Subclinical Mastitis and Infant Growth in The Western Highlands of Guatemala

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Abstract

Three studies were designed to explore: (i) cultural determinants of optimal breastfeeding practices among Indigenous *Mam*-Mayan women in the Western Highlands of Guatemala, (ii) the relationship between subclinical mastitis and impaired infant anthropometric indicators and (iii) the role of infection, inflammation, hygiene and lactation practices in underscoring the complex relationship between subclinical mastitis and infant growth.

Manuscript 1 explored whether cultural influences were associated with achievement of the World Health Organization infant feeding recommendations and if these cultural practices served as moderators of the relationship between breastfeeding practices and infant weight-for-age (WAZ). Mothers who delivered at the traditional midwife's house and those that did not believe in the transmission of *susto* (fright) through breast milk were more likely to initiate breastfeeding within 1 hour postpartum. Higher breastfeeding frequency was observed among mothers who spent more time in the *temascal* (sauna). Initiating breastfeeding within 1 hour postpartum was the sole infant feeding practice positively associated with exclusive breastfeeding and infant WAZ. This work highlighted that health practitioners need to understand how local cultural practices influence early initiation of breastfeeding to promote adequate infant weight.

Manuscript 2 compared mothers with SCM (breast milk Na/K ratio >0.6) and without SCM for selected factors and practices and evaluated if SCM increased the likelihood of infant stunting (LAZ<-2SD), underweight (WAZ<-2SD) and microcephaly (HCZ<-2SD) in a cross sectional study. Older maternal age, higher parity, higher breastfeeding frequency, and increased hours of maternal daily walking characterized mothers with SCM. In a multiple logistic regression, the presence of maternal stool *Entamoeba coli* increased the odds of infant

microcephaly. Importantly, SCM increased the likelihood of infant stunting, underweight and microcephaly. Given the demonstrated associations between SCM and anthropometric indicators of poor infant growth, improving maternal breast health may be required to improve infant growth.

Manuscript 3 explored the prevalence and determinants of SCM at 0-6 weeks (early) and 4-6 months (established) in a longitudinal cohort of mothers and investigated the contribution of infection, inflammation, mammary gland permeability, and growth factors present in milk to infant WAZ, LAZ, and HCZ at both stages of lactation. Prevalence of SCM (Na/K ratio >0.6) differed between early and established stages of lactation demonstrating that SCM did not persist. Determinants of SCM at 0-6 wks were lack of home faucet and parity and at 4-6 mos were lack of home faucet, lower feeding frequency, and higher early milk Na/K ratio. In early lactation, increased mammary gland permeability and maternal stool *B.hominis* impaired anthropometric measurements WAZ and HCZ whereas leukocytes in urine and the pro-inflammatory cytokine IL-6 in milk were associated with increased LAZ and HCZ, respectively. In established lactation, maternal stool *E.coli* impaired WAZ and LAZ, milk EGF was negatively associated with LAZ and HCZ whereas milk pro-inflammatory cytokines TNF-□ and IL-6 were associated with increased WAZ and HCZ. This study suggests that breast milk may be a pathway through which non-genetic transmission of growth faltering is passed although an inflammatory response in the mammary gland may provide a measure of protection to the infant.

Taken together, these studies re-enforce the promotion of optimal breastfeeding practices among Indigenous *Mam*-Mayan mothers and but caution health practitioners not to underestimate subclinical mastitis and the importance of breast milk as a vehicle for transmission of poor growth.

Résumé

Trois études ont été conçues pour explorer : (i) les déterminants culturels des pratiques optimales d'allaitement maternel chez les femmes Mam-mayas indigènes des hauts plateaux occidentaux du Guatemala, (ii) la relation entre la mammite subclinique et l'altération des indicateurs anthropométriques du nourrissons et (iii) le rôle des infections, de l'inflammation, de l'hygiène et des pratiques d'allaitement en soulignant la relation complexe entre la mammite subclinique et la croissance du nourrisson.

La manuscrit 1 a examiné si des influences culturelles étaient associées à l'atteinte des recommandations d'alimentation du nourrisson de l'Organisation mondiale de la santé et si ces pratiques culturelles servaient de modérateurs de la relation entre les pratiques d'allaitement maternel et le score du poids-pour-âge du nourrisson. Les mères qui ont accouché à la maison de la sage-femme traditionnelle et celles qui ne croyaient pas à la transmission de *susto* (peur) par le lait maternel étaient plus susceptibles de commencer l'allaitement dans l'heure qui suit l'accouchement. Une fréquence plus élevée d'allaitement maternel a été observé chez les mères qui ont passé plus de temps dans le *temascal* (sauna). L'initiation de l'allaitement maternel dans l'heure qui suit l'accouchement était la seule pratique de l'alimentation infantile associée positivement à l'allaitement maternel exclusif et au score du poids-pour-âge du nourrisson. Ce travail a mis en évidence que les agents de la santé ont besoin de comprendre comment les pratiques culturelles locales influencent l'initiation précoce de l'allaitement pour promouvoir un poids adéquat du nourrisson.

Le manuscrit 2 a comparé les mères avec MSC (ratio Na/K du lait maternel > 0,6) et celles sans MSC pour certains facteurs et pratiques et a évalué si la MSC augmentait la probabilité de retard de croissance infantile (TPA< -2ET), d'insuffisance pondérale (PPA< -2ET)

et de microcéphalie (PC < -2ET) dans une étude transversale. L'âge avancé de la mère, la parité élevée, la fréquence d'allaitement maternel élevée et l'augmentation des heures de marche quotidienne maternelle caractérisent les mères avec MSC. Dans une régression logistique multiple, la présence dans les selles maternelles d'*Entamoeba coli* augmente les risques de microcéphalie infantile. Surtout, la MSC augmente la probabilité de retard de croissance du nourrisson, d'insuffisance pondérale et de microcéphalie. Compte tenu de l'association démontrée entre la MSC et les indicateurs anthropométriques d'une faible croissance du nourrisson, l'amélioration de la santé du sein maternelle pourrait être nécessaire pour améliorer la croissance du nourrisson.

Le manuscrit 3 a exploré la prévalence et les déterminants de la MSC à 0-6 semaines et 4-6 mois dans une cohorte longitudinale de mères et a étudié la contribution des infections, de l'inflammation, de la perméabilité de la glande mammaire et des facteurs de croissance présents dans le lait au PPA, à la TPA et au PC du nourrisson à deux stades de la lactation. La prévalence de la MSC (ratio Na/K> 0,6) diffère entre les stades précoce et établie de lactation; ce qui démontre que la MSC n'est pas persistante. Les déterminants de la MSC à 0-6 semaines étaient le manque d'un robinet dans la maison et la parité et à 4-6 mois étaient le manque d'un robinet dans la maison, la faible fréquence d'allaitement et un ratio Na/K élevé dans le lait précoce, à 0-6 semaine**.** Pendant la lactation précoce, entre 0-6 semaines, la perméabilité accrue de la glande mammaire et la présence de *B. hominis* dans les selles maternelles ont affectés les mesures anthropométriques PPA et PC; tandis que les leucocytes dans l'urine et la cytokine pro-inflammatoire IL-6 dans le lait étaient associés à une augmentation de la TPA et du PC respectivement. Pendant la lactation entre 4-6 mois, la présence d'*E. coli* dans les selles maternelles a altéré le PPA et la TPA; le FCE du lait était négativement associé à la TPA et au

PC tandis que les cytokines pro-inflammatoires TNF-α et de l'IL-6 du lait étaient associés à une augmentation du PPA et du PC. Cette étude suggère que le lait maternel est une voie non génétique de la transmission de la croissance altérée bien qu'une réponse inflammatoire dans la glande mammaire pourrait fournir une mesure de protection pour le nourrisson.

Pris ensemble, ces études renforcent la promotion des pratiques optimales d'allaitement chez les mères Mam-mayas indigènes et approuvent les pratiques culturelles pendant l'allaitement; mais avertis les professionnels de la santé de ne pas sous-estimer la mammite subclinique et l'importance du lait maternel comme un vecteur pour la transmission de la faible croissance.

Abréviations : **ET**: Ecart-type; **FCE**: Facteur de croissance épidermal; **MSC**: Mammite subclinique; **PC**: Périmètre crânien; **PPA**: Poids-pour-l'âge; **TPA**: Taille-pour-l'âge

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Contribution of Authors

This thesis is written in manuscript format according to the Guidelines Concerning Thesis Preparation stipulated by McGill University. Hilary M. Wren wrote this thesis.

I, Hilary Wren, was responsible for study design, questionnaire development for all human milk and lactation questions, breast milk sample collection, breast milk sample laboratory analysis using ICP-MS (Na, K) and Luminex (pro-inflammatory cytokines, growth factors), statistical analysis of the data and data interpretation within the context of available literature. In collaboration with CeSSIAM, I was responsible for all lactation research in Guatemala during the pilot, cross sectional and longitudinal studies. The thesis contains three manuscripts, all of which were co-authored and developed in collaboration with my academic advisors, Dr. Kristine G. Koski and Dr. Marilyn E. Scott. In addition, all three manuscripts are co-authored with my field supervisor from CeSSIAM in Guatemala, Dr. Noel W. Solomons. Dr. Anne Marie Chomat co-authored the first manuscript (Chapter III) as she participated in data collection and supervision in Guatemala. The first manuscript (Chapter III) is published; the second manuscript (Chapter IV) and the third manuscript (Chapter V) will be submitted in the near future. Permission to reproduce the manuscripts in this thesis has been granted by all co-authors. McGill students Chen Li, Andreanne Leblanc and Ailee Shinoki assisted laboratory analysis. Dr. M. E. Scott and Dr. K. G. Koski served as thesis supervisors, provided financial resources for the laboratory work, and pre-edited all written manuscripts and this thesis.

Statement of Originality

1. Early initiation of breastfeeding was delayed by use of *agüitas,* by beliefs surrounding transmission of *susto* and was enhanced by delivery at the *comadrona's* house.
2. The cultural practice of using a *temascal* was associated with higher breastfeeding frequency, which is consistent with indigenous mothers' perception that the *temascal* stimulates milk flow. The potential benefit of applying heat to the breast to stimulate milk flow is also biologically plausible.
3. Initiating early breastfeeding within one hour postpartum was the sole infant feeding practice positively associated with exclusive breastfeeding and WAZ. Given, that the rationale by WHO to initiate breastfeeding within one hour postpartum does not explicitly address infant growth, our novel finding should be considered in order to promote adequate infant weight.
4. In a cross sectional study, fourteen percent of all mothers had SCM (Na/K ratio >0.6) and as expected, the mean breast milk Na/K ratio was higher for mothers with SCM compared to mothers without SCM. This indicator did not differ between transitional (4-17d) and early milk (18-46d).
5. Older maternal age, higher parity, higher breastfeeding frequency, and increased hours of maternal daily walking characterized mothers with a breast milk Na/K ratio >0.6. Cultural practices, deeply embedded in Guatemalan culture, including the feeding of *agüitas* (ritual fluids) and frequency and duration of *temascal* (sauna) use did not differ between mothers with and without SCM.

6. SCM was associated with higher odds of infant stunting, whereas increasing maternal height was associated with a decreased likelihood of stunting. Although the association between poor maternal height and stunting is well known, SCM may need to be added to a list of emerging subclinical conditions contributing to stunting.
7. SCM emerged as the only factor to increase the likelihood of infant underweight.
8. SCM and non-pathogenic *Entamoeba coli* were associated with infant microcephaly. It is not inconceivable that fecal contamination of the breast through poor sanitation could put the infant in direct contact with exposure to non-pathogenic protozoa during breastfeeding.
9. In a longitudinal study, SCM was associated with impaired infant WAZ and HCZ during 0-6 weeks postpartum. This finding suggests that increased mammary gland permeability and the opening of tight junctions may decrease milk volume and/or change milk composition to compromise anthropometry.
10. Interestingly, biomarkers of inflammation in breast milk including pro-inflammatory cytokines IL-6 and TNF-α were positively associated with infant HCZ and WAZ respectively. This finding suggests that an inflammatory response, which results in higher concentrations of pro-inflammatory cytokines in milk, may provide a measure of protection to the infant.
11. Breast milk EGF was negatively associated with LAZ and HCZ during established lactation. If milk EGF is sequestered in the mammary gland and not reaching the infant, the lack of delivery to the infant could impact the development of the intestinal function and contribute to increased susceptibility to poor growth.

12. Compromised infant anthropometry was associated with higher rates of non-pathogenic protozoa in maternal stool. Specifically, maternal stool *B.hominis* was negatively associated with infant WAZ and HCZ during early postpartum, whereas, *E.coli* was negatively associated with WAZ and LAZ during established. These finding cautions health practitioners against underestimating the importance of breast milk as a vehicle for transmission of poor anthropometry and highlights the complexity of the infection-inflammation paradigm as it relates to milk composition.

