrations in TM and EMM than in MM (Results – Table 4.3). These findings are supported by a number of longitudinal studies of healthy breast milk over an extended lactation period (Feeley *et al.*, 1983b; Hannan *et al.*, 2005; Krachler *et al.*, 1998; Silvestre *et al.*, 2001). The decreasing trend in minerals is believed to be associated with changes of infant needs for them (Silvestre *et al.*, 2001; Vaughan *et al.*, 1979), as well as a consequence of a decrease in protein and ligand concentrations which lead to their reduced binding capacity in milk (Rossipal & Krachler, 1998). In contradiction to this trend, the magnesium concentrations in Guatemalan mother's milk were higher in MM than in TM and EMM. However, the underlying reasons for this observation remain to be determined.

Among the many potentially active immunologic components present in milk, the pro-inflammatory cytokines have been identified to play a crucial role in mastitis (Oviedo-Boyso *et al.*, 2007). They are potent inducers of acute-phase response, fever, and vascular endothelial activation (Goldman *et al.*, 1996). Summary table 5.3 provides an overview of previous studies that have measured the concentrations of IL-1β, IL-6, IL-8, and TNF-α in breast milk. The method of detection, postpartum time, sample size, mean concentrations as well as concentration ranges are given where possible. The huge inter-individual variability of cytokines in breast milk is evident in the reported

values (Discussion – Table 5.3). This broad range of cytokine concentrations during lactation increase the difficulty of meaningful data interpretation and inter-study comparison. Our finding of IL-1β in about half of the samples is contradictory to the data described by both Srivastava *et al.* and Basolo *et al.* who were unable to detect any IL-1β. In general, our data contrast significantly with previous literature values. Most studies reported cytokine concentrations that were at least 10 folds higher than our measurements (Buescher & Malinowska, 1996; Hawkes, Bryan, James, & Gibson, 1999; Sone *et al.*, 1997).

The considerable variability of cytokines between individual mothers can explain, to some degree, the huge discrepancy among studies. Differences in methods (Luminex vs. ELISA) used for determination and analysis errors can also explain the discrepancies. Luminex uses fluorescence as a reporter system, whereas ELISA uses enzymatically induced colorimetric changes to indicate the relative presence of an analyte. Luminex is based on multiplex bead array assay which is multiplexed, and therefore may be subjected to cross-reactivities between multiple ligands. By contrast, ELISA methodologies generally study one analyte at a time, and thus avoiding any concerns arising from multiplexing (Elshal & McCoy, 2006). Moreover, the difficulties of accurate measurements increase as the concentration of target analytes lowers. In this respect, cytokines, which are known to have low concentrations and fluctuating levels in milk can be tricky to measure. Milk is also one of the most problematic matrices to study as it contains molecules of various sizes ranging from large fat globules to small cytokine proteins. The lipids in milk can also hinder (interferences) the determination of cytokines when using luminex.

Bovine research has shown that during early phases of lactation, cytokines are enriched in the milk, possibly to provide protection for the neonate during the period when the hormonal and immune systems are still developing (Bannerman *et al.*, 2004). A study by Erbağcı *et al.* demonstrated that breast milk cytokines exhibit biological variability at different periods of human lactation, with higher levels of IL-1β, IL-6, IL-8 and TNF-α in the colostrum versus mature milk following normal pregnancy (Erbağcı *et al.*, 2005). Our results, on the other hand, provided information regarding milk cytokine concentrations in TM vs. EMM vs. MM. In the present study, both IL-1β and IL-6 concentrations were indeed higher in TM and EMM than in MM, but contrary to earlier findings, IL-8 levels were lower in these earlier lactation groups and TNF-α showed no difference throughout lactation. The physiological changes of cytokines profile may reflect the changes in the breast immune system (e.g., intra-mammary infections or in response to the needs of recipient infant) (Ustundag *et*

al., 2005). There has been evidence that IL-8 and TNF-α are produced by mammary tissues primarily for the purpose of maternal protection and hence might be down-regulated in early breast milk to prevent adverse effects on infant health (Mulokozi & Bilotta, 1999).

The inflammatory effects associated with SCM are the results of increased production of pro-inflammatory cytokine and chemokine by stimulated lymphocytes and leukocytes (Osman *et al.*, 2010). Bonferroni post hoc multiple comparisons (Results – Table 4.3) depicted that concentrations of IL-6, IL-8 and TNF-α in TM from SCM mothers were significantly higher than the non-SCM mothers, suggesting TM could be a crucial time for the establishment of SCM. The increase in cytokine concentrations during SCM is in line with responses seen in earlier studies (Bannerman, 2009; Bannerman *et al.*, 2004; Blum *et al.*, 2000; Fitzgerald *et al.*, 2007; Li *et al.*, 2009; Persson Waller *et al.*, 2003). IL-6 is often recognized as a marker for systemic activation of pro-inflammatory cytokines (Ustundag *et al.*, 2005). Mizuno *et al.* found that IL-6 concentrations in human breast milk expressed from the mastitic mammary lobe were significantly increased compared to the healthy breast lobe, suggesting that IL-6 production could be in response to localized effects rather than systemic, as elevated IL-6 mRNA synthesis only occurred in the mastitic mammary lobe (Mizuno *et al.*, 2012).

A study of *S. aureus*-induced mastitis in cows by Osman *et al.* examined 25 cows with SCM and 15 cows with clinical mastitis. They showed that IL-6 concentrations in the subclinical animals were significantly higher (30.8 ng/mL) than the clinically manifested animals (18.0 ng/mL). They concluded that the establishment of physical symptoms associated with clinical mastitis marked the peak concentrations of IL-6 (Osman *et al.*, 2010). This is a fascinating observation, and based on this, one could postulate that the degree of elevation in the cytokine concentrations during SCM might be associated with subsequent developments of clinical symptoms, and ultimately dictate the disease progression from SCM to clinical mastitis. Perhaps clinical mastitis occurs when a threshold concentration of cytokine is reached, where the effects of cytokines become so strong that the mammary gland starts to exhibit the physical signs of inflammation.

Similar to IL-6, TNF-α was present in significant amount in *E. coli* challenged quarters, whereas its concentration in non-challenged (contralateral) quarters was barely measurable, indicating that its production was also localized and restricted to challenged quarters (Blum et al., 2000). TNF-α is a physiologically significant regulator of mammary gland development, stimulating growth and branching morphogenesis of mammary epithelial cells. It enhances NF-κB activity in bovine mammary epithelial monolayers, and thereby induces serosal IL-8 release via binding of putative κB consensus

sequence in bovine IL-8 promoter to TNF-α inducible heterodimer. High dose of IL-1β can also induce IL-8 release, but less potently than TNF-α (Fitzgerald *et al.*, 2007). Stimulated by both TNF-α and IL-1β could explain why IL-8 had the highest concentration in milk amongst the cytokines studied with noticeably higher concentrations in SCM than in non-SCM milk samples (Fig 4.5C). High levels of IL-8 are responsible for the trafficking of neutrophils, monocytes and lymphocytes from the maternal circulation to the breast milk (Ustundag *et al.*, 2005).