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
AGA	Appropriate-for-Gestational Age
ANOVA	Analysis of Variance
CeSSIAM	Center for Studies of Sensory Impairment, Aging and Metabolism
CRH	Corticotrophin-Releasing Hormone
d	Day
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Early Milk
GA	Gestational Age
GC	Glucocorticoid
GD	Gestational Day
GH	Growth Hormone
HCZ	Head Circumference z-score
HPA	Hypothalamus-Pituitary-Adrenal
HIV	Human Immunodeficiency Virus
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IGF	Insulin-like Growth Factor
IL	Interleukin
JHL	Journal of Human Lactation

LAZ Length-for-Age Z-score

LMP Last Menstrual Period

LOD Limit of Detection

MM Mature Milk

Na/K Sodium: Potassium ratio

NEC Necrotizing Enterocolitis

PBF Predominant Breastfeeding

PBS Phosphate Buffered Saline

RIA Radioimmunoassay

SCC Somatic Cell Count

SCM Subclinical Mastitis

SD Standard Deviation

SGA Small for Gestational Age

sIgA Secretory Immunoglobulin A

SRM Standard Reference Material

TM Transitional Milk

TNF-α Tumour Necrosis Factor Alpha

VEGF Vascular Endothelial Growth Factor

WAZ Weight-for-Age Z-score

WHO World Health Organization

WLZ Weight-for-Length Z-score

CHAPTER I

Introduction

Background

Subclinical mastitis (SCM) is a common asymptomatic inflammatory condition of the lactating mammary gland (Filteau et al., 1999; Lunney et al., 2010; Willumsen et al., 2002) which has only recently been introduced to human lactation literature due to its association with elevated risk of mother-to-child transmission of HIV through breast milk (Gomo et al., 2003; Kantarci et al., 2007; Kasonka et al., 2006) and association with infant growth faltering (Aryeetey et al., 2009; Filteau et al., 1999; Gomo et al., 2003; Morton, 1994). In the dairy industry, SCM has been extensively studied because of its economic importance as cows with SCM have a compromised quality and quantity of milk (Bannerman, 2009; Batavani et al., 2007; Bruckmaier et al., 2004). However, human studies on SCM are scarce making SCM an under-researched problem in public health.

SCM is understood to be more prevalent worldwide than *clinical* mastitis, which is usually under 10% in most populations (WHO, 2000). Reported rates of SCM range from 2% to 66% from lactating women in African countries (Arsenault et al., 2010; Nussenblatt et al., 2005; Semba & Neville, 1999). The wide variation in rates occurs because there is no “gold standard” definition of SCM, different assay methods have been used to diagnosis SCM and many women studied with SCM are also HIV-infected which may impact the inflammatory condition to an unknown magnitude. Although there is no standard definition of SCM in human lactation literature, breast milk Na/K ratio has been used by researchers as a diagnostic biomarker for SCM (Arsenault et al., 2010; Aryeetey et al., 2009; Filteau et al., 1999; Lunney et al., 2010; Rasmussen et al., 2008; Richards et al., 2010).

Currently, research suggests that SCM is most prevalent during early lactation and occurs for several reasons including milk stasis, physical breast tissue trauma, micronutrient deficiency, and poor lactation practices by the mother-infant dyad (Abou-Dakn et al., 2010; Dorosko, 2005; Filteau et al., 1999; Kasonka et al., 2006; Neville et al., 1991; WHO, 2000). Milk stasis and infection are the two main causes of *clinical* mastitis (WHO, 2000). On one hand, milk stasis can increase the risk of bacterial infection in the mammary gland from stagnated milk (Sordillo, 2002; WHO, 2000). The most common bacterium associated with SCM and *clinical* mastitis are *Staphylococcus aureus* and *Escherichia coli* (Collado et al., 2009; Delgado et al., 2008). On the other hand, milk stasis increases luminal pressure in the mammary epithelium, which directly contributes to the opening of tight junctions in the mammary gland (Nguyen & Neville, 1998).

Milk stasis, a dominant risk factor for the development of SCM, may result from delayed breastfeeding initiation, decreased breastfeeding frequency, and decreased breastfeeding duration. The World Health Organization (WHO) currently recommends that infants less than six months be exclusively breastfed with no other solids or liquids with subsequent introduction of complementary feeding starting around the six month of life (WHO, 2003). There is also concordance within the international pediatric community that mothers should breastfeed their infants 8-12 times per 24 hours to ensure adequate removal of milk from mammary ducts (AAP, 2012; Kramer, 2009; WHO, 2003). In the dairy industry, once daily milking of cows can reduce labor costs, however, it is not a common practice as it leads to lower milk yield (Holmes et al., 1992). Studies in cows have shown that the accumulation of milk, or milk stasis, is associated with a decline in milk secretion (Nguyen & Neville, 1998) and that tight junction permeability in cows increased after only 18 hours of milk stasis (Stelwagen et al., 1997).

In human literature, an older study found that junctional complexes likely start to open when milk volume falls below 400 mL/day (Neville et al., 1991). The study observed a significant increase in sodium, chloride, and protein and a decrease in lactose concentration in milk only when milk volume had fallen bellow 400 mL/day, consistent with opening of the paracellular pathway. This finding was similar with the observation of Garza et al. (1983), which demonstrated that a 66% reduction in feeding frequency was associated with a significant rise in milk sodium, although volume was not measure in that study. High sodium concentrations in milk, associated with increased mammary gland permeability, were also found in an older study in women with low milk volumes (Dewey et al., 1984). A reduction in the frequency of feeds may thus predispose mothers to milk stasis and/or engorgement, both of which may increase the risk of SCM (WHO, 2000).

During SCM, tight epithelial junctions become “leaky”, allowing movement of plasma constituents, including sodium, into milk (Contreras et al., 2011;Filteau et al., 1999). The term “subclinical” is thus used to describe those changes that occur in breast milk in the absence of external changes to the breast. This compares to *clinical* mastitis, which can easily be diagnosed by visual assessment of the breast due to its clinical features - redness, increased gland size, hardness – and often is accompanied by systemic illness including flu-like symptoms, hyperemia, pain, and fever (WHO, 2000). The challenge of diagnosing SCM is therefore the asymptomatic nature of the condition, however, recent findings have demonstrated that SCM is accompanied by the presence of the inflammatory cytokine interleukin-8 (IL-8) (Filteau et al., 1999; Rasmussen et al., 2008; Semba et al., 1999; Willumsen et al., 2000) suggesting a “true” inflammatory condition. This complements research from dairy literature where cytokines, as intercellular mediators, have been identified in infected bovine mammary glands (Wenz et al., 2010).

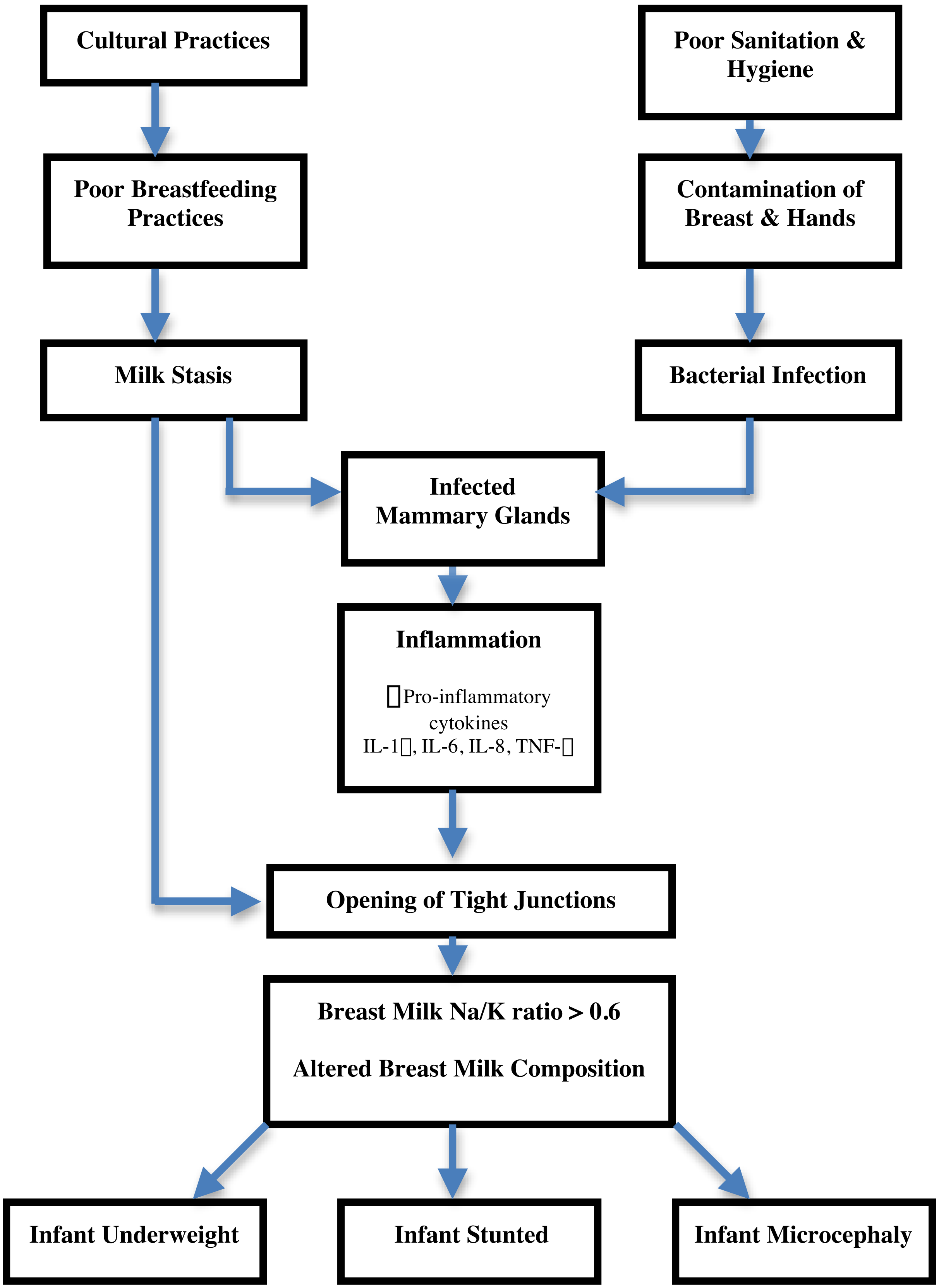
An inflammatory reaction that increases permeability may be an important factor in the process as it allows immune components to reach the infection site in the breast (Nguyen et al., 1998). However, SCM mediated changes in human milk composition as a result of inflammation is an under-researched problem in public health. One study found that increased mammary permeability, defined as an elevated Na/K ratio, was associated with higher concentrations of immune factors including sIgA (Filteau et al., 1999). Yet, there is relatively little evidence on the impact of inflammation on infant health outcomes. Inflammation may impact bone regulation specifically inhibiting bone formation through decreased insulin-like growth factor 1 (IGF-1) levels and inducing bone resorption as demonstrated in animal models (Odiere et al., 2010; Odiere et al., 2013). Specifically, pups of *H.bakeri* infected dams had higher levels of IL-1beta and these pups had lower levels of IGF-1 (Odiere, 2010). IGF-1 is a hormone synthesized by the liver that mediates the effects of growth hormone (GH) by stimulating proliferation of chondrocytes at the epiphyseal growth plate (Jones et al., 2015). Inflammatory cytokines may impair linear growth through induction of osteoclast differentiation, proliferation and activity and inhibition of oseteoblast function (Gao et al., 2007; Odiere et al., 2010). In human literature, one recent study in Zimbabwe found that inflammatory markers were higher in stunted children than in non-stunted children from 6 wk of age and were associated with lower concentrations of IGF-1 throughout infancy (Prendergast et al., 2014).

Inflammation that arises from infection is of concern given strong existing evidence that underlying infections during childhood directly underscore growth faltering (de Onis et al., 2013; Dewey & Mayers, 2011). Infants with infections suffer from a decrease in nutrient intake and increase in nutrient loss due to decreased intestinal absorption (de Onis et al., 2013; Salam et al., 2015). Increased diarrhea, as a result of infection, further leads to direct nutrient losses from the body, including micronutrients such as zinc and vitamin A from the intestine or urinary tract (Palmer, 2011). On the other hand, it has recently been proposed that poor sanitation and hygiene, and not diarrhea *per se*, is a primary cause of infant undernutrition (Humphrey, 2010). Fecal contamination of the maternal breast and infant hands during breastfeeding can lead to increased fecal ingestion by infants via breast milk. High pathogenic bacteria ingestion may overwhelm the small-intestinal lumen, leading to increased intestinal permeability and altered gut barrier function, promoting microbial translocation and increasing the risk of infant undernutrition (Dewey & Mayers, 2011;Humphrey, 2010).

Conceptual Framework

Based on the literature, a conceptual framework was proposed in order to explore the complex relationship between SCM and infant growth in Guatemala (Figure 1-1). Both milk stasis and infection may increase the odds of SCM. The elevated inflammatory reaction in the breast and cytokine production during SCM can promote the opening of tight junctions in mammary epithelial cells, leading to altered milk composition, and impact on infant growth.

Figure 1-1: A conceptual framework for the complex relationship between SCM, milk composition and infant growth



Rationale, Research Context and Objectives

SCM remains an under-researched problem in public health. In contrast to dairy literature, where SCM is extensively studied, gaps in our current understanding of this asymptomatic inflammatory condition exist in human lactation literature. Given that the condition may underscore early cessation of breastfeeding and that the effects of SCM are not confined to the maternal system but may be evident in the offspring through reduced weight and length for age (Aryeetey et al., 2009; Kasonka et al., 2006; Filteau et al., 1999; Gomo et al., 2003; Morton, 1994) it is critical to understand this breast condition and how public health practitioners may be able to construct and scale up interventions aimed the prevention of SCM.

In Guatemala, exclusive breastfeeding is rare and early introduction of *agüitas* (ritual fluids) is associated with a higher risk of infant stunting (Doak et al., 2013). Among Indigenous communities, the feeding of *agüitas* represents one of the first disruptions in exclusive breastfeeding to infants less than six months of age (Campos et al., 2010; Doak et al., 2013). It is unknown if the replacement of necessary breast milk with *agüitas* may underscore milk stasis via a reduction in the frequency of feeds, which could increase the risk of SCM. In addition, previous literature has suggested that the period of lactation is a "cold" condition, and many Indigenous women use a *temascal* (sauna) to restore balance (i.e. warm up a cold condition) (Adams et al., 2007; Cominsky, 1980). Lactating women use *temascals* because of their perceived ability to increase milk flow when experiencing milk stasis. This cultural practice may be grounded in physiology as it has been shown that direct application of heat to the breast, increases blood flow in capillaries and may unplug clogged mammary ducts (Walker, 2008) thus suggesting that heat from the *temascal* may be beneficial to milk stasis. On the other hand, use of *temascals* immediately postpartum may delay initiation of breastfeeding and prolonged time periods in the *temascal* may reduce the frequency of feedings, both of which may increase milk stasis and the risk of SCM.