The innate immune system has evolved to recognize microorganisms via a limited number of pattern-recognition receptors (PRRs) (Oviedo-Boyso *et al.*, 2007; Rainard & Riollet, 2006) and to respond accordingly for specific pathogens. For example, intra-mammary infection with *E. coli*, but not *S. aureus*, induces the up-regulation of TNF-α, IL-1β, and IL-8 (Rainard & Riollet, 2006) resulting in acute mastitis rather than the more chronic infectious state characteristic of *S. aureus*-induced mastitis (Bannerman *et al.*, 2004). The range of pathogen-induced cytokine responses could account for the much higher SEM values observed for cytokine concentrations in SCM milk (Results – Table 4.3 and Fig 4.5). Therefore, a next step after the current study would be to analyze maternal cytokine profiles on an individual basis with an attempt to match their immune response to their milk bacterial profile. This would allow a closer and more precise examination of maternal immunity during SCM.

A study by Lönnerdal and colleagues that assessed the impact of maternal inframammary infection on trace minerals in colostrum and early milk has found no significant differences in mineral concentrations between ill and non-ill women (Lönnerdal *et al.*, 1996). However, in our study of SCM, phosphorus and selenium concentrations were both affected by maternal SCM status, but the effects did not reach significance after using Bonferroni correction ($P = 0.0029$) and further post-hoc multiple comparisons failed to demonstrate any difference in the mineral concentrations between SCM and non-SCM group (Results – Table 4.2). Nevertheless, a trend towards higher selenium and lower phosphorus in milk of SCM mothers was observed.

Selenium is one of the most widely studied elements in bovine research. Animal supplementation studies have shown that selenium is crucial in preventing impaired function of the immune system. Selenium in the form of selenoproteins, specifically glutathione peroxidase (GPx), can affect three broad areas of cellular function: antioxidant activities, thyroid hormone metabolism, and the regulation of redox-active protein activity (Salman et al., 2009). Neutrophils from selenium-deficient animals such as mice, rats and cattle were able to ingest pathogens *in vitro*, but they were significantly less effective at intracellular killing of mastitis pathogens (Meglia et al., 2001; Salman et

al., 2009). Conversely, the role of phosphorus in SCM is unknown and has not been studied before. Its changes in concentration might be related to the lowered milk production during SCM or the increased incorporation of phosphorus into ATP for energy production due to inflammation.

Further regression analysis again showed that SCM was associated with higher selenium concentrations in milk. Although this association did not reach statistical significance when using Bonferroni correction, the observation is supported by findings from numerous bovine works. Heifers supplemented with selenium were associated with reduced prevalence of infection, lowered incidence of mastitis, shorter infection duration, and lowered milk SCC (Semba & Neville, 1999). Selenium also appears to strengthen antibacterial activity in milk by enhancing bacterial killing in neutrophils (Smith *et al.*, 1997). With this knowledge in mind, the higher selenium concentrations in SCM milk might be a result of increased uptake of selenium (hyper-accumulation) from serum as part of host defense mechanisms to combat inflammation. A somewhat similar mechanism to the hyper-accumulation of zinc in malignant breast tissues (Lopez, Foolad, & Kelleher, 2011). Selenium exhibit its anti-inflammatory effect by promoting signal transduction pathways in the immune cells and macrophage (Duntas, 2009). *In vivo*, the level of selenium directly correlates with GPx. Overexpressed GPx decreases ROS levels by inhibiting IκB-α phosphorylation and consequently the translocation of NF-κB into the nucleus (Kretz-Remy & Arrigo, 2001). Therefore, expression of genes encoding for inflammatory cytokines is hindered by increased selenium levels (Duntas, 2009).

To our knowledge, this study is the first to make an attempt to provide some insight on the complex relationship between milk minerals and cytokines in humans. Multiple regression models revealed that several cytokines were associated with higher mineral concentrations in milk. (Results – Table 4.4). Bovine studies suggest that cytokine-mediated inflammatory responses during clinical and subclinical mastitis can trigger physical damages to the epithelial layer, decreasing its synthetic and secretory capacity as well as enhancing mammary epithelium permeability via opening of tight junctions between cells (Li *et al.*, 2009). The leaky tight junctions can lead to changes in the milk mineral concentrations during SCM.

In addition, the inflammatory cytokines have the ability to initiate inflammation-mediated changes in the expression levels of transporters (Ling, 2011). Experimental evidence from rodent models showed that temporal changes in milk zinc, copper and iron concentrations are regulated through coordinated changes in gene expression, protein levels and localization of several specific transporters (Almeida, Lopes, Silva, & Barrado, 2008). For example, intracellular zinc pools are constantly redistributed for specific cellular functions, such as during lactogenic stimulation or disease

state. Zinc homeostasis is tightly regulated through 24 zinc transporters (Hennigar & Kelleher, 2012). Recent studies have demonstrated that pro-inflammatory cytokines, such as IL-1β and TNF-α, can regulate zinc transporter expression and redistribute zinc pools (Besecker *et al.*, 2008; Lichten, Liuzzi, & Cousins, 2009). Furthermore, Smirnov's group has found that TNF-α and IL-1β enhanced iron uptake by type 2 alveolar epithelial cells (A549) with differing effects on non-transferrin-bound and transferrin-bound iron (Smirnov, Bailey, Flowers, Garrigues, & Wesselius, 1999).

One of the main challenges of examining possible cytokine and mineral interactions in SCM and non-SCM milk is the direction of causality. Inflammation occurrences during SCM can cause involuntary opening of tight junction between mammary epithelial cells (Aryeetey, 2007) via cytokine signaling, which down-regulates the expression of tight junction strand proteins such as occludin (Dorosko, 2005). The increased permeability of mammary epithelial tissues allows leukocytes and other plasma constituents such as minerals to enter freely into the milk, thereby altering the overall milk mineral concentrations. On the other hand, deficiencies in minerals such as selenium, copper, iron, and zinc can lead to prolonged inflammation, increased accumulation of ROS, reduced immune cell proliferation and activity, as well as diminished ability for intracellular kill of mastitis pathogens (Lönnerdal et al., 1996; Meglia et al., 2001; Oviedo-Boyso et al., 2007; Wintergerst et al., 2007). SCM occurs as the result of the reduced host immune resistance.

A few limitations to this study include small sample size for SCM groups, diurnal variations in minerals, and the lack of abilities to control for possible confounding factors such as gestational age, mode of delivery, maternal diet, stress, and hormone levels. Small sample size of the SCM groups (n=5) could be the reason for the lack of statistical significance observed in many post-hoc multiple comparisons and after Bonferroni correction. Further study with larger sample sizes is warranted. Earlier studies have identified diurnal variations in a few minerals such as iron, sodium and potassium (Feeley *et al.*, 1983b; Silvestre *et al.*, 2001; Vaughan *et al.*, 1979). To minimize the variations, milk collections should be made at a fixed time point during the day. Mode of delivery is a factor that has been associated with changes in maternal milk microbiome (Cabrera-Rubio *et al.*, 2012), and therefore can potentially alter the host's cytokine response. Moreover, peripartum events can induce several changes in circulating hormone levels, and varying degrees of stress experienced by these women at delivery could also have an effect on the hormonal levels and serum trace elements independent of the effect of infection (Lönnerdal *et al.*, 1996).