The relationship between poor lactation practices and impaired growth is of interest given that Guatemala has the highest prevalence of stunting in Latin America and amongst the ten worst worldwide (MSPAS, 2010). The population of interest was the Indigenous *Mam*-Mayan of rural Guatemala where, in some communities, over 80% of children under five years of age are stunted (MSPAS, 2010). To date, research in Guatemala has largely focused on the nutritional status of breastfeeding mothers as contributing to poor infant growth (Frojo et al., 2012; Oyesiku et al., 2013). Although, recently a broad range of factors including psychosocial stressors as possible determinants of infant growth were evaluated, demonstrating a more complex web of intersecting factors than previously understood (Chomat et al., 2015).

The first six months postpartum, when an infant is most dependent on her mother for nutrition via exclusive breastfeeding (WHO, 2003), is an opportune period of time that deserves extra attention yet remains under researched among Indigenous *Mam*-Mayan communities. Therefore, it was plausible to propose that poor lactation practices underscoring SCM may be a risk factor for growth faltering in this population although it had not yet been investigated. The goals of this dissertation were as follows:

In Manuscript 1, the objective was to identify culture-specific factors that may act as potential barriers to optimal breastfeeding practices (i.e. decrease in feeding frequency, decrease in exclusiveness, and/or delayed initiation). In Manuscript 2, using a cross sectional study design, the objectives were to (i) compare mothers with SCM (breast milk Na/K ratio >0.6) and without SCM for cultural practices and other selected factors and (ii) to evaluate if SCM increased the likelihood of infant stunting, underweight and/or microcephaly. In Manuscript 3, using a longitudinal study design, the objectives were to (i) describe the prevalence of SCM at two stages of lactation, (ii) identify the determinants of SCM, and (iii) investigate the contribution of maternal stool non-pathogenic protozoa, breast inflammation and increased mammary gland permeability, and growth factors present in milk to infant weight-for-age (WAZ), height-for-age (LAZ), and head-circumference-for-age (HCZ) at both stages of lactation.

The findings from Manuscripts 1-3 will add substantially to our understanding of SCM and add to a growing body of literature on the complex interaction between nutrition, infection, and inflammation. As such, a comprehensive assessment concerning the influence of SCM, together with factors that underscore optimal breastfeeding and its impact on milk composition, will provide a better understanding of the importance of breast milk as a vehicle for transmission of factors in milk that may contribute to poor growth. This research could be used to identify public health interventions aimed at the prevention of SCM, which could have a profound impact on infant growth.

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CHAPTER II

Review of the Literature

1.0 Description of Subclinical Mastitis (SCM)

SCM is an asymptomatic inflammatory condition of the lactating breast (WHO, 2000). Recent curiosity with human SCM has developed because of its adverse association with an increased risk of mother-to-child transmission of HIV (Semba et al., 1999; Willumsen et al., 2003; Nussenblatt et al., 2005; Kantarci et al., 2007; Lunney et al., 2010). During SCM, tight epithelial junctions become “leaky”, allowing movement of plasma constituents, including HIV RNA, leucocytes, and sodium, into breast milk (Dorosko, 2005; Contreras et al., 2011). The term “subclinical” is thus used to describe changes that might occur in breast milk in the absence of external changes to the breast. This compares to *clinical* mastitis, which can be easily diagnosed by visual assessment of the breast due to its clinical features - redness, increased gland size, hardness – and is often accompanied by systemic illness including flu-like symptoms, hyperemia, pain, and fever (WHO, 2000). The challenge of diagnosing SCM is therefore the asymptomatic nature of the condition.

Drawing from our understanding in cow literature, elevated somatic cell counts (SCC) in milk, higher than 200,000 cells/mL, is the “gold standard” by which to diagnosis SCM (Contreras et al., 2011). Somatic cells are milk-secreting epithelial cells that have been shed from the lining of the mammary gland and white blood cells (leukocytes) that have entered the mammary gland in response to infection (Sharma et al., 2011). Few studies have implicated elevated cells in breast milk to SCM in humans. A classical study by Thomsen et al. (1983) demonstrated an association between mothers with symptoms of breast redness, tenderness and swelling in the first week postpartum and elevated cells in human milk. The study concluded by classifying symptomatic breast inflammation based on milk leukocyte and bacterial cells as follows: (1) milk stasis: leukocytes < 10^6 and bacteria < 10^3 per mL of human milk; (2) noninfectious inflammation: leukocytes > 10^6 but bacteria are still < 10^3 per mL of human milk; and (3) infectious *clinical* mastitis: leukocytes > 10^6 and bacteria are still > 10^3 per mL of human milk. Following research by Thomsen, independent studies by Semba et al. (1999) and Nussenblatt et al. (2005) classified women with a breast milk leukocyte count > 1 million cells/mL as having SCM, however, both studies were conducted among HIV-infected women which may have altered milk composition to an unknown magnitude. Studies in humans have also suggested that high leukocytes or SCC in milk cannot be used as the sole indicator for SCM because women from areas where poor hygiene practices are common may have naturally elevated baseline leukocytes counts in milk (Abou-Dakn et al., 2010).

Historically, the diagnosis of SCM in humans has centered on breast leakiness and raised sodium (Na) concentrations in breast milk (Morton, 1994). Normal human milk sodium contains 5 to 6 mmol/L of sodium which increases to 12-20 mmol during SCM (Michie et al., 2003). In an older study by Semba et al. (1999), elevated milk sodium levels consistent with mastitis (>12 mmol/liter) were present in 16% of lactating women in Malawi. Moreover, sodium values in breast milk were not affected by a mother's dietary sodium intake or the method of milk expression (Neville et al., 1984; Ereman et al., 1987). However, the use of milk sodium levels alone makes it difficult to determine the precision of SCM as there are potentially other causes for high sodium levels in breast milk including stage of lactation. Colostrum has the highest levels of milk sodium which decreases rapidly during the first 2 days postpartum as the paracellular pathway closes (Nguyen & Neville, 1998). Other causes of of increased breast milk sodium include early weaning of the infant, insuffient milk supply, and poor feeding by a malnourished or dehydrated infant (Galipeau et al., 2012). Recent studies have concluded that the sodium-potassium (Na/K) ratio, rather that sodium alone, is a more accurate indicator for SCM.

An elevated breast milk sodium/potassium (Na/K) ratio is considered indicative of SCM and both ratios of Na/K >0.6 (Filteau, 1999; Gomo, 2003; Kantarci, 2007; Flores-Quijano, 2008; Rasmussen, 2008; Lunney, 2010, Richards, 2010, Thurnham, 2010) and Na/K >1.0 (Willumsen, 2003; Kasonka, 2006; Makasa, 2007; Aryeetey, 2008; Aryeetey, 2009) have been used to indicate SCM in humans. The most recent study classified SCM as follows: (1) Na/K ratio >0.6 as "Any SCM" (2) Na/K ratio >0.6 and ≤ 1.0 as "Moderate SCM" and (3) Na/K ratio >1.0 as "Severe SCM" (Arsenault et al., 2010). Use of the ratio also permits the collection of spot milk samples without consideration of time of sampling or time since the infant was last fed (Filteau *et al.*, 1999), controls for assay variations that may occur due to the unequal distributions of the ions in the aqueous and lipid phases of milk (Aryeetey et al., 2008) and accounts for the normal and almost parallel decline in both Na and K ions during lactation (Allen et al., 1991; Richards et al., 2010).

2.0 Prevalence of Subclinical Mastitis and Risk Factors

While there is no question that SCM exists among lactating women worldwide, the precise prevalence in developing countries is unknown. Estimates for the prevalence of SCM vary among the literature with the most recent reported prevalence ranging from 2% to 66% from lactating women in African countries (Nussenblatt et al., 2005; Arsenault et al., 2010). The wide variation in rates occurs because the asymptomatic nature of the condition makes it challenging to identify. However, most studies suggest that the prevalence of SCM is highest in the early stages of lactation. An older study in Bangladesh (Filteau et al., 1999) reported prevalence of SCM of 25% at 2 weeks and 12% at 3 months postpartum, respectively. It is also generally understood that the prevalence of SCM among lactating mothers is much higher than *clinical* mastitis, with estimates for the prevalence of *clinical* mastitis ranging from 1%-17% (Arsenault et al., 2010; Nussenblatt et al., 2005; Semba et al., 2000; WHO, 2000).

Maternal factors positively associated with SCM include younger age (Aryeetey et al., 2009) and primiparity (Kasonka et al., 2006; Aryeetey et al., 2009). Low to moderate grades of maternal undernutrition (Kasonka et al., 2006) and micronutrient deficiency (Filteau et al., 1999; Arsenault et al., 2010) have been previously implicated but not clearly established. Additionally, the World Health Organization (2000) has identified a number of factors that may increase the risk of *clinical* mastitis including: previous case of mastitis, complications during delivery, maternal stress and fatigue, employment outside the home, and/or breast trauma.

Previous research has also shown that non-exclusive breastfeeding is positively associated with SCM (Willumsen et al., 2003; Kasonka et al., 2006). Poor lactation practices may underscore milk stasis, which provides an ideal medium for infection. WHO recommends exclusive breastfeeding for infants <6 mo of age and "breastfeeding on demand" or as often as the infant wants, day and night (WHO, 1999; WHO, 2003). There is also concordance within the international pediatric community that mothers should breastfeed their infants 8-12 times per 24 hr to ensure adequate milk intake and maintenance of lactation (de Carvalho et al., 1982; AAP, 1997; Matias et al., 2010). Older studies have found that junctional complexes likely start to open when milk volume falls below 400 mL/day (Neville et al., 1991) and that both a reduction in feeding frequency (Garza et al., 1983) and low milk volumes (Dewey et al., 1984) are associated with an increase in mammary gland permeability.

However, with efficient removal of milk, the condition may be prevented and thus mothers are encouraged to express milk from the affected breast and avoid causes of milk stasis including poor attachment of the infant at the breast, ineffective suckling, and restriction of the frequency or duration of feeds, overabundant milk supply and blockage of milk ducts (WHO, 2000). One study in Bangladesh found that mothers who received one breastfeeding counseling session at any time around delivery had significantly lower rates of SCM (milk Na/K ratio >0.6) than those without counseling suggesting that counseling on the improvement of lactation practices can improve maternal breast health (Flores & Filteau, 2002).

3.0 Physiology and Pathophysiology of Subclinical Mastitis

3.1 Human Milk Secretion Pathways

Solutes can enter milk through both transcellular and paracellular pathways (McManaman & Neville, 2003). There are five general secretion pathways for transport of solutes into milk including: (1) Exocytotic Pathway (2) Lipid Secretion Pathway (3) Transcytotic Pathway (4) Membrane Transport Pathway (5) Paracellular Transport Pathway.

<u>Exocytotic Pathway:</u> The exocytotic secretion pathway is the mechanism by which alveolar cells primarily secrete proteins, oligosaccharides, lactose, phosphate, calcium and citrate. Exocytotic secretion is a mechanism where substrates are packaged into secretory vesicles within the Golgi and then transported to the apical region of the cells. At the apical region, the vesicles fuse with the apical plasma membrane and discharge the contents into the extracellular space. This exocytotic secretion mechanism is similar to mechanisms found in other cells, however, there is a unique feature of this pathway in alveolar cells. Namely, there are high concentrations of

lactose within the vesicles. In humans, lactose is synthesized from UDP-galactose and glucose within the Golgi by lactose synthase. The high concentrations of lactose in the Golgi during lactation lead to osmotic influx of water that contributes to the fluidity of milk (McManaman & Neville, 2003).

Lipid Secretion Pathway: Milk lipids secreted from mammary epithelial cells are primarily tricylgylcerides and phospholipids. Synthesized in the smooth endoplasmic reticulum in the basal region of the cell, milk lipids are formed from precursor fatty acids and glycerol. Lipids travel to the apical plasma membrane as part of a storage structure known as a "lipid body" yet are secreted by a unique budding process as new enveloped structures known as "milk fat globules" (MFG) (Mather & Keenan, 1998). The MFG is a major energy source for the infant and the unique structure of the MFG prevents fat globules from coalescing into large fat droplets that may be difficult to expel during lactation (Patton & Keenan, 1975).

Transcytotic Pathway: The transcytosis pathway exists for transport of exogenous substances into milk including serum proteins albumin and transferrin (Monks & Neville, 2001), endocrine hormones such as insulin, prolactin and estrogen (Koldovsky, 1995) and stromal derived factors such as sIgA (Goldman et al., 1996).

Membrane Transport Pathway**:** The membrane transport pathway is used for the direct movement of small molecules including water and glucose across the apical and basal membranes of the cell. Transcellular transfer of these factors from blood to milk requires the presence of specific transporters at the basal and/or apical plasma membranes of alveolar cells (McManaman & Neville, 2003).

Paracellular Transport Pathway**:** Transport through the paracellular pathway accounts for movement of low-molecular weight substances between the alveolar lumen and the interstitial space. This pathway is only open during pregnancy or after involution (McManaman & Neville, 2003). This pathway is closed during lactation in humans due to the closure of tight junction between epithelial cells. The result is that, in general, only the transcellular pathways 1 through 4 function to transfer nutrients and bioactive factors into milk. The paracellular transport pathway is open during inflammatory states including both *clinical* and subclinical mastitis. This pathway is therefore affected by the functional state of the mammary gland and regulated by both direct and indirect actions (McManaman & Neville, 2003).

3.2 Regulation of Tight Junctions in the Mammary Gland

The paracellular pathway, associated with the bi-directional transfer of substrates between the alveolar lumen and the interstitial space in humans, is normally closed during lactation (McManaman & Neville, 2003). This can be explained by closure of the tight junctions that block the pathway. Closure of tight junction complexes occurs during Lactogenesis Phase 2 (Neville & Morton, 2001). Changes to tight junctions occur after delivery and are largely complete by 72 hour postpartum (Neville & Morton, 2001). Closure of the junctional complexes between mammary alveolar cells is reflected in changes in milk sodium, chloride and lactose concentrations. There is an increase in sodium and chloride and a decrease in lactose concentrations in milk composition (Neville et al., 1991). The hormonal regulation of tight junction permeability is not yet fully understood in human lactation literature. However, it is commonly believed that high levels of progesterone maintain pregnancy and a decrease of this hormone with parturition, specifically with the removal of the placenta as the major source of progesterone, initiates lactation (Neifert et al., 1981). A decrease in progesterone following parturition is therefore a key trigger for tight junction closure in humans (McManaman & Neville, 2003). The paracellular pathway will remain closed throughout lactation and will begin to open only with involution when the infant starts to wean.

Animal studies using the mouse mammary gland have provided strong evidence to confirm that the loss of progesterone is a trigger for tight junction closure (Nguyen et al., 2001). Glucocorticoids have also been implicated in the regulation of mammary tight junction permeability in the mouse (Nguyen et al., 2001) and other animals. A study by Thompson (1996) found that injection of cortisol into the mammary gland of goats decreased tight junction permeability. Similarly, lactating cows displayed a decrease in the permeability of the mammary gland when the levels of cortisol were increased by ACTH treatments (Stelwagen et al., 1995). Additionally, *in vitro* studies in mouse mammary epithelial cell lines have also shown that glucocorticoids can induce closure of tight junctions (Zettl et al., 1992). The mechanism underlying how the interaction between these two hormones signal closure of the tight junction structures remains unknown. In humans, an older study by Keenan et al. (1983) found that milk sodium and potassium may be regulated in part by glucocorticoids in human milk. Specifically, the study showed that dexamethasone administration caused a significant decrease in milk Na and an increase in milk K. On the other hand, cortisol may play a more indirect role. Previous research has also shown that high cortisol levels caused by prolonged external stress in mothers were associated with a delay in lactogenesis (Chen et al., 1998; Grajeda & Perez-Escamilla, 2002). There is limited clarity in human lactation literature although it is plausible that cortisol and tight junction permeability may be interrelated using different reciprocal pathways.

3.3 Milk Stasis

Studies in both cows (Stelwagen & Lacy-Hulbert, 1996) and goats (Fleet & Peaker, 1978) provide evidence for the involvement of intramammary pressure, and not a regulatory factor in milk, for the decline in milk yield as a result of milk stasis. In humans, research examining the relationship between milk stasis and tight junction permeability is scarce. An older study found that junctional complexes likely start to open when milk volume falls below 400 mL/day (Neville et al., 1991). Poor lactation practices including non-exclusive breastfeeding and a reduction in feeding frequency are therefore often used as a proxy for stasis given that they likely underscore the condition

Tight junctions of the alveolar epithelial cells in the mammary gland are impermeable during lactation in most species (Nguyen & Neville, 1998). Nevertheless, the continuous seal provided by the tight junctions, which under normal conditions does not allow leakage of milk components from the lumen, can be regulated by certain external stimuli including milk stasis (McManaman & Neville, 2003). In the dairy industry, once daily milking of cows can reduce labor costs, however, it is not a common practice as it leads to lower milk yield (Holmes et al., 1992). Studies in cows have shown that the accumulation of milk, or milk stasis, is associated with a decline in milk secretion (Nguyen & Neville, 1998). Using lactose concentration as measures of tight junction permeability, since lactose is synthesized only in the mammary gland, a study in cows showed that tight junction permeability increased after only 18 hours of milk stasis (Stelwagen et al., 1997). Interestingly, the same study found that tight junctions reverted to the closed state after milking, showing that tight junction permeability was transient (Stelwagen et al., 1997; Nguyen & Neville, 1998). The exact mechanism by which milk stasis regulates opening of tight junctions is undefined. Although, it is likely that increasing luminal pressure damages the mammary epithelium (Nguyen & Neville, 1998).

3.4 Inflammation

Inflammation leads to down-regulation of tight junction proteins, allowing plasma constituents to traverse the basement membrane into the lumen via the paracellular route (Dorosko, 2005). One potential mechanism by which this occurs is via inflammatory cytokine signaling, specifically tumor necrosis factor alpha and interferon gamma, which have been shown to down-regulate the expression of tight junction proteins such as occludin (Mankertz et al., 2000). An inflammatory reaction that increases permeability may be an important factor in the process as it allows immune components to reach the infection site (Nguyen et al., 1998).

Cytokines in human milk are peptides that act in an autocrine/ paracrine fashion by binding to specific cellular receptors (Garofalo, 2010) or commonly understood as signaling molecules used in cellular communication (Agarwal et al., 2011). In response to foreign organisms such as viruses or bacteria that enter the mammary gland, cytokines act to mediate the effector phase of both innate and adaptive immunity by regulating inflammatory responses that are associated with the overall immune response (Garofalo, 1998; Goldman, 1993). The theoretical ideal is balance of the host's (i.e. mammary gland) ability to respond to different stimuli (i.e. bacteria) with an appropriate response (i.e. secretion of pro-inflammatory cytokines) without excessive inflammation or damage to the host, thus, maintaining immunological homeostasis (Lawrence et al., 2007). If mothers with subclinical mastitis have higher levels of pro-inflammatory in their milk due to an inflammatory reaction of the lactating mammary gland, milk nutrients may be negatively influenced by the inflammatory response leading to impaired growth. On the other hand, the threatened mammary gland might act defensively to protect the infant including through the secretion of higher amounts of immune factors, including sIgA, in human milk (Filteau et al., 1999). It is therefore not surprising that the inflammatory response in the mammary gland has been described as a "two-edge sword" (Thurnham, 2010).

3.5 Infection

The World Health Organization has cited infection as one of the principle causes of *clinical* mastitis (WHO, 2000). Without effective removal of milk, milk stasis progresses to infectious mastitis, and infectious mastitis to the formation of an abscess (Barbosa-Cesnik et al., 2003). The most common infectious organism found in clinical mastitis is *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) (Osterman et al. 2000; Delgado et al., 2009). How infectious organisms enter the breast is uncertain, however, several routes have been suggested including: (1) via the lactiferous ducts into a lobe; (2) by haematogenous spread; and/or (3) through a nipple fissure into periductal lymphatic system (WHO, 2000). Different bacterial species may elicit different inflammatory responses in the mammary gland; *E.coli* usually associated with acute response that resolves quickly whereas *S.aureus* often results in chronic and low-grade infections in the mammary gland (Ling, 2010). However, bacteria are often found in milk from asymptomatic breasts and the presence of bacteria in milk does not necessarily indicate infection (Hassiotou et al., 2013) suggesting that the characterization of SCM based on bacteria strains alone may not be the most reliable method.

3.6 Fecal-Oral Transmission

It has recently been proposed that poor sanitation and hygiene, and not diarrhea *per se*, is a primary cause of infant undernutrition (Humphrey, 2010). The role of poor hygiene and sanitation has previously been implicated in relation to *clinical* mastitis (Fetherston et al., 1998; Michie et al., 2003; Peters et al., 1992). Fecal contamination of the maternal breast and infant hands during breastfeeding can lead to increased fecal ingestion by infants via breast milk (Dewy & Mayers, 2011; Prendergast & Humphrey, 2014; Mbuya & Humphrey, 2015). It is therefore possible that high pathogenic bacteria ingestion may overwhelm the small-intestinal lumen, leading to increased intestinal permeability and altered gut barrier function, promoting microbial translocation and increasing the risk of infant undernutrition (Humphrey, 2010; Dewey & Mayers, 2011). Previous research has demonstrated the sharing of bacterial strains between breast milk and infant feces (Martin et al., 2012). In addition, previous research has shown that maternal gut bacteria may be vertically transferred from mother to neonate via breastfeeding (Jost et al., 2014) further supporting the notion of fecal-oral transmission between mother and infant.

4.0 Subclinical Mastitis and Milk Composition

4.1 Stages of Lactation and Milk Composition

Breast milk is a unique fluid that provides the “gold standard” for infant nutrition. It includes bioactive components that promote healthy development and infant survival. But, unlike infant formula that is standardized, human milk is a non-uniform fluid that can vary by stage of lactation, within a feeding cycle, diurnally, and as a result of maternal individual differences (Ballard et al., 2013). Although recent reviews on “human milk composition” reveal a fluid with a myriad of components (Ballard et al., 2013; Andreas et al., 2015), interestingly, components of human milk are still being identified.

There are three stages of milk synthesis: colostrum, transitional milk and mature milk (Ballard et al., 2013). Colostrum is the first fluid after birth and is distinct by its yellowish colour and thicker, creamy appearance. It is rich in immunological components such as secretory immunoglobulin A (sIgA) and appears to provide a more immunologic rather than nutritional function. Levels of sodium, chloride and magnesium are higher in colostrum, whereas, levels of potassium and calcium are lower (Kulski et al., 1981; Pang et al., 2007; Ballard et al., 2013). The second stage is transitional milk, which appears with the timing of secretory activation, or lactogenesis stage two. Delayed onset of this phase is defined as greater than 72 hours postpartum, indicating the closure of tight junctions in the mammary epithelium (Neville et al, 2001). The phase may be delayed with maternal health conditions such as obesity (Nommsen-Rivers et al., 2012) or diabetes during pregnancy (De Bortoli et al., 2015). By 2 weeks postpartum, human milk is considered mature milk, which is then produced throughout the period of lactation. Mature human milk is a complex fluid, about 90% in the aqueous phase, and contains many different nutritional and bioactive components (Ballard et al, 2013).

4.2 Impact of Subclinical Mastitis on Breast Milk Composition

SCM is known to have an adverse effect on the quality of cow milk (Batavani et al., 2007) and given the economic implications, dairy scientists have an invested interest in researching changes in milk composition as a result of SCM. However, limited research on SCM mediated changes in human milk exists.

4.2.1 Nutritive Composition

Carbohydrate: Lactose is the principal carbohydrate in milk, is the least variable of the macronutrients and is accompanied by large amounts of water making it a key determinant in osmotic pressure and milk volume (Neville, 1991). During SCM, concentrations of lactose significantly decrease in human milk (Fetherston et al., 2006a). If SCM decreases lactose synthesis in the mammary gland, this implies that SCM changes milk volume as lactose helps to regulate water content in milk (Fetherston et al., 2006). In animal literature, SCM causes a decrease in milk lactose in cows (Batavani et al., 2007), camels (Aljumaah et al., 2011), sheep and goats (Merin et al., 2004).

Fat: Consisting mainly of triglycerides (98-99%), milk fat is the most variable component of human milk and is directly related to breastfeeding frequency with decreased frequency producing lower fat concentrations in the subsequent milk (Nommsen et al., 1991). In a classical study by Ramadan et al. (1972) a marked decrease in total lipid content of milk was demonstrated in women with mastitis. Contradictory to the older study, a recent study by Hunt et al. (2012) found no alteration to total lipid content but reported a significant elevation in overall concentration of free fatty acids in human milk from mastitic breasts ($p<0.05$). These recent findings suggest that SCM may be associated with increased lipolysis in the human breast but not alterations in milk fat synthesis. In literature with animals, SCM causes an increase in milk fat in cows (Bruckmaier et al., 2004) and camels (Aljumaah et al., 2011) but a decrease in sheep and goats (Merin et al., 2004).

Protein: There is little information on the impact of SCM on protein content in humans. One study by Filteau et al. (2003) showed a borderline significant ($p=0.06$) increase in milk lysozyme in women with SCM. Likewise, research has suggested that lactoferrin, a potent iron-binding protein, is increased in women with SCM (Semba et al., 1999; Fetherston et al., 2006a), although a study by Buescher & Hair (2001) did not find an increase in lactoferrin in milk during mastitis despite an increase in other milk components such as IL-1ß and IL-6. Animal research has suggested that whey protein content in milk is increased in cows (Batavani et al., 2007), sheep and goats (Merin et al., 2004) with SCM.

Vitamins: To date, there are no studies on how SCM influences vitamin content of human milk. However, an older study found that maternal dietary supplementation with vitamin E-rich sunflower oil but not pro-vitamin A-containing red palm oil decreased levels of milk Na/K ratios suggesting that if SCM results in part from systemic inflammation, breast health may be improved with maternal nutritional intake of vitamin E (Filteau et al., 1999). On the contrary, others have found evidence that multi-vitamins, including vitamin A, increase the risk of subclinical mastitis (Arsenault et al., 2010; Thurnham, 2010).