Future human longitudinal studies should be conducted while controlling for the above-mentioned confounding factors. Longitudinal studies will provide an opportunity to track the changes

in milk minerals and cytokines, as well as their interactions throughout lactation in the context of SCM. At the moment, a considerable amount of work is needed in order to bridge the existing knowledge gap in literature on SCM and to determine how these findings can be translated into clinical significances.

TABLE 5.1. Overview of studies that measured breast milk macromineral concentrations via ICP-AES or ICP-MS.

Macromineral	Measurement Method	Postpartum Time (n)	Mean Concentration (mmol/mL)	Reference
Calcium	ICP-AES	1-365d (1170)	6.24±1.77	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	7.09±1.90	(Schramel, Lill, Hasse, & Klose, 1988)
	ICP-MS	14-21d (60)	7.61±1.12	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	7.00	(Deng *et al.*, 2009)
Magnesium	ICP-AES	1-365d (1170)	1.11±0.37	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	1.41±0.31	(Schramel *et al.*, 1988)
	ICP-MS	14-21d (60)	1.15±0.20	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	1.16	(Deng *et al.*, 2009)
Phosphorus	ICP-AES	1-365d (1170)	4.84±1.23	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	4.52±1.13	(Schramel *et al.*, 1988)
	ICP-MS	14-21d (60)	5.55±0.74	(Björklund *et al.*, 2012)
Potassium	ICP-AES	1-365d (1170)	12.02±3.09	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	17.14±2.17	(Schramel *et al.*, 1988)
	ICP-MS	14-21d (60)	16.20±1.02	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	12.75	(Deng *et al.*, 2009)
Sodium	ICP-AES	1-365d (1170)	5.87±3.78	(Yamawaki *et al.*, 2005)
	ICP-MS	14-21d (60)	9.44±3.35	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	8.21	(Deng *et al.*, 2009)

TABLE 5.2. Overview of studies that measured breast milk trace mineral concentrations via ICP-AES or ICP-MS.

Trace Mineral	Measurement method	Postpartum Time (n)	Mean Concentration (μmol/mL)	Reference
Barium	ICP-MS	14-21d (60)	0.087±0.013	(Björklund *et al.*, 2012)
Chromium	ICP-AES	1-365d (1170)	1.13±0.90	(Yamawaki *et al.*, 2005)
	ICP-MS	14-21d (60)	0.0058±0.0.0052	(Björklund *et al.*, 2012)
Copper	ICP-AES	1-365d (1170)	5.51±3.30	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	5.82±2.68	(Schramel *et al.*, 1988)
	ICP-AES	10-30d (41)	4.25±1.42	(Wasowicz *et al.*, 2001)
	ICP-MS	14-21d (60)	7.41±1.42	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	5.33	(Deng *et al.*, 2009)
Iron	ICP-AES	1-365d (1170)	21.31±44.95	(Yamawaki *et al.*, 2005)
	ICP-MS	14-21d (60)	6.07±2.40	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	6.43	(Deng *et al.*, 2009)
Manganese	ICP-AES	1-365d (1170)	0.200±0.419	(Yamawaki *et al.*, 2005)
	ICP-MS	14-21d (60)	0.055±0.025	(Björklund *et al.*, 2012)
Rubidium	ICP-MS	14-21d (60)	8.35±1.26	(Björklund *et al.*, 2012)
Selenium	ICP-AES	1-365d (1170)	0.215±0.076	(Yamawaki *et al.*, 2005)
	Fluorometry	10-30d (41)	0.0092± 0.0036	(Wasowicz *et al.*, 2001)
	ICP-MS	14-21d (60)	0.165±0.033	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	0.105	(Deng *et al.*, 2009)
Strontium	ICP-MS	14-21d (60)	0.377±0.148	(Björklund *et al.*, 2012)
Zinc	ICP-AES	1-365d (1170)	22.18±20.65	(Yamawaki *et al.*, 2005)
	ICP-AES	N/A (30)	76.48±26.00	(Schramel *et al.*, 1988)
	ICP-AES	10-30d (41)	21.41±10.71	(Wasowicz *et al.*, 2001)
	ICP-MS	14-21d (60)	53.01±14.97	(Björklund *et al.*, 2012)
	ICP-MS/ICP-AES	3-8wk (60)	35.02	(Deng *et al.*, 2009)

TABLE 5.3. Overview of studies that measured IL-1β, IL-6, IL-8 and TNF-α concentrations in human milk using ELISA or Luminex.[1]

Cytokines	Method	Postpartum Time (n)	Mean Concentration (pg/mL)	Concentration Range (pg/mL)	Reference
IL-1β	ELISA	6-30d (4)	ND	ND	(Srivastava *et al.*, 1996)
		>30d (29)	2±4 (SD)	ND-7	
	ELISA	3d (20)	192	ND-500	(Sone *et al.*, 1997)
	ELISA	2-6d (49)	17±4 (SEM)	ND-147	(Hawkes *et al.*, 1999)
		2wk (48)	23±10 (SEM)	ND-400	
		4wk (45)	10±2 (SEM)	ND-66	
		6wk (42)	8±1 (SEM)	ND-27	
		8wk (37)	9±1 (SEM)	ND-37	
	Luminex	4-6 wk (57)	14.28±39.31 (SD)	n/a	(Groer & Beckstead, 2011)
IL-6	RIA	1-2d (7)	150±90 (SD)	n/a	(Nakajima *et al.*, 1997)
	ELISA	6-30d (4)	6±5 (SD)	ND-10	(Srivastava *et al.*, 1996)
		>30d (29)	4±9 (SD)	ND-42	
	ELISA	3-4d (35)	818±107 (SEM)	n/a	(Na, Daniels, & Seelig, 1997)
	ELISA	3d (20)	143	ND-500	(Sone *et al.*, 1997)
	ELISA	2-6d (49)	51±17 (SEM)	ND-742	(Hawkes *et al.*, 1999)
		2wk (48)	75±31 (SEM)	ND-1032	
		4wk (45)	13±4 (SEM)	ND-189	
		6wk (42)	14±4 (SEM)	ND-180	
		8wk (37)	14±4 (SEM)	ND-137	
	Luminex	4-6 wk (57)	148.58±231.49 (SD)	n/a	(Groer & Beckstead, 2011)
IL-8	ELISA	1-10d (20)	585.7±30.75 (SEM)	n/a	(Meki *et al.*, 2003)
		10-30d (20)	308.1±35.47 (SEM)	n/a	
		1-6mo (15)	200.3±25.01 (SEM)	n/a	
	ELISA	n/a (14)	302	n/a	(Hunt *et al.*, 2013)
	Luminex	4-6 wk (57)	416.59±1450.34 (SD)	n/a	(Groer & Beckstead, 2011)
TNF-α	RIA	1-2d (7)	620±183 (SD)	250-1700	(Rudloff *et al.*, 1992)
	ELISA	6-30d (4)	5±4 (SD)	ND-12	(Srivastava *et al.*, 1996)
		>30d (29)	4±3 (SD)	ND-13	
	ELISA	1-200d (87)	99±20 (SEM)	ND-1200	(Buescher & Malinowska, 1996)
	ELISA	3d (20)	80	ND-250	(Sone *et al.*, 1997)
	ELISA	2-6d (49)	151±65 (SEM)	ND-2933	(Hawkes *et al.*, 1999)
		2wk (48)	47±16 (SEM)	ND-570	
		4wk (45)	42±18 (SEM)	ND-724	
		6wk (42)	34±17 (SEM)	ND-722	
		8wk (37)	51±26 (SEM)	ND-944	
	Luminex	4-6 wk (57)	7.78±15.79 (SD)	n/a	(Groer & Beckstead, 2011)