Minerals: SCM directly affects the concentration of minerals in human milk including an increase in Na and a decrease in K, which is why the Na/K ratio is routinely used in the diagnosis of SCM (Arsenault et al., 2010). In the milk of mothers with SCM or mastitis, others have reported a change in milk minerals Cl (Fetherston et al., 2006a) and Zn (Neville et al., 1984). A marked reduction in Zn concentration is of particular interest as Zn concentration in milk is not influenced by maternal Zn deficiency, age or parity and because Zn helps maintain the epithelial integrity of skin due to its role in cellular repair and replacement (Kelleher et al., 2011) which may provide interesting avenues for novel therapeutic interventions in the prevention of SCM in women. In dairy literature, SCM in cows has been shown to decrease milk minerals Cu, Zn, Fe, and Ca but have no effect on Mg (Yildiz et al., 2005). Further, dietary studies in cows found that mastitis is linked to inadequate intake of selenium (Hogan et al., 1993).

4.2.2 Bioactive Composition

4.2.2.1. Cytokines: In both dairy and human studies, SCM influences milk cytokines IL-1□, IL-6, TNF□, and TGF□ (Filteau et al., 1999; Bannermann, 2009). Inflammatory cytokines in human milk are known to stimulate immune activation including moving lymphocytes into human milk in response to infection (Ballard, 2013). Recent findings have also demonstrated that SCM in humans is accompanied by the presence of the inflammatory cytokine interleukin-8 (IL-8) (Filteau et al., 1999; Semba et al., 1999; Willumsen et al., 2000; Rasmussen et al., 2008; Filteau et al., 2009) suggesting a “true” inflammatory condition and not merely leakiness of tight junctions between mammary epithelial cells. In addition, a recent study by Mizuno et al. (2012) found pro-inflammatory cytokine IL-6 significantly increased in milk of mothers with *clinical* mastitis compared to milk from healthy mothers.

Given their known roles - IL-6 as a marker for systemic activation of cytokines and responsible for promoting the movement of monocytes to the mammary gland (Oviedo-Boyso et al., 2007), whereas, IL-8 mediates the recruitment of granulocytes and accumulation of macrophages from the maternal circulation to the breast milk (Michie et al., 1998) – it has been logical to suggest their potential roles as early indicators of human SCM. However, variations in the concentration of some pro-inflammatory cytokines, including both IL-8 and IL-6, throughout lactation have been observed (Hawkes et al., 2002; Groër et al., 2011) suggesting that the use of milk cytokines as early indicators of SCM without consideration of stage of lactation should be done with caution. The following section briefly reviews human milk cytokines applicable to this dissertation.

Interleukin-1□: Interestingly, IL-1□ is considered the first cytokine to be quantified in human milk (Munoz et al., 1990). In general, IL-1□ is involved in the mediation of the inflammatory response as well as cell proliferation, differentiation, and apoptosis (Agarwal et al., 2011). IL-1□ is higher in the first 3 months postpartum and declines gradually during lactation (Hawkes et al., 1999). A unique study by Groër et al. (2009) found that human milk cytokines including IL-17, IL-2, IFN-□, and IL-1□ were increased in mothers with increasing exercise, specifically, increasing metabolic-equivalent tasks and caloric expenditure. This study led to the speculation that exercise in the early postpartum period by be physiologically interpreted as a "danger signal" to the infant, although it is generally believed that the postnatal period is designed to be stress-resistant.

Interleukin-6: In human milk, IL-6 acts as a potent pyrogen (Rudloff et al., 1993; Agarwal et al., 2011) and therefore is often used as a marker for systemic inflammation and fever (Meki et al., 2003). It is a pro-inflammatory cytokine that stimulates the acute phase response as well as B-cell activation (Ustundag et al., 2005; Meki et al., 2003). It has been suggested that the concentration of IL-6 in milk is higher during the first three months postpartum (Hawkes et al., 1999) although a previous study found that the levels of IL-6 relatively constant throughout lactation (Agarwal et al., 2011). Human milk IL-6 appears to be distinct from other pro-inflammatory cytokines as an important signal for the production of the major immunoglobulin (sIgA) in the mammary gland (Garofalo, 2010). Moreover, in a cross sectional study of milk samples collected on day 3 postpartum from diverse ethnic backgrounds, Asian women had the highest IL-6 levels in milk, suggesting that women from different locations are exposed to different environmental pathogens in their gut, which then stimulates different immune responses in the mammary gland, resulting in breast milk immune components specific to environment (Ciardell et al., 2007).

<u>Tumor Necrosis Factor-α:</u> In human milk, TNF-α is the main pro-inflammatory cytokine to stimulate inflammatory immune activation, including the recruitment of leukocytes, during periods of infection (Agarwal et al., 2011). Levels of milk TNF-α correlate positively with other inflammatory cytokines, including cytokine IL-6, and are independent of infant age (Meki et al., 2003). There are substantial variations in the reported amount of milk TNF-α although it generally decreases over the period of lactation (Rudloff, 2002). The levels of TNF-α in milk have been associated with timing of delivery (Ustundag et al., 2005) and were reportedly modestly increased in mature milk of mothers who had preeclampsia (Erbagci et al., 2005).

<u>Interleukin-8:</u> IL-8 is a pro-inflammatory cytokine that belongs to the CXC family of chemokines (Baggiolini et al., 1994). Chemokines are a novel class of small chemotactic cytokines with cell selectivity able to activate leukocytes and therefore important mediators of inflammation (Groër, 2011). IL-8 was previously identified as a chemoattractant solely targeting neutrophils, but it was later discovered to attract basophils and eosinophils (Erger, 1995). It can be found in high concentrations in milk when the mammary gland is disturbed such as during

SCM (Agarwal et al., 2011). Interestingly, the ability of IL-8 to recruit neutrophils makes it an important component in the innate immune response system for both mother and infant. In a longitudinal study of bioactive factors in human milk from women who experienced preeclampsia compared to healthy women, it was found that at 48 hours postpartum, mothers with preeclampsia had elevated levels of milk IL-8 which remained elevated at 30 days postpartum (Erbagci et al., 2005). As IL-8 facilitates the migration of milk leukocytes across the infant gut epithelium, and is relatively resistant to certain digestive processes, it has been suggested that elevated concentrations will boost the immune response of the infant gut although not elucidated (Field, 2005; Garofalo, 2010).

<u>4.2.2.2. Immunoglobulins:</u> Secretory IgA (sIgA), which is both synthesized and stored in the breast, is the major immunoglobulin in human milk. A study by Filteau et al. (1999) found a significant ($p<0.001$) increase in sIgA with increased severity of SCM. Likewise, Fetherston et al. (2006a) found an increase in sIgA in women with hyper acute breast inflammation, which is in contrast to an older study by Prentice et al. (1985) reporting lower concentrations of sIgA in milk of mothers with mastitis. To date, there are no studies on the association between SCM and the other immunoglobulins (IgG, IgM) in human milk.

<u>4.2.2.3. Cortisol:</u> To date, there are no studies on the interaction between SCM and human milk cortisol, however, it is plausible that SCM and cortisol are interrelated using different reciprocal pathways. On one hand, SCM may act as a stressor on the mammary epithelium causing an increase in human milk cortisol (Palmer, 2011). On the other hand, elevated human milk cortisol might influence tight junction permeability in the mammary gland. This is seen in studies with

animals, where glucocorticoids appear to play a role in the regulation of mammary tight junctions (Nguyen & Neville, 1998; Thompson, 1996). Elevated human milk cortisol might be a result of external stress experienced by the mother. Furthermore, increased levels of maternal cortisol have well-known inhibitory effects on the letdown reflex in the lactating breast (Nguyen & Neville, 1998). A negative-feedback loop of cortisol on prolactin prevents milk production, causing delayed lactogenesis, and results in milk stasis in the mammary gland (Nguyen et al., 2001), possibly promoting SCM. Elevated cortisol has also been associated with decreased confidence of how to breastfeed, poorer infant latch to the breast, less frequent maternal touch during breastfeeding, and less sensitive positioning at the breast by the mother; further compromising breastfeeding frequency and promoting SCM (Hart et al., 2004; Flores-Quijano et al., 2008).

A steroid hormone, cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Field & Diego, 2008). Activation of the HPA axis prompts a cascade of hormonal events starting with the secretion of corticotrophin releasing hormone (CRH) from the hypothalamus, which leads to the release of adrenocorticotropic hormone (ACTH) by the anterior pituitary, resulting in adrenal cortex release of glucocorticoids (cortisol), mineralcorticoids and adrenal medulla release of catecholamines (norepinephrine and epinephrine) (Marieb, 2009). During pregnancy, cortisol helps to ensure proper mammary development for approaching lactation by working with insulin and prolactin to ensure the final differentiation of the alveolar epithelial cell into a mature milk cells (Neville, 2001). Specifically, in order for prolactin to have an effect on mammary tissue, mammary cells must express prolactin receptors and thus cortisol induces the expression of prolactin receptors so that prolactin can exert its lactogenic effects (Marieb, 2009). During lactation, cortisol is present in relatively high concentrations in colostrum but declines rapidly by the second day (Kulski & Hartmann, 1981). The specific role of cortisol in human milk is unclear although older studies provide theories as to the function of cortisol in milk including that it may play a role in the growth of an infant's pancreas (Morrisset & Jolicoeur, 1980) or that it may control the transport of fluids and salts in an infant's gastrointestinal tract (Kulski & Hartmann, 1981). Most recent studies measuring cortisol in human milk stem from the field of psychoneuroimmunology which is research proposing that maternal stress and immune responses have the ability to change breast milk composition. One study by Gröer, Humenick and Hill (1994) found that cortisol in human milk was associated with maternal mood and that there was an inverse correlation between secretory sIgA and cortisol in human milk thus concluding that stress in the lives of lactating women may interfere with the production of immune factors in human milk.

4.2.2.4 Growth Factors: There are no studies on how SCM influences growth factors in human milk. However, in human milk, several growth factors – bioactive peptides that promote cellular proliferation and permit trophy effects – have been identified including insulin-like-growth factor (IGF), transforming growth factor beta (TGF-β) and hepatocyte growth factor (HGF) (Ballard et al., 2013). Epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) are two growth factors that have also been identified in human breast milk and occur in relatively high concentrations (Dvorak et al., 2003; Kobata et al., 2008). Although the role of these growth factors in human milk has yet to be elucidated, animal studies and *in vitro* research suggests these growth factors are protected against proteolytic cleavage and low pH, thus capable of remaining intact throughout the infant's gastrointestinal tract and exerting a trophic effect (Hirai et al., 2002; Playford et al., 2000). Moreover, in the infant, lower neonatal protease activity and higher gastric pH in the gut are contributing factors that increase the likelihood that these growth factors are absorbed intact and retain their biological significance (Playford et al., 2000). The following section briefly reviews growth factors applicable to this dissertation.

Vascular Endothelial Growth Factor (VEGF): In human milk, VEGF is found at high concentrations and it believed to assist with the promotion of angiogenesis (formation of blood vessels) and tissue repair (Nishimura et al., 2002; Loui et al., 2012; Patki et al., 2012). This growth factor is found at concentrations that are 100 fold higher in human milk than in maternal serum (Ozgurtas et al., 2011; Kobata et al., 2008). The concentration of VEGF is highest in colostrum and declines within the first two weeks postpartum (Kobata et al., 2008). Interestingly, previous research has shown that mothers who gave birth to small-for-gestational age (SGA) infants had significantly lower concentrations of VEGF in their milk than mothers of appropriate-for-gestational age (AGA) infants (Loui et al., 2012). The same authors found that VEGF was significantly lower in preterm infants compared to term infants (Loui et al., 2012).

Epidermal Growth Factor (EGF): The concentration of EGF in human milk is highest in colostrum and declines over lactation (Dvorak et al., 2003). The average concentration of EGF in mature milk is 100 fold higher than in maternal serum (Dvorak et al., 2004). Interestingly, previous research has shown that EGF was significantly higher in extremely preterm infants (23-27 wk) compared to preterm (32-36 wk) and term (48-42 wk) infants (Dvorak et al., 2003). In human milk, it is believed that EGF stimulates cell proliferation and maturation (Ballard et al., 2011; Kobata et al., 2008). Resistant to low pH and digestive enzymes, EGF easily passes into the infant's intestine where it assists with the absorbance of water and glucose and protein synthesis (Chang et al., 2002). It has also recently been suggested that EGF in human milk may protect against necrotizing enterocolitis (NEC) in infants and may play an important role in the maintenance and the repair of the gastrointestinal epithelial barrier (Coursodon et al., 2012).

5.0 Subclinical Mastitis and Infant Growth

5.1 Determinants of Stunting, Underweight, and Microcephaly in Developing Countries

Stunting: Stunting in infants is defined as length for age-Z-score <-2 standard deviations (WHO, 2006). Evidence suggests that stunting begins *in utero* (Prendergast & Humphrey, 2014) and that stunting in early life is associated with adverse consequences including poor cognition, poor educational performance, low adult earning and low productivity in adulthood (Victora et al., 2008; de Onis et al, 2013). Stunting also appears to be multi-generational as stunted mothers are more likely to give birth to stunted children (Ramakrishnan et al., 1999). The post-natal period appears to be a “window of opportunity” (Victora et al., 2010) where exclusive breastfeeding is recommended during 0-6 months (WHO, 2006; Bhutta et al., 2008). It is plausible that breast milk with high concentrations of growth-promoting factors will promote linear growth, although surprisingly, there is little evidence to date of an impact on stunting based on randomized trials to promote breastfeeding (Black et al., 2013, de Onis et al., 2013). Specifically, in a cluster-randomized trail to promote exclusive breastfeeding in 3 African countries, increased rates of EBF were not associated with improved HAZ scores at 6 months (Engebretsen et al., 2014). In a meta-analysis of data from nine studies, 25% of infant stunting was attributed to having five or more episodes of diarrhea in the first 2 year of life (Checkley et al., 2008) and it has been reported that most of the cases of moderate to severe diarrhea in infants are attributable to 4 pathogens: rotavirus, cryptosporidium, enterotoxigenic *Escherichia coli* and *Shigella* (Kotloff et al., 2013)

Underweight: Underweight in infants is defined as weight for age-Z-score <-2 standard deviations (WHO, 2006). Low weight for age is an underlying cause of early child death associated with diarrhea, pneumonia, malaria, and measles (Caulfield et al., 2004). Several papers, including a recent Lancet series to tackle undernutrition, have acknowledged that the underlying causes of undernutrition are numerous including, but not limited to, environmental conditions, economic and political states, food insecurity, poverty, disease burden, micronutrient deficiency and preterm birth (Black et al., 2008). Suboptimum breastfeeding during the first 6 months is one of the greatest risk factors for undernutrition (Bhutta et al., 2008). Increased morbidity and mortality from non-exclusive breastfeeding is well documented (Bahl et al., 2005; Edmond et al., 2006; Black et al., 2013). In addition, delayed initiation of breastfeeding > 1 hour postpartum, deprivation from colostrum and improper complementary feeding are significant risk factors for an infant to be underweight (Kumar et al., 2006).