[1] Table adapted from Hawkes *et al.* (1999).

*ND = Not detectable. Samples were below the limit of detection.

CHAPTER VI. CONCLUSION

In conclusion, we have found that most of the milk minerals and cytokines studied were present at higher concentrations in TM and EMM than in MM, except for magnesium and IL-8, which were higher in MM. The impact of SCM was only observed in phosphorus, selenium and IL-6, IL-8 and TNF-α. Further analysis showed that IL-6, IL-8 and TNF-α were higher in SCM milk than in non-SCM milk. The regression analyses for each mineral demonstrated that lactation stages were negatively associated with most minerals but positively associated with magnesium. SCM was also positively associated with selenium. Regression analyses also revealed that several cytokines were associated with higher milk mineral concentrations: IL-1β with phosphorus, iron and manganese; IL-6 with potassium, calcium and copper; IL-8 with zinc; and TNF-α with Mn. To our knowledge, this is the first human study that examine the potential associations of SCM with concentrations of select minerals and cytokines in breast milk.

REFERENCES CITED

Abou-Dakn, M., Richardt, A., Schaefer-Graf, U., & Wöckel, A. (2010). Inflammatory breast diseases during lactation: milk stasis, puerperal mastitis, abscesses of the breast, and malignant tumors–current and evidence-based strategies for diagnosis and therapy. *Breast Care, 5*(1), 33-37.

Aitken, S. L., Corl, C. M., & Sordillo, L. M. (2011). Immunopathology of mastitis: insights into disease recognition and resolution. *Journal of mammary gland biology and neoplasia, 16*(4), 291-304.

Arsenault, J. E., Aboud, S., Manji, K. P., Fawzi, W. W., & Villamor, E. (2010). Vitamin supplementation increases risk of subclinical mastitis in HIV-infected women. *Journal of Nutrition, 140*(10), 1788-1792.

Aryeetey, R. (2007). *Effects of maternal subclinical mammary inflammation on infant growth.* (Ph.D.), Iowa State Univerisity.

Aryeetey, R., Marquis, G., Brakohiapa, L., Timms, L., & Lartey, A. (2009). Subclinical mastitis may not reduce breastmilk intake during established lactation. *Breastfeeding Medicine, 4*(3), 161-166.

Aryeetey, R., Marquis, G., Timms, L., Lartey, A., & Brakohiapa, L. (2008). Subclinical mastitis is common among Ghanaian women lactating 3 to 4 months postpartum. *Journal of Human Lactation, 24*(3), 263-267.

Aryeetey, R., Marquis, G., Timms, L. L., Lartley, A., & Brakohiapa, L. (2008). Relationship of subclinical mastitis in Ghanaian women and breast milk intake by infants. *Animal Industry Report, 654*(1), 65.

Atakisi, O., Oral, H., Atakisi, E., Merhan, O., Metin, P. S., Ozcan, A., Marasli, S., Polat, B., Colak, A., & Kaya, S. (2010). Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk. *Research in veterinary science, 89*(1), 10-13.

Aydin, S., Aydin, S., Ozkan, Y., & Kumru, S. (2006). Ghrelin is present in human colostrum, transitional and mature milk. *Peptides, 27*(4), 878-882.

Ballard, O., & Morrow, A. L. (2013). Human milk composition. *Pediatric Clinics of North America, 60*, 49-74.

Bannerman, D. D. (2009). Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. *Journal of Animal Science, 87*(13 suppl), 10-25.

Bannerman, D. D., Paape, M. J., Hare, W. R., & Hope, J. C. (2004). Characterization of the bovine innate immune response to intramammary infection with *Klebsiella pneumoniae*. *Journal of dairy science, 87*(8), 2420-2432.

Barbosa-Cesnik, C., Schwartz, K., & Foxman, B. (2003). Lactation mastitis. *Journal of the American Medical Association, 289*(13), 1609-1612.

Batavani, R. A., Asri, S., & Naebzadeh, H. (2007). The effect of subclinical mastitis on milk composition in dairy cows. *Iranian Journal of Veterinary Research, 8*(3), 205-211.

Björklund, K. L., Vahter, M., Palm, B., Grandér, M., Lignell, S., & Berglund, M. (2012). Metals and trace element concentrations in breast milk of first time healthy mothers: a biological monitoring study. *Environmental Health, 11*(1), 92.

Blum, J. W., Dosogne, H., Hoeben, D., Vangroenweghe, F., Hammon, H. M., Bruckmaier, R. M., & Burvenich, C. (2000). Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domestic Animal Endocrinology, 19*(4), 223-235.

Boersma, E. R., Offringa, P. J., Muskiet, F. A., Chase, W. M., & Simmons, I. J. (1991). Vitamin E, lipid fractions, and fatty acid composition of colostrum, transitional milk, and mature milk: an international comparative study. *American Journal of Clinical Nutrition, 53*(5), 1197-1204.

Bruckmaier, R. M., Ontasouka, C. E., & Blum, J. W. (2004). Fractionized milk composition in dairy cows with subclinical mastitis. *Veterinarni Medicina*.

Buescher, E. S., & Malinowska, I. (1996). Soluble receptors and cytokine antagonists in human milk. *Pediatric research, 40*(6), 839-844.

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *American Journal of Clinical Nutrition, 96*(3), 544-551.

Castellote, C., Casillas, R., Ramírez-Santana, C., Pérez-Cano, F. J., Castell, M., Moretones, M. G., López-Sabater, M. C., & Franch, À. (2011). Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. *Journal of Nutrition, 141*(6), 1181-1187.

Chomat, A. M., Solomons, N. W., Koski, K. G., Pedersen, D., Wren, H., Vossenaar, M., & Scott, M. E. (2014). *Influence of maternal malnutrition, clinically and locally defined illnesses and psychosocial stressors on early infant growth in rural Mam-Mayan communities in Guatemala: mixed methods design and population characteristics* Manuscript submitted for publication.

Collado, M. C., Delgado, S., Maldonado, A., & Rodriguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real ‐ time PCR. *Letters in applied microbiology, 48*(5), 523-528.

Contreras, G. A., & Rodríguez, J. M. (2011). Mastitis: comparative etiology and epidemiology. *Journal of mammary gland biology and neoplasia, 16*(4), 339-356.

Methods of dealing with values below the limit of detection using SAS (2003).

Delgado, S., Arroyo, R., Martín, R., & Rodríguez, J. M. (2008). PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. *BMC infectious diseases, 8*(1), 51.

Delgado, S., Collado, C., Fernández, L., & Rodríguez, J. (2009). Bacterial analysis of breast milk: a tool to differentiate Raynaud's phenomenon from infectious mastitis during lactation. *Current microbiology, 59*(1), 59-64.

Deng, B., Zhang, H., Yan, C., & Zhang, L. (2009). Levels of mineral elements composition and heavy metal pollution in human breast milk in Shenzhen City. *Journal of hygiene research, 38*(3), 293-295.

Dorosko, S. M. (2005). Vitamin A, mastitis, and mother-to-child transmission of HIV-1 through breast-feeding: current information and gaps in knowledge. *Nutrition reviews, 63*(10), 332-346.

Elshal, M. F., & McCoy, J. P. (2006). Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods, 38*(4), 317-323.

EMD Millipore. (2012). MILLIPLEX® MAP High sensitivity human cytokine magnetic bead. In EMD Millipore (Ed.).

Emmett, P. M., & Rogers, I. S. (1997). Properties of human milk and their relationship with maternal nutrition. *Early human development, 49*, S7-S28.

Erbağcı, A. B., Çekmen, M. B., Balat, Ö., Balat, A., Aksoy, F., & Tarakçıoğlu, M. (2005). Persistency of high proinflammatory cytokine levels from colostrum to mature milk in preeclampsia. *Clinical biochemistry, 38*(8), 712-716.

Feeley, R. M., Eitenmiller, R. R., Jones, J. B., & Barnhart, H. (1983a). Calcium, phosphorus, and magnesium contents of human milk during early lactation. *Journal of pediatric gastroenterology and nutrition, 2*(2), 262-267.

Feeley, R. M., Eitenmiller, R. R., Jones, J. B., & Barnhart, H. (1983b). Copper, iron, and zinc contents of human milk at early stages of lactation. *American Journal of Clinical Nutrition, 37*(3), 443-448.

Field, C. J. (2005). The immunological components of human milk and their effect on immune development in infants. *Journal of Nutrition, 135*(1), 1-4.

Filteau, S. M. (2009). Measuring trace immune factors in human milk *Breast-Feeding: Early Influences on Later Health* (pp. 331-337): Springer.

Filteau, S. M., Rice, A. L., Ball, J. J., Chakraborty, J., Stoltzfus, R., de Francisco, A., & Willumsen, J. F. (1999). Breast milk immune factors in Bangladeshi women supplemented postpartum with retinol or β-carotene. *American Journal of Clinical Nutrition, 69*(5), 953-958.

Fitzgerald, D. C., Meade, K. G., McEvoy, A. N., Lillis, L., Murphy, E. P., MacHugh, D. E., & Baird, A. W. (2007). Tumour necrosis factor-α (TNF-α) increases nuclear factor κB (NFκB) activity in and interleukin-8 (IL-8) release from bovine mammary epithelial cells. *Veterinary Immunology and Immunopathology, 116*(1), 59-68.

Foxman, B., D'Arcy, H., Gillespie, B., Bobo, J. K., & Schwartz, K. (2002). Lactation mastitis: occurrence and medical management among 946 breastfeeding women in the United States. *American Journal of Epidemiology, 155*(2), 103-114.

Foxman, B., Schwartz, K., & Looman, S. J. (1994). Breastfeeding practices and lactation mastitis. *Social Science and Medicine, 38*(5), 755-761.

Garofalo, R., Chheda, S., Mei, F., Palkowetz, K. H., Rudloff, H. E., Schmalstieg, F. C., Rassin, D. K., & Goldman, A. S. (1995). Interleukin-10 in human milk. *Pediatric research, 37*(4), 444-449.

Georgeson, J. C., & Filteau, S. M. (2000). Physiology, immunology, and disease transmission in human breast milk. *AIDS patient care and STDs, 14*(10), 533-539.

Goldman, A. S., Chheda, S., Garofalo, R., & Schmalstieg, F. C. (1996). Cytokines in human milk: properties and potential effects upon the mammary gland and the neonate. *Journal of mammary gland biology and neoplasia, 1*(3), 251-258.

Gomo, E., Filteau, S. M., Tomkins, A. M., Ndhlovu, P., Michaelsen, K. F., & Friiss, H. (2003). Subclinical mastitis among HIV-infected and uninfected Zimbabwean women participating in a multimicronutrient supplementation trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 97*(2), 212-216.

Grasso, P. J., Scholz, R. W., Erskine, R. J., & Eberhart, R. J. (1990). Phagocytosis, bactericidal activity, and oxidative metabolism of milk neutrophils from dairy cows fed selenium-supplemented and selenium-deficient diets. *American journal of veterinary research, 51*(2), 269-274.

Groer, M. W., & Beckstead, J. W. (2011). Multidimensional scaling of multiplex data: human milk cytokines. *Biological Research for Nursing, 13*(3), 289-296.

Grönlund, U., Johannisson, A., & Persson Waller, K. (2006). Changes in blood and milk lymphocyte sub-populations during acute and chronic phases of *Staphylococcus aureus* induced bovine mastitis. *Research in veterinary science, 80*(2), 147-154.

Günther, J., Liu, S., Esch, K., Schuberth, H. M., & Seyfert, H. M. (2010). Stimulated expression of TNF-α and IL-8, but not of lingual antimicrobial peptide reflects the concentration of pathogens contacting bovine mammary epithelial cells. *Veterinary Immunology and Immunopathology, 135*(1), 152-157.

Hannan, M. A., Dogadkin, N. N., Ashur, I. A., & Markus, W. M. (2005). Copper, selenium, and zinc concentrations in human milk during the first three weeks of lactation. *Biological trace element research, 107*(1), 11-20.

Hawkes, J. S., Bryan, D. L., James, M. J., & Gibson, R. A. (1999). Cytokines (IL-1β, IL-6, TNF-α, TGF-β1, and TGF-β2) and prostaglandin E2 in human milk during the first three months postpartum. *Pediatric research, 46*(2), 194-199.

Heinrichs, A. J., Costello, S. S., & Jones, C. M. (2009). Control of heifer mastitis by nutrition. *Veterinary microbiology, 134*(1), 172-176.

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M., Beck, D. L., Abdo, Z., Fox, L. K., Williams, J. E., McGuire, M. K., & McGuire, M. A. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. *PLoS One, 6*(6), e21313.

Hunt, K. M., Williams, J. E., Shafii, B., Hunt, M. K., Behre, R., Ting, R., McGuire, M. K., & McGuire, M. A. (2013). Mastitis is associated with increased free fatty acids, somatic cell count, and interleukin-8 concentrations in human milk. *Breastfeeding Medicine, 8*(1), 105-110.