Microcephaly: Microcephaly in infants is defined as head circumference for age-Z-score <-2 standard deviations (WHO, 2006). Measurement of the occipitofrontal head circumference is a surrogate for brain volume and thus microcephaly is associated with a reduced brain volume (Ivanovic et al., 2004). It is also an indicator for nutritional status and undernutrition during infancy is associated with delayed health growth (Olusanya, 2012). A number of maternal factors are associated with microcephaly including younger maternal age, first parity, smoking, and drug use (Kallen, 2014). During pregnancy, mothers with intrauterine infections including rubella (De

Santis et al., 2006) and cytomegalovirus (Natacha et al., 2014) have been associated with having microcephalic infants. Interestingly, in a mouse model, cytomegalovirus was transmitted from infected mothers via breast milk to neonates with the infectious virus present in multiple organs including the brain (Wu et al., 2011). The relationship between breast milk and post-natal onset of microcephaly is unknown in human literature although one study found that microcephalic infants not exclusively breastfed were unlikely to experience catch up growth (Olusanya, 2012).

5.2 Subclinical Mastitis and Infant Anthropometry

To date, only 6 studies in the human lactation literature have reported on the relationship between subclinical mastitis and infant anthropometry (see Chapter II: Table 2-1). Two current theories propose to explain the relationship of SCM with infant growth faltering. The first theory suggests that SCM increases milk sodium, resulting in a "salty" taste of milk that decreases milk acceptability and milk intake by the infant. One study reported the case of an infant who preferred sweet milk produced from a healthy breast as opposed to "salty" milk produced by the other breast with raised sodium (Conner, 1979). The second theory arises from dairy literature where lactating cows with SCM produce less milk (Bruckmaier et al., 2004; Batavani et al., 2007). Together, these theories suggest that SCM compromises milk volume and/or composition, which may underscore poor infant anthropometry.

The first study to report a negative association between SCM and infant anthropometry showed that infants breastfeeding from mothers with breast milk sodium concentrations >16 mmol/L gained less weight (818 g vs 994g, $p=0.001$) compared to the average weight gain of infants above initial birth weight (Morton, 1994). A second study conducted in Bangladesh (Filteau et al., 1999) found that subclinical mastitis, using the Na/K ratio as an indicator, was positively correlated with concentrations of breast milk pro-inflammatory cytokine IL-8 in human milk. In addition, elevated milk Na/K ratio was correlated with reduced infant weight between (baseline) 1-3wks and 3 mo and that infant weight gain between baseline and 3mo was also negatively correlated with concentrations of milk IL-8 (p=0.001). A third study in Zimbabwe (Gomo et al., 2003) showed that infant weight in mothers with milk Na/K ratio >1.0 was significantly lower than infant weight from mothers with Na/K ratio <0.6 (normal) (5.5 kg vs. 6.2 kg, p=0.049).

Moreover, studies five and six to report an association between subclinical mastitis and infant anthropometry were both from the same large study investigating the early growth of infants of HIV-infected and uninfected mothers in the Zambia (Kasonka et al., 2006; Makasa et al., 2007). Of the two studies, the fist published report showed that a milk Na/K ratio at days 3 and 7 (early lactation) was negatively associated with infant 6 week weight (-0.08, p=0.001), 6 week length (-0.08, <0.001) and 16 week weight (-0.05, p=0.046) whereas the Na/K ratio at day10-week 16 (established lactation) was negatively associated with only infant 16 week weight (-0.04, p=0.019). The second published report demonstrated that in multivariate linear regression analyses, a breast milk Na/K ratio, using milk collected only from the established lactation period, was negatively associated with infant WAZ at 6 weeks (-0.37,p=0.006), infant WAZ at 16 weeks (-0.44, p=0.007) and with infant LAZ at 6 weeks (-0.48,p=0.026) but not with LAZ at 16 weeks (-0.38, p=0.086). From these studies the authors concluded that early growth of infants of HIV-infected mothers is less than that of uninfected mothers, which is in part associated with subclinical mastitis.

Lastly, the most recent study to report an association between subclinical mastitis and infant anthropometry was conducted in Ghana with 60 lactating mother-infant dyads between 3 and 6 months postpartum (Aryeetey et al., 2009). The study found that 27% of mothers has a breast milk Na/K ratio >1.0 (SCM) and in univariate analyses, mothers with milk Na/K ratio >1.0 had infants with lower length (59.5 cm vs. 60.5, p=0.04) compared to mothers with a milk Na/K ratio ≤ 1.0. A non-statistical trend was also observed in this study between mothers with and without SCM for having lower infant weight (5.8 kg vs. 6.2 kg, p=0.05) and lower infant head circumference (39.6 vs. 40.1 cm, p=0.05). Multiple regression analyses were not modeled for anthropometric indicators in this study although alternatively, the study concluded that maternal SCM was not a predictor of total grams of infant milk intake at 3-6 months.

5.3 Conclusion

In conclusion, SCM remains an under-researched problem in public health. In contrast to dairy literature, where SCM is extensively studied, gaps in our current understanding of this asymptomatic inflammatory condition exist in human lactation literature. This review of the literature has highlighted the sparse research on SCM. Given that the condition may underscore early cessation of breastfeeding and that the effects of SCM are not confined to the maternal system but may be evident in the offspring through reduced weight and length for age (Aryeetey et al., 2009; Kasonka et al., 2006; Filteau et al., 1999; Gomo et al., 2003; Morton, 1994) it is critical to understand this breast condition and how public health practitioners may be able to construct and scale up interventions aimed the prevention of SCM.

Table 2-1. Summary of subclinical mastitis and infant growth literature

Year & Author	**Study Population**	**N**	**Stage of Lactation**	**Key Findings**
1994; Morton	California	130	3-8 days	Infants gained less weight (818 g vs 994g, p=0.001) when feeding from mothers with concentrations of Na>16 mmol/L compared to the average weight gain of infants above birth weight.
1999: Filteau	Bangladesh	212	1-3 weeks 3 months	- Infant weight gain between baseline (1-3wks) and 3mo was negatively correlated with concentrations of milk IL-8 (p=0.007) - Milk IL8 correlated with Na/K ratio (p=0.001) - Elevated milk Na/K ratio was correlated with reduced infant weight between 1-3wks and 3 mo (no data shown)
2003: Gomo	Zimbabwe	68	1.5-4.5 mo	Infant weight in mothers with Na/K ratio >1.0 was significantly lower than infant weight from mothers with Na/K ratio <0.6 (normal) (p=0.049) Normal: 6.2 kg infant weight Na/K >0.6: 5.8 kg Na/K>1.0: 5.5 kg
2006; Kasonka	Zambia	387	Early (d3&7) Established (d10–wk16)	<u>Early:</u> The Na/K ratio at days 3 and 7 was negatively associated with infant 6 wk weight (-0.08, p=0.001), 6 wk length (-0.08, <0.001) and 16 wk weight (-0.05, p=0.046) <u>Established:</u> The Na/K ratio at day10-week 16 was negatively associated with infant 16 wk weight (-0.04, p=0.019).
2007; Makasa	Zambia	270	Established (d10–wk16)	In multivariate linear regression analyses, milk Na/K ratio (continuous variable from milk collected during established lactation) was negatively associated with infant WAZ at 6 wks (-0.37,p=0.006) and WAZ at 16 wks (-0.44, p=0.007) and with LAZ at 6 wks (-0.48,p=0.026) but not with LAZ at 16 wk (-0.38,p=0.086).
2009; Aryeetey	Ghana	60	3 - 6 mo	SCM status as Na/K ratio >1.0 University Analysis showed mothers with SCM had infants with: -Lower infant length (59.5 vs. 60.5 cm, p=0.04) Non-significant trend for: - Lower infant weight (5.8 vs 6.2 kg, p=0.05) - Lower infant head circumference (39.6 vs 40.1 cm, p=0.05)

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CHAPTER III

Cultural Determinants of Optimal Breastfeeding Practices among Indigenous Mam-Mayan Women in the Western Highlands of Guatemala

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Well Established

In developing countries, a variety of cultural factors are known to impact breastfeeding exclusivity and its early initiation. These include colostrum avoidance, feeding of ritual fluids, and practices associated with the hot/cold humoral theory of disease.

Newly Expressed

This study demonstrates that cultural practices used by indigenous *Mam*-Mayan mothers in the Western Highlands of Guatemala are associated with initiation of breastfeeding and increased breastfeeding frequency and may modify infant weight.

Abstract

Background: Among indigenous *Mam*-Mayan women, breastfeeding practices may be intertwined with cultural influences during the early postpartum period.

Objectives: Our study explored whether beliefs regarding transmission of emotions through breast milk, the feeding of *agüitas* or *temascal* (traditional sauna) use were associated with achievement of WHO infant feeding recommendations and if these cultural practices served as moderators of the relationship between optimal breastfeeding practices and infant anthropometry.

Methods: We recruited 190 mother-infant dyads at infant age < 46 days. Data on breastfeeding and cultural practices were collected via questionnaire. Infant length, weight and head circumference were measured and Z scores calculated. Multiple linear and logistic regression analyses were used to examine determinants of initiation of breastfeeding within 1 hr, breastfeeding frequency, breastfeeding exclusivity and infant weight-for-age z score (WAZ).

Results: Mothers who delivered at the traditional midwife's house (OR =2.5) and those who did not believe in the transmission of *susto* (fright) through breast milk (OR=2.4) were more likely to initiate breastfeeding within one hour postpartum. Higher breastfeeding frequency was observed among mothers who spent more time in the *temascal*. Initiating early breastfeeding within one hour postpartum was the sole infant feeding practice positively associated with exclusive breastfeeding and WAZ.

Conclusions: Our investigation in the Western Highlands of Guatemala has highlighted the link between cultural practices and beliefs during lactation, breastfeeding practices and infant growth. Public health practitioners need to understand how local cultural practices influence early initiation of breastfeeding in order to promote adequate infant weight.

BACKGROUND

The first 1,000 days of life, from conception to the second birthday, are critical to infant and young child development, adequate growth and survival.[1] Optimal infant feeding practices during this most vulnerable period have important implications for the immediate and future health of newborns.[2-4] The World Health Organization (WHO), while recommending exclusive breastfeeding (EBF) for infants <6 mo of age, also recognizes an alternative category of predominant breastfeeding (PBF) in which breast milk remains the predominant source of nourishment; PBF can include certain liquids (water, water-based drinks, fruit juice), ritual fluids and oral rehydration salts, drops or syrups. PBF is considered a better infant feeding option than mixed feeding, where food is given within the first 6 mo of life.[5,6] The WHO also recommends early initiation of breastfeeding, i.e. within 1 hr of birth. Evidence suggests that this practice can reduce diarrhea, respiratory tract infections and neonatal mortality.[7-10] There is also concordance within the international pediatric community that mothers should breastfeed their infants 8-12 times per 24 hr to ensure adequate milk intake.[11-14] Although the benefits of breastfeeding in reducing infant morbidity and mortality are indisputable, the relationship between infant feeding practices and growth has so far been inconclusively delineated.[15-18]

Globally, a variety of cultural factors are known to impact infant feeding practices[19-21] including cultural taboos against colostrum on the basis that colostrum is "dirty milk"[22] or "bad luck for the family"[23] and participation in *quarantena* (40-day postpartum period of rest).[24] Among the indigenous Mayan in Guatemala, cultural influences including the feeding of *agüitas* (ritual fluids)[25], the use of a *temascal* (traditional sauna) and beliefs about breast milk could impact infant feeding practices, and consequently infant weight.

In Guatemala, the feeding of prelacteal or postlacteal liquids is a common cultural practice. In the Mayan village of Santa Domingo Xenacoj, 74% of 6-12 mo infants were given *agüitas* (sweetened water, herbal infusion)[26] and other studies in the urban city of Quetzaltenango have confirmed the high prevalence of feeding *agüitas* to infants and young children.[27,25] Interestingly, a recent study also found that infants given *agüitas* before 2.9 wks of age were 1.8 times more likely to be stunted in comparison to all other infants including those never fed *agüitas* and those fed later than 2.9 wks of age.[25]

Another Mayan cultural practice is associated with the humoral theory that disease occurs when the body is out of balance between hot and cold.[28-30] As lactation is seen as a "cold" condition, many mothers use a *temascal* throughout the postpartum period to restore balance (i.e. warm up a cold condition toward neutrality).[31] Mothers also believe that heat from the *temascal* will increase milk flow. Reportedly, more than 86% of indigenous households in the Western Highlands of Guatemala use a wood-fired *temascal*.[32,33]

Transmission of maternal emotions through breast milk is a cultural belief held by some mothers worldwide. Mothers report that they limit breastfeeding during periods of high emotion because they believe emotions transferred via milk to their infant cause harm or illness.[34,21] In Mexico, mothers who experienced *susto* or *coraje*, local folk illnesses associated with fright or anger, respectively, reduced or stopped breastfeeding.[35] In Peru, mothers avoided breastfeeding to prevent transferring *sorrow* in their milk.[36] It is not known whether such beliefs influence breastfeeding practices in *Mam*-Mayan women.