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition, 110*(7), 1253-1262.

Kantarci, S., Koulinska, I. N., Aboud, S., Fawzi, W. W., & Villamor, E. (2007). Subclinical mastitis, cell-associated HIV-1 shedding in breast milk, and breast-feeding transmission of HIV-1. *Journal of Acquired Immune Deficiency Syndromes, 46*(5), 651-654.

Kasonka, L., Makasa, M., Marshall, T., Chisenga, M., Sinkala, M., Chintu, C., Kaseba, C., Kasolo, F., Gitau, R., & Tomkins, A. (2006). Risk factors for subclinical mastitis among HIV ‐ infected and uninfected women in Lusaka, Zambia. *Paediatric and perinatal epidemiology, 20*(5), 379-391.

Kaufmann, R., & Foxman, B. (1991). Mastitis among lactating women: occurrence and risk factors. *Social Science & Medicine, 33*(6), 701-705.

Keane, O. M., Budd, K. E., Flynn, J., & McCoy, F. (2013). Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture. *Veterinary Record, 173*(11), 268.

Krachler, M., Shi Li, F., Rossipal, E., & Irgolic, K. J. (1998). Changes in the concentrations of trace elements in human milk during lactation. *Journal of trace elements in medicine and biology, 12*(3), 159-176.

Kudi, A. C., Bray, M. P., Niba, A. T., & Kalla, D. J. (2009). Mastitis causing pathogens within the dairy cattle environment. *International Journal of Biology, 1*(1), P3.

Kuehn, J. S., Gorden, P. J., Munro, D., Rong, R., Dong, Q., Plummer, P. J., Wang, C., & Phillips, G. J. (2013). Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. *PLoS One, 8*(4), e61959.

Latshaw, J. D. (1991). Nutrition—mechanisms of immunosuppression. *Veterinary Immunology and Immunopathology, 30*(1), 111-120.

Leitner, G., Merin, U., & Silanikove, N. (2004). Changes in milk composition as affected by subclinical mastitis in goats. *Journal of dairy science, 87*(6), 1719-1726.

Lemons, J. A., Moye, L., Hall, D., & Simmons, M. (1982). Differences in the composition of preterm and term human milk during early lactation. *Pediatric research, 16*(2), 113-117.

Leotsinidis, M., Alexopoulos, A., & Kostopoulou-Farri, E. (2005). Toxic and essential trace elements in human milk from Greek lactating women: association with dietary habits and other factors. *Chemosphere, 61*(2), 238-247.

Li, J., Zhou, H., Yuan, L., He, T., & Hu, S. (2009). Prevalence, genetic diversity, and antimicrobial susceptibility profiles of Staphylococcus aureus isolated from bovine mastitis in Zhejiang Province, China. *Journal of Zhejiang University SCIENCE B, 10*(10), 753-760.

Ling, B. (2011). *Drug/inflammation nutrient transport interaction in the lactating mother-neonate dyad.*

Lönnerdal, B. (2000). Regulation of mineral and trace elements in human milk: exogenous and endogenous factors. *Nutrition reviews, 58*(8), 223-229.

Lönnerdal, B., Zavaleta, N., Kusunoki, L., Lanata, C. F., Peerson, J. M., & Brown, K. H. (1996). Effect of postpartum maternal infection on proteins and trace elements in colostrum and early milk. *Acta Paediatrica, 85*(5), 537-542.

Luminex Corporation. (2010). The Luminex® 200 System. In Luminex Corporation (Ed.).
Lunney, K. M., Iliff, P., Mutasa, K., Ntozini, R., Magder, L. S., Moulton, L. H., & Humphrey, J. H. (2010). Associations between breast milk viral load, mastitis, exclusive breast-feeding, and postnatal transmission of HIV. *Clinical Infectious Diseases, 50*(5), 762-769.
Martín, R., Heilig, H., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., & Rodríguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Research in microbiology, 158*(1), 31-37.
Martineau, F., Picard, F. J., Ke, D., Paradis, S., Roy, P. H., Ouellette, M., & Bergeron, M. G. (2001). Development of a PCR assay for identification of staphylococci at genus and species levels. *Journal of clinical microbiology, 39*(7), 2541-2547.
Matos, C., Moutinho, C., Almeida, C., Guerra, A., & Balcão, V. (2014). Trace element compositional changes in human milk during the first four months of lactation. *International journal of food sciences and nutrition*(0), 1-5.
Matos, C., Moutinho, C., Balcão, V., Almeida, C., Ribeiro, M., Marques, A. F., & Guerra, A. (2009). Total antioxidant activity and trace elements in human milk: the first 4 months of breast-feeding. *European Food Research and Technology, 230*(2), 201-208.
McClellan, H. L., Miller, S. J., & Hartmann, P. E. (2008). Evolution of lactation: nutrition v. protection with special reference to five mammalian species. *Nutrition Research Reviews, 21*(2), 97-116.
Meglia, G. E., Johannisson, A., Petersson, L., & Persson Waller, K. (2001). Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. *Acta Veterinaria Scandinavica, 42*(1), 139-150.
Meki, A. R., Saleem, T., Al-Ghazali, M., & Sayed, A. (2003). Interleukins-6,-8 and-10 and tumor necrosis factor-alpha and its soluble receptor I in human milk at different periods of lactation. *Nutrition Research, 23*(7), 845-855.
Michie, C., Lockie, F., & Lynn, W. (2003). The challenge of mastitis. *Archives of disease in childhood, 88*(9), 818-821.
Michie, C., Tantscher, E., Schall, T., & Rot, A. (1998). Physiological secretion of chemokines in human breast milk. *European cytokine network, 9*(2), 123-129.
Mizuno, K., Hatsuno, M., Aikawa, K., Takeichi, H., Himi, T., Kaneko, A., Kodaira, K., Takahashi, H., & Itabashi, K. (2012). Mastitis is associated with IL-6 levels and milk fat globule size in breast milk. *Journal of Human Lactation, 28*(4), 529-534.
Morton, J. A. (1994). The clinical usefulness of breast milk sodium in the assessment of lactogenesis. *Pediatrics, 93*(5), 802-806.
Mulokozi, G., & Bilotta, S. (1999). Milk cytokines and subclinical breast inflammation in Tanzanian women: effects of dietary red palm oil or sunflower oil supplementation. *Immunology, 97*(4), 595-600.
Muñoz, C., Schlesinger, L., & Cavaillon, J. M. (1995). Interaction between cytokines, nutrition and infection. *Nutrition Research, 15*(12), 1815-1844.
Na, H. R., Daniels, L. C., & Seelig, L. L. (1997). Preliminary study of how alcohol consumption during pregnancy affects immune components in breast milk and blood of postpartum women. *Alcohol and alcoholism, 32*(5), 581-589.
Nakajima, Y., Mikami, O., Yoshioka, M., Motoi, Y., Ito, T., Ishikawa, Y., Fuse, M., Nakano, K., & Yasukawa, K. (1997). Elevated levels of tumor necrosis factor-α,(TNF-α) and interleukin-6 (IL-6) activities in the sera and milk of cows with naturally occurring coliform mastitis. *Research in veterinary science, 62*(3), 297-298.
Nakamori, M., Ninh, N. X., Isomura, H., Yoshiike, N., Hien, V. T. T., Nhug, B. T., Nhien, N. V., Nakano, T., Khan, N. C., & Yamamoto, S. (2009). Nutritional status of lactating mothers and

their breast milk concentration of iron, zinc and copper in rural Vietnam. *Journal of nutritional science and vitaminology, 55*(4), 338-345.

Neville, M. C., Allen, J. C., Archer, P. C., Casey, C. E., Seacat, J., Keller, R. P., Lutes, V., Rasbach, J., & Neifert, M. (1991). Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis. *American Journal of Clinical Nutrition, 54*(1), 81-92.

Nussenblatt, V., Lema, V., Kumwenda, N., Broadhead, R., Neville, M. C., Taha, T. E., & Semba, R. D. (2005). Epidemiology and microbiology of subclinical mastitis among HIV-infected women in Malawi. *International Jurnal of STD and AIDS, 16*(3), 227-232.

Osman, K. M., Hassan, H. M., Ibrahim, I. M., & Mikhail, M. (2010). The impact of staphylococcal mastitis on the level of milk IL-6, lysozyme and nitric oxide. *Comparative immunology, microbiology and infectious diseases, 33*(1), 85-93.

Osterman, K. L., & Rahm, V. A. (2000). Lactation mastitis: bacterial cultivation of breast milk, symptoms, treatment, and outcome. *Journal of Human Lactation, 16*(4), 297-302.

Overton, T. R., & Yasui, T. (2014). Practical applications of trace minerals for dairy cattle. *Journal of Animal Science, 92*(2), 416-426.

Oviedo-Boyso, J., Valdez-Alarcón, J. J., Cajero-Juárez, M., Ochoa-Zarzosa, A., López-Meza, J. E., Bravo-Patino, A., & Baizabal-Aguirre, V. M. (2007). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of infection, 54*(4), 399-409.

Persson Waller, K., Colditz, I. G., Lun, S., & Östensson, K. (2003). Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. *Research in veterinary science, 74*(1), 31-36.

Pyörälä, S. (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research, 34*(5), 565-578.

Rainard, P., & Riollet, C. (2006). Innate immunity of the bovine mammary gland. *Veterinary Research, 37*(3), 369-400.

Rambeaud, M., Almeida, R., Pighetti, G., & Oliver, S. (2003). Dynamics of leukocytes and cytokines during experimentally induced Streptococcus uberis mastitis. *Veterinary Immunology and Immunopathology, 96*(3), 193-205.

Rasmussen, L. B. W., Hansen, D. H., Kæstel, P., Michaelsen, K. F., Friis, H., & Larsen, T. (2008). Milk enzyme activities and subclinical mastitis among women in Guinea-Bissau. *Breastfeeding Medicine, 3*(4), 215-219.

Richards, A. A., Darboe, M. K., Tilling, K., Smith, G. D., Prentice, A. M., & Lawlor, D. A. (2010). Breast milk sodium content in rural Gambian women: between‐and within‐women variation in the first 6 months after delivery. *Paediatric and perinatal epidemiology, 24*(3), 255-261.

Rinaldi, M., Li, R. W., Bannerman, D. D., Daniels, K. M., Evock-Clover, C., Silva, M. V. B., Paape, M. J., Van Ryssen, B., Burvenich, C., & Capuco, A. V. (2010). A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to *E.coli* mastitis. *Functional and Integrative Genomics, 10*(1), 21-38.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. J. (2004). Development of an extensive set of 16S rDNA‐targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real‐time PCR. *Journal of applied microbiology, 97*(6), 1166-1177.

Rossipal, E., & Krachler, M. (1998). Pattern of trace elements in human milk during the course of lactation. *Nutrition Research, 18*(1), 11-24.

Rudloff, H. E., Schmalstieg, F. C., Mushtaha, A. A., Palkowetz, K. H., Liu, S. K., & Goldman, A. S. (1992). Tumor necrosis factor-α in human milk. *Pediatric research, 31*(1), 29-33.

Rudloff, H. E., Schmalstieg, F. C., Palkowetz, K. H., Paszkiewicz, E. J., & Goldman, A. S. (1993). Interleukin-6 in human milk. *Journal of reproductive immunology, 23*(1), 13-20.

Saito, S., Maruyama, M., Kato, Y., Moriyama, I., & Ichijo, M. (1991). Detection of IL-6 in human milk and its involvement in IgA production. *Journal of reproductive immunology, 20*(3), 267-276.

Salamon, S., & Csapó, J. (2009). Composition of the mother's milk: III. Macro and micro element contents. A review. *Acta Universitatis Sapientiae - Alimentaria, 2*(2), 235-275.

Salman, S., Khol-Parisini, A., Schafft, H., Lahrssen-Wiederholt, M., Hulan, H. W., Dinse, D., & Zentek, J. (2009). The role of dietary selenium in bovine mammary gland health and immune function. *Animal Health Research Reviews, 10*(01), 21-34.

Scaletti, R. W., Trammell, D. S., Smith, B. A., & Harmon, R. J. (2003). Role of dietary copper in enhancing resistance to *Escherichia coli m*astitis. *Journal of dairy science, 86*(4), 1240-1249.

Schramel, P., Lill, G., Hasse, S., & Klose, B. J. (1988). Mineral- and trace element concentrations in human breast milk, placenta, maternal blood, and the blood of the newborn. *Biological trace element research, 16*(1), 67-75.

Schukken, Y. H., Günther, J., Fitzpatrick, J., Fontaine, M. C., Goetze, L., Holst, O., Leigh, J., Petzl, W., Schuberth, H. J., & Sipka, A. (2011). Host-response patterns of intramammary infections in dairy cows. *Veterinary Immunology and Immunopathology, 144*(3), 270-289.

Semba, R. D. (2000). Mastitis and transmission of human immunodeficiency virus through breast milk. *Annals of the New York Academy of Sciences, 918*(1), 156-162.

Semba, R. D., Kumwenda, N., Hoover, D. R., Taha, T. E., Quinn, T. C., Mtimavalye, L., Biggar, R. J., Broadhead, R., Miotti, P. G., & Sokoll, L. J. (1999). Human immunodeficiency virus load in breast milk, mastitis, and mother-to-child transmission of human immunodeficiency virus type 1. *Journal of Infectious Diseases, 180*(1), 93-98.

Semba, R. D., Kumwenda, N., Taha, T. E., Hoover, D. R., Quinn, T. C., Lan, Y., Mtimavalye, L., Broadhead, R., Miotti, P. G., & van der Hoeven, L. (1999). Mastitis and immunological factors in breast milk of human immunodeficiency virus-infected women. *Journal of Human Lactation, 15*(4), 301-306.