The goal of our study was to explore whether beliefs regarding transmission of emotions through breast milk, the feeding of *agüitas* or *temascal* use was associated with compliance with WHO infant feeding recommendations[5,6] among indigenous *Mam*-Mayan mothers. We hypothesized that:

1) mothers who believe that emotions are transmitted through breast milk will be less likely to initiate breastfeeding within one hour postpartum and to exclusively breastfeed during the first 46 d;
2) the feeding of *agüitas* within the first day postpartum will be associated with delayed breastfeeding initiation and use of *agüitas* will be associated with decreased breastfeeding frequency;
3) use of the *temascal* will be associated with increased breastfeeding frequency; and
4) these cultural practices will modify the impact of optimal breastfeeding practices on infant anthropometry.

METHODS

Study Design and Protocol

This observational cross-sectional study of full breastfeeding (EBF and PBF) [27] *Mam*-Mayan mothers was part of a larger observational field project (Chomat et al in preparation). The majority of mothers within this indigenous population had no more than primary schooling, worked as housewives and lived within a context of food insecurity. A structured in-depth questionnaire about independent factors (maternal, infant, and cultural practices and beliefs) that may influence breastfeeding initiation, breastfeeding exclusivity and frequency was administered to mothers and anthropometry of both mother and infant was measured at a local health centre or at the participants' home.

Study Population and Recruitment

From June 2012 - January 2013, breastfeeding mother-infant dyads at infant age <46d were recruited by *comadronas* and health workers residing in eight rural *Mam*-Mayan communities in the Western Highlands of Guatemala. Of the 201 dyads recruited, the 11 (6%) mixed feeders were excluded. The final sample size was 190 full breastfeeding mother-infant dyads. Ethical approval for the protocol was obtained from Institutional Review Boards of McGill University and CeSSIAM (Center for Studies of Sensory Impairment, Aging and Metabolism), a Guatemala–based research organization. All mothers provided fully informed consent and were informed of their rights to withdraw from participation at any time during the study.

Questionnaire

The study questionnaire was developed in June 2011 and pilot tested for cultural appropriateness in 50 non-participant mothers with similar characteristics to the intended study population. Then, trained local health care workers administered the questionnaire orally in either Spanish or *Mam* to participants during a 30-40 min interview that addressed the following topics.

Maternal Characteristics: maternal age (confirmed with mother's identification document when available), languages (Spanish, *Mam*), marital status (married/union/divorced/widowed), highest level of education (none/primary/secondary) and current occupation (housewife/farmer).

Delivery Experience: age at first pregnancy; frequency of antenatal care; delivery at home, home of *comadrona* (traditional midwife), hospital or private clinic; birth attendant (*comadrona*, physician); and parity.

Breastfeeding Category: Although WHO categorizes breastfeeding practices based on recall during the previous 24 hrs, recall of feeding practices for offering breast milk, fluids and foods since birth has been shown to be more accurate among mothers in the Western Highlands of Guatemala.[27] Therefore feeding patterns were defined as exclusive breastfeeding (EBF) or predominant breastfeeding (PBF) based on cumulative recall since birth.

Breastfeeding Frequency: Frequency of breastfeeding during the day and at night were recorded and categorized as <8, 8-12 or >12 times/24 hr.

Breastfeeding Initiation: Mothers were asked when they initiated breastfeeding (min, hr, d). Those who reported that they put their infant to the breast within one hr after birth were classified as early initiators.[37]

Other Breastfeeding Practices: Mothers were asked if they fed their infant colostrum/the first milk (yes/no). Mothers were asked if they ever received support with breastfeeding (yes/no). If yes, the type of support provider(s) (*comadrona*, mother, mother-in-law, sister, health centre worker, physician, other) were queried. They were also asked if they had ever experienced a problem with breastfeeding (yes/no). If yes, specific types of problems (pain in breast, insufficient milk, infant illness, occupation, other) were queried.

Cultural Practices and Beliefs:

Agüitas: Mothers were asked if they ever fed their infant an *agüitas* since birth (yes/no). If yes, timing of *agüitas* initiation was queried (d, hr, wks postpartum). Likewise, mothers were asked to identify from a list the type of *agüitas* given to the infant and provide an open-ended reason for *agüitas* administration. Method of administration (cup, spoon, bottle) was also recorded.

Temascal: Mothers were asked if they had used a *temascal* since birth of the study infant (yes/no). If yes, frequency of *temascal* use (times/wk) and duration of *temascal* use (min/session) were queried.

Emotions: Mothers were asked if they believed that emotions could be transferred through their breast milk to the infant (yes/no). If yes, types of emotion (susto and *enojo*) were queried. According to local beliefs, *susto* is understood to result from a frightening experience while *enojo* is understood as anger. Both emotions may disrupt the normal equilibrium of the body and result in a diverse array of symptoms (stomach pain, irritability, fatigue) and illness.

Anthropometry

Two trained Guatemalan nutritionists measured maternal weight (kg) in triplicate using a digital scale (Seca 803) with a precision of 100 g and height in duplicate to the nearest 0.5 cm using a wall stadiometer. The age of the infant was calculated based on the date of birth and date of interview. Recumbent length (cm) was measured three times, to the nearest 0.5 cm, using an infantometer (Seca 210). Weight (kg) was measured using a digital infant scale (Seca 354) to the nearest 100 g. Head circumference was measured with a head circumference baby band (Seca 212).[38] Infants were classified as stunted (length-for-age Z score (HAZ) < -2SD), underweight (weight-for-age Z score (WAZ) < -2SD) or wasted (weight-for-height Z score (WHZ) < -2SD) according to WHO Growth Reference Standards[39] using WHO Anthro Software (3.1). Head circumference-for-age (HCZ score) was used to identify infants with microcephaly (<-2HCZ) or macrocephaly (>2HCZ).

Statistical Analysis

Data were analyzed using IBM SPSS statistical software version 22.0 (SPSS Inc./IBM Chicago IL, USA). Descriptive statistics (mean ± S.D, percentages ± 95% C.I.) were computed and chi-squares (x^2) and Fisher's exact test (categorical) and student t-tests were used to determine whether maternal characteristics, practices or anthropometry differed between those who did and did not initiate breastfeeding within 1 hr and between EBF and PBF. ANOVA was used to compare women who breastfed <8, 8-12 or >12 times / 24hr. In all cases, significance was set at $p \leq 0.05$. Multiple logistic regression analysis was used for categorical dependent variables (early initiation and EBF) and multiple linear regression analysis was used for continuous dependent variables (feeding frequency and infant WAZ) and in both types of modeling, all cultural practices, statistically significant variables from the bivariate analyses and other variables (p □ 0.25) were considered for inclusion. For the multivariate logistic regression model for breastfeeding initiation within one hour postpartum the following variables were included: infant age, infant sex, primiparity, delivery at *comadrona's* house and *susto* transmission and for EBF, infant age, infant sex, maternal weight, parity, *enojo* transmission and breastfeeding within one hour were included in the model. Multiple linear regression was used to generate a model for breastfeeding frequency while controlling for ever fed *agüitas, susto* transmission, *temascal* frequency, infant sex, infant age, mother's weight, and breastfeeding within 1 hr postpartum. In the second model for infant WAZ, we controlled for ever fed *agüitas,* breastfeeding frequency, *temascal* duration, infant sex and infant age.

RESULTS

Study Response Rate

Of the 190 full breastfeeding mother-infant dyads, we have data pertaining to breastfeeding initiation within one hr postpartum (n=187), exclusive breastfeeding (n=190), and feeding frequency (n=170), respectively.

Population Characteristics

The mean age of infants was 19.1 ± 9.8 d with an age distribution shown in Fig. 3-1a. Rates of EBF and PBF were 59% and 41%, respectively. Slightly more than half (56%) had initiated breastfeeding within the first hr postpartum and 97% fed colostrum. Mothers breastfed 11.9 ± 5.0 times/24 hr with 6.9±3.0 daytime and 5.0 ± 2.5 nighttime feedings. Almost all mothers (86%) reported receiving breastfeeding support from one or more sources: mother or mother-in-law

(43%), health centre (32%), physician (16%) and *comadrona* (11%). Only 9% (n=17) reported problems with breastfeeding among whom insufficient milk was reported by 52%, breast pain by 48%, infant illness by 12% and small breasts by 4%. Mothers could report more than one problem.

Three cultural practices were examined. Almost all mothers (98%) reported using the *temascal* on a weekly basis. On average, mothers used the *temascal* 2.4 ±0.7 times/wk for a mean duration of 29.2 ±10.6 min/session. Less than half of mothers (41%) reported ever feeding *agüitas* to their infant and all administered *agüitas* by teaspoon or bottle. *Agüitas* had been given to 29% of infants <1 wk old, increasing to 58% by 29-46 d (Fig. 3-1b). Reasons for feeding *agüitas* were based on maternal and infant conditions (Table 3-1). Maternal reasons included no or insufficient milk or tiny breasts. In response, mothers gave the infants boiled, sugar or corn-paste water, or chamomile tea (Table 3-1). Infant reasons included crying, colic, stomach pain, bloating, and several culturally-defined illnesses. In response, anise, orange-leaf, mint or sage water were given. Regarding cultural beliefs, 55% of mothers believed that "*susto*" might be transferred and 48% believed that "*enojo*" might be transferred through their breast milk.

Bivariate Comparisons for Infant Feeding Practices

Early vs Later Initiation (Table 3-2): Mothers who initiated breastfeeding within 1 hr breastfed more frequently during the day and were more likely to exclusively breastfeed compared with mothers delaying initiation. They also were more likely to have delivered at the *comadrona's* house. Interestingly, fewer mothers who initiated breastfeeding within 1 hr fed *agüitas* to their infants and fewer believed that *susto* or *enojo* could be transferred through milk. Early initiators had infants with significantly higher WAZ, WHZ and HCZ scores.

EBF vs PBF (Table 3-3): EBF mothers were more likely to receive breastfeeding support from a *comadrona* and to initiate breastfeeding within one hr postpartum. EBF mothers were less likely to report problems or to believe that *enojo* would be transmitted through their breast milk compared with PBF mothers. Comparisons between EBF and PBF showed no differences in infant characteristics except that EBF infants were, on average, 4 days younger.

Breastfeeding Frequency (Table 3-4): Mothers who fed <8 times/24 hr had lower BMI than those who breastfed more frequently. A higher percentage of those who breastfed >12 times/24 hr had initiated early breastfeeding, and had used the *temascal* more frequently per week. They also had higher infant WHZ scores.

Regression Models

Early breastfeeding initiation was more likely in mothers who delivered at the *comadrona's* home (OR=2.5) and who did not believe in the transmission of *susto* through breast milk (OR=2.4) (Table 3-5). Ever feeding *agüitas* did not enter the model.

EBF was less likely in older infants (OR=0.9) but more likely in infants who had initiated breastfeeding within one hour (OR=6.1) (Table 3-5).

Breastfeeding frequency was positively related only to *temascal* duration and to infant WHZ with the overall model Adjusted R^2 = 14.4% (p <0.001) (Table 3-6).

Infant WAZ was positively associated with both breastfeeding initiation within one hr postpartum and maternal weight with the overall model Adjusted R^2 = 14.5% (p <0.001) (Table 3-6).

DISCUSSION

Our study in the Western Highlands of Guatemala provides the first report on associations between cultural practices and beliefs used by indigenous *Mam*-Mayan mothers on optimal breastfeeding practices. We showed that early initiation of breastfeeding was associated with delivery at the *comadrona's* house, not believing in transmission of *susto* through breast milk and not offering *agüitas*, and was a determinant of EBF. More time per week in the *temascal* was associated with increased breastfeeding frequency. Early initiation of breastfeeding was also the only infant feeding practice positively associated with infant WAZ.

Literature from Latin American countries has shown that EBF is positively associated with maternal characteristics including older age, lower education, lower socioeconomic status, not being employed and single motherhood.[40-42] In Guatemala, previous research had identified maternal work outside the home[43] and increased years of schooling[44] as negative determinants of EBF. In contrast, we did not establish a difference between EBF and PBF for any of these characteristics or for infant gender as had been reported in Brazil and Honduras[40] perhaps because of the relative homogeneity in social variables within this indigenous population.

Early initiation of breastfeeding was delayed by use of *agüitas,* by beliefs surrounding transmission of *susto* and was enhanced by delivery at the *comadrona's* house. The interruption of breastfeeding with the feeding of ritual fluids is not unique to Guatemala.[22,45,46] In our study, many mothers reported that they delayed initiation and fed *agüitas* because of perceived insufficient milk. Medical anthropologists describe this transcultural phenomenon as the "insufficient milk syndrome" where mothers who lack understanding of the stages of milk production lose confidence, and thus supplement or terminate breastfeeding.[47-49] Use of *agüitas* did not emerge as a determinant of early initiation when controlling for other cultural practices in the regression analysis. However, mother's belief in *susto* transmission through breast milk was negatively associated with early initiation both in bivariate and regression analyses. This finding supports previous research that suggests a connection between a mother's belief surrounding transmission of maternal emotions through breast milk and breastfeeding practices.[35]

Early initiation of breastfeeding emerged as the only factor that increased the odds of EBF confirming studies in developing countries that show that breastfeeding initiation within one hr postpartum had a demonstrably positive impact on duration of exclusive breastfeeding.[50,51] EBF was also more common among mothers who received breastfeeding support from a *comadrona* but less common in those who believed in *enojo* transmission although neither emerged in the final regression analysis for EBF.