Semba, R. D., & Neville, M. C. (1999). Breast‐feeding, mastitis, and HIV transmission: Nutritional implications. *Nutrition reviews, 57*(5), 146-153.

Silvestre, D., Martìnez-Costa, C., Lagarda, M. J., Brines, J., Farré, R., & Clemente, G. (2001). Copper, iron, and zinc contents in human milk during the first three months of lactation. *Biological trace element research, 80*(1), 1-11.

Slebodziński, A. B., Malinowski, E., & Lipczak, W. (2002). Concentrations of triiodothyronine (T3), tumour necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) in milk from healthy and naturally infected quarters of cows. *Research in veterinary science, 72*(1), 17-21.

Smith, K. L., Hogan, J. S., & Weiss, W. P. (1997). Dietary vitamin E and selenium affect mastitis and milk quality. *Journal of Animal Science, 75*(6), 1659-1665.

Sone, S., Tsutsumi, H., Takeuchi, R., Matsuda, K., Imai, S., Ogra, P. L., & Chiba, S. (1997). Enhanced cytokine production by milk macrophages following infection with respiratory syncytial virus. *Journal of leukocyte biology, 61*(5), 630-636.

Sordillo, L. M. (2005). Factors affecting mammary gland immunity and mastitis susceptibility. *Livestock Production Science, 98*(1), 89-99.

Sordillo, L. M. (2011). New concepts in the causes and control of mastitis. *Journal of mammary gland biology and neoplasia, 16*(4), 271-273.

Sordillo, L. M. (2013). Selenium-dependent regulation of oxidative stress and immunity in periparturient dairy cattle. *Veterinary medicine international, 2013*.

Sordillo, L. M., Pighetti, G. M., & Davis, M. R. (1995). Enhanced production of bovine tumor necrosis factor-α during the periparturient period. *Veterinary Immunology and Immunopathology, 49*(3), 263-270.

Sordillo, L. M., & Streicher, K. L. (2002). Mammary gland immunity and mastitis susceptibility. *Journal of mammary gland biology and neoplasia, 7*(2), 135-146.

Sorg, D., Danowski, K., Korenkova, V., Rusnakova, V., Küffner, R., Zimmer, R., Meyer, H. H. D., & Kliem, H. (2012). Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens. *animal, 1*(1), 1-7.

Spain, J. (1993). *Tissue integrity: A key defense against mastitis infection: The role of zinc proteinates and a theory for mode of action.* Paper presented at the Proc. of Alltech's 9th Ann. Symp., 53-60. p.

Spears, J. W., & Weiss, W. P. (2008). Role of antioxidants and trace elements in health and immunity of transition dairy cows. *The Veterinary Journal, 176*(1), 70-76.

Srivastava, M. D., Srivastava, A., Brouhard, B., Saneto, R., Groh-Wargo, S., & Kubit, J. (1996). Cytokines in human milk. *Research communications in molecular pathology and pharmacology, 93*(3), 263-287.

Taponen, S., Salmikivi, L., Simojoki, H., Koskinen, M. T., & Pyörälä, S. (2009). Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *Journal of dairy science, 92*(6), 2610-2617.

Thomsen, A. C., Espersen, T., & Maigaard, S. (1984). Course and treatment of milk stasis, noninfectious inflammation of the breast, and infectious mastitis in nursing women. *American journal of obstetrics and gynecology, 149*(5), 492-495.

Ustundag, B., Yilmaz, E., Dogan, Y., Akarsu, S., Canatan, H., Halifeoglu, I., Cikim, G., & Aygun, A. D. (2005). Levels of cytokines (IL-1 β, IL-2, IL-6, IL-8, TNF-α) and trace elements (Zn, Cu) in breast milk from mothers of preterm and term Infants. *Mediators of inflammation, 2005*(6), 331-336.

Vaughan, L. A., Weber, C. W., & Kemberling, S. R. (1979). Longitudinal changes in the mineral content of human milk. *American Journal of Clinical Nutrition, 32*(11), 2301-2306.

Vels, L., Røntved, C. M., Bjerring, M., & Ingvartsen, K. L. (2009). Cytokine and acute phase protein gene expression in repeated liver biopsies of dairy cows with a lipopolysaccharide-induced mastitis. *Journal of dairy science, 92*(3), 922-934.

Vogel, A., Hutchison, B. L., & Mitchell, E. A. (1999). Mastitis in the first year postpartum. *Birth, 26*(4), 218-225.

Wasowicz, W., Gromadzinska, J., Szram, K., Rydzynski, K., Cieslak, J., & Pietrzak, z. (2001). Selenium, zinc, and copper concentrations in the blood and milk of lactating women. *Biological trace element research, 79*(3), 221-233.

Wegner, T. N., & Stull, J. W. (1978). Relation between mastitis test score, mineral composition of milk, and blood electrolyte profiles in Holstein cows. *Journal of dairy science, 61*(12), 1755-1759.

Weiss, W. P., & Spears, J. W. (2006). Vitamin and trace mineral effects on immune function of ruminants *Ruminant Physiology* (pp. 473-496). Utrecht, The Netherlands: Wageningen Academic.

Wenz, J. R., Fox, L. K., Muller, F. J., Rinaldi, M., Zeng, R., & Bannerman, D. D. (2010). Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis. *Journal of dairy science, 93*(6), 2458-2470.

WHO. (2000). Mastitis: causes and management. *Geneva: World Health Organization*.

Willumsen, J. F., Filteau, S. M., Coutsoudis, A., Uebel, K. E., Newell, M. L., & Tomkins, A. M. (2002). Subclinical mastitis as a risk factor for mother-infant HIV transmission *Short and Long Term Effects of Breast Feeding on Child Health* (pp. 211-223): Springer.

Wintergerst, E. S., Maggini, S., & Hornig, D. H. (2007). Contribution of selected vitamins and trace elements to immune function. *Annals of Nutrition and Metabolism, 51*(4), 301-323.

Wöckel, A., Abou-Dakn, M., Beggel, A., & Arck, P. (2008). Inflammatory breast diseases during lactation: health effects on the newborn-a literature review. *Mediators of inflammation, 2008*.

Yamawaki, N., Yamada, M., Kan-no, T., Kojima, T., Kaneko, T., & Yonekubo, A. (2005). Macronutrient, mineral and trace element composition of breast milk from Japanese women. *Journal of trace elements in medicine and biology, 19*(2), 171-181.

Yang, W., Zerbe, H., Petzl, W., Brunner, R. M., Günther, J., Draing, C., von Aulock, S., Schuberth, H. J., & Seyfert, H. M. (2008). Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E.coli,* but *S.aureus* fails to both activate NF-ϰB in mammary epithelial cells and to quickly induce TNFα and interleukin-8 (CXCL8) expression in the udder. *Molecular immunology, 45*(5), 1385-1397.

Zhu, Y., Berg, M., Fossum, C., & Magnusson, U. (2007). Proinflammatory cytokine mRNA expression in mammary tissue of sows following intramammary inoculation with *Escherichia coli*. *Veterinary Immunology and Immunopathology, 116*(1), 98-103.