Very little research exists within a developing country context on cultural factors that influence breastfeeding frequency. The cultural practice of using a *temascal* was associated with higher breastfeeding frequency, which is consistent with indigenous mothers' perception that the *temascal* stimulates milk flow. The potential benefit of applying heat to the breast to stimulate milk flow has been documented.[52,53] However, *temascal* use is also a potentially dangerous source of carbon monoxide exposure.[54,55] Previous work in Guatemala has demonstrated a negative relationship between exposure to wood smoke and infant birth weight[56,57] but we did not find an association between a mother's weekly duration in the *temascal* and infant WAZ. Additionally, WHZ emerged as a determinant of feeding frequency likely because infant size is a determinant of breast milk demand to support the higher caloric needs of a larger infant.

When considering the impact of cultural practices and beliefs together with breastfeeding practices on infant anthropometry, we found that early initiation of breastfeeding was a significant predictor of infant WAZ within 46 days postpartum. Use of *agüitas* was more common in those who delayed initiation and therefore it may have had an indirect negative effect on WAZ. Previous research has suggested that infants introduced to *agüitas* before 2.9 weeks of age were more likely to be stunted[25], but we did not detect an effect on HAZ. We also found that WAZ, WHZ and HCZ were related to early initiation but not EBF, consistent with a recent meta-analysis showing that EBF is not associated with growth deficits within the first 6 mo[58]. A few studies have demonstrated that initiation within one hour was associated with reduced risk of underweight.[17,18,59]

Limitations and Strengths

Reliance on self-reported data on infant feeding practices was a limitation. We queried mothers with infants <46d which may have skewed data on the ever feeding of ritual fluids due to recall bias and therefore we included age of infant in all models to possibly correct for this limitation. We were only able to collect information on *agüita* use within the first day and not the first hour. Likewise, we did not ask mothers about their belief in the transmission of *susto* or *enojo* within the first hour, which may limit temporal associations. Missing data can be an impediment for data analysis although we found no differences in maternal, infant and cultural characteristics between dyads with full and missing data. Acknowledging that indigenous cultural practices during lactation are complex, our findings would benefit from further qualitative investigation. A major advantage to this study was our precise infant growth measurements and that we could associate cultural practices with optimal breastfeeding within 46 days postpartum.

CONCLUSION

The rationale by WHO to initiate breastfeeding within one hour postpartum does not explicitly address infant growth.[37] Our investigation in the Western Highlands of Guatemala has highlighted the link between cultural practices and beliefs during lactation, breastfeeding practices and infant growth. In our study, increased duration of *temascal* use was associated with increased breastfeeding frequency. Delivery at a *comadrona's* house promoted early breastfeeding initiation but the early feeding of *agüitas* and a mother's belief in *susto* transmission through breast milk were associated with delayed early initiation. Breastfeeding within one hour of birth was associated with exclusive breastfeeding which supports a recent finding in the US [60] but also with higher infant WAZ in our study. Further longitudinal research is warranted to explore the link between cultural practices throughout lactation and early infant growth.

DECLARATION OF CONFLICICITING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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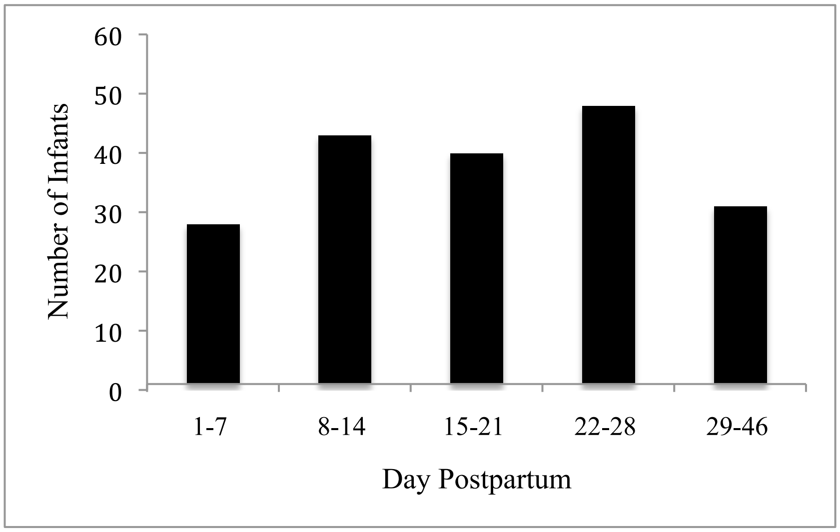
Figure 3-1. Age distribution of 190 *Mam*-Mayan infants in study (a) and percent per infant age group in days postpartum that have ever been fed *agüitas* (b).

(a)



(b)

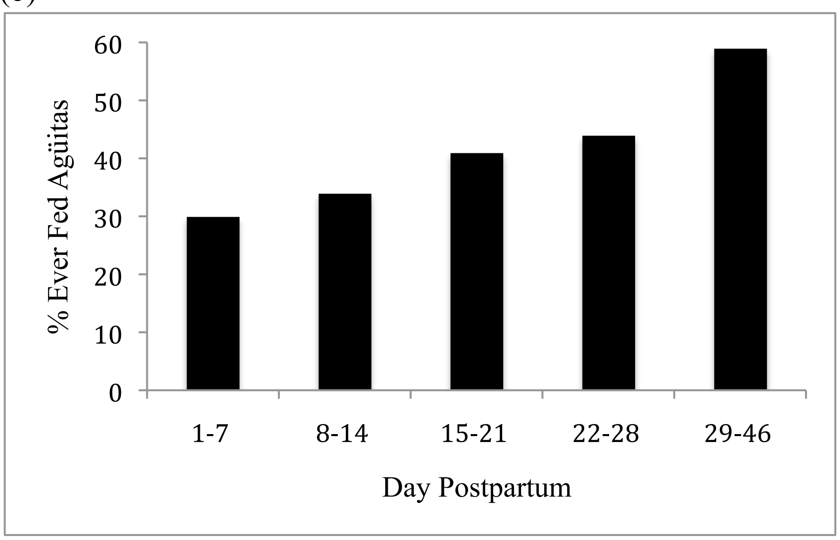


Table 3-1. Maternal and infant conditions for feeding *agüitas* to *Mam*-Mayan infants aged 2-46 d and associated types of *agüitas* used.

	Maternal Condition	**Infant Condition**
Reasons for feeding *agüitas*	*no tiene leche* has no milk	*porque solo lloraba* baby cried
	no tiene suficiente leche has insufficient milk	*cólico* colic
	el pecho es demasiado chiquito breast is too tiny	*porque tiene dolor de estómago* has stomach ache
		hinchado su estómago bloated stomach
		aire ailment from air and/or a distention from gas (culturally defined)
		frío excessive cold from loss of a hot-cold equilibrium (culturally defined)
		pujo infant exhibits grimaces as if straining to defecate (culturally defined)
Types of	*agua hervida* boiled water	*agua de anis* anise water

agüitas used	*agua azucarada* sugar water	*agua de naranja* orange-leaf water
	agua de manzanilla chamomile tea	*agua de hierba-buena* mint water
	agua de masa corn-paste water	*salvia santa* sage water

Table 3-2: Comparison between *Mam*-Mayan maternal-infant dyads who initiated breastfeeding < 1 hr (n=104) and > 1 hr (n=83) postpartum

	Initiation ≤ 1 hr Mean ± S.D or % (95% C.I.)	**Initiation > 1 hr** Mean ± S.D or % (95% C.I.)	**p-value**
Maternal characteristics and delivery experience			
Age category [a]			0.22
≤ 18 years (%)	20 (12.5-28.9)	23 (14.5-33.8)	
19-29 years (%)	64 (54.2-73.6)	52 (41.1-63.5)	
≥ 30 years (%)	16 (9.3-24.4)	24 (15.5-35.1)	
Weight (kg)	51.4 ± 8.2	50.6 ± 7.6	0.42
Height (cm)	146.1 ± 5.4	146.6 ± 5.1	0.48
Height < 145 cm (%)	41 (31.7-51.4)	40 (29.1-51.1)	0.82
BMI (kg/m^2)	24.0 ± 3.4	23.4 ± 2.9	0.23
Education			0.52
No education (%)	22 (14.5-31.3)	16 (8.6-25.2)	
Primary (%)	63 (52.4-71.8)	66 (55.0-76.2)	
Secondary or higher (%)	15 (9.0-23.7)	18 (10.4-28.0)	
Marital status			
Married/Union (%)	91 (84.2-95.9)	94 (86.5-98.0)	0.49
Occupation			
Housewife (%)	98 (93.2-99.7)	99 (93.4-99.9)	0.69

Language			
Spanish spoken (%)	55 (44.7-64.5)	55 (44.1-66.3)	0.93
Mam spoken (%)	95 (89.1-98.4)	96 (89.8-99.2)	0.68
Age at first pregnancy (yr)	18.3 ± 2.5	18.0 ± 3.0	0.48
Antenatal care (ANC)			
ANC during pregnancy (%)	98 (93.2-99.7)	99 (93.4-99.9)	0.69
ANC (times/pregnancy) [b]	5.4 ± 3.3	5.6 ± 3.1	0.76
Location of delivery			0.03
Home (%)	41 (30.8-50.4)	56 (45.2-67.4)	
Comadrona's house (%)	44 (34.5-54.3)	26 (17.4-37.3)	
Hospital/ Clinic (%)	15 (9.0-23.7)	18 (10.4-28.0)	
Birth attendant			
Comadrona (%)	82 (72.9-88.6)	81 (70.5-88.5)	0.86
Physician (%)	15 (9.0-23.7)	18 (10.4-28.0)	0.62
Parity			0.06
1st born (%)	31 (22.0-40.5)	42 (31.4-53.5)	
2nd-4th born (%)	54 (43.8-63.6)	37 (26.9-48.6)	
5th born or higher (%)	14 (8.3-22.6)	20 (12.4-30.7)	
Maternal breastfeeding experience			
Fed colostrum (%)	99 (94.7-99.9)	95 (88.1-98.6)	0.10
Breastfeeding frequency [c]			
Total (#/24 hr)	12.6 ± 5.2	11.1 ± 4.8	0.06
Daytime (#)	7.3 ± 2.9	6.4 ± 3.0	0.05

Nighttime (#)	5.3 ± 2.6	4.6 ± 2.4	0.06
Breastfeeding categories			<0.001
Exclusive (%)	75 (65.5-82.9)	39 (28.0-49.8)	
Predominant (%)	25 (17.0-34.4)	61 (50.1-71.9)	
Breastfeeding support	86 (77.7-91.7)	86 (76.1-92.3)	0.86
From health provider (%)	34 (24.6-43.5)	47 (35.9-58.2)	0.06
From *comadrona* (%)	11 (5.4-18.1)	8 (3.4-16.6)	0.62
From Mother / Mother-in-law (%)	42 (32.6-52.3)	31 (21.5-42.4)	0.12
Breastfeeding problems (%)	8 (3.3-14.6)	12 (5.9-21.0)	0.32
Cultural practices and beliefs during lactation			
Breast milk beliefs			
Susto (fright) in milk (%)	48 (38.1-58.0)	65 (53.8-75.2)	0.02
Enojo (anger) in milk (%)	41 (31.7-51.4)	57 (45.2-67.4)	0.03
Agüitas (ritual fluids)			
Ever fed to infant (%)	25 (17.0-34.4)	61 (50.0-71.9)	<0.001
Fed day 1 postpartum (%) [d]	24 (8.2-47.1)	73 (58.0-85.4)	<0.001
Reason for *agüitas* [e]			0.01
Maternal (eg. no milk) (%)	15 (4.3-34.8)	53 (38.4-67.0)	
Infant (eg. crying) (%)	70 (48.2-85.6)	31 (19.1-45.8)	
No reason/ Other (%)	15 (4.3-34.8)	16 (7.0-28.5)	
Infant characteristics			
Sex (male) (%)	57 (46.6-66.4)	53 (41.7-64.0)	0.61

Age (days)	18.9 ± 9.5	18.8 ± 9.8	0.92
HAZ score [f]	-1.50 ± 1.26	-1.78 ± 1.38	0.14
Stunted (< -2 SD) (%)	30 (21.2-39.5)	40 (30.1-50.9)	0.10
WAZ score [f]	-0.56 ± 1.09	-1.07 ± 1.14	<0.001
Underweight (< -2 SD) (%)	9 (4.1-16.0)	22 (13.3-32.0)	0.01
WHZ score [f]	0.82 ± 1.22	0.40 ± 1.18	0.02
Wasted (< -2 SD) (%)	3 (0.6-8.2)	2 (0.3-8.4)	0.65
HCZ score [f]	-0.33 ± 1.37	-0.76 ± 1.43	0.03
Microcephalay (< -2 SD)(%)	14 (7.7-21.9)	22 (13.3-32.0)	0.15

[a] maternal age (n=186)

[b] ANC frequency (n=174)

[c] feeding frequency (n=170)

[d] timing of *agüitas* (n=66)

[e] reason for *agüitas* (n=77)

[f] HAZ = height-for-age Z score; WAZ = weight-for-age Z score; WHZ = weight-for-height Z score; HCZ = head-circumference-for-age

Table 3-3: Comparison between *Mam*-Mayan maternal-infant dyads who fed by exclusive breastfeeding (n=113) and predominant breastfeeding (n=77) postpartum

	Exclusive Breastfeeding Mean ± S.D or % (95% C.I.)	**Predominant Breastfeeding** Mean ± S.D or % (95% C.I.)	**p-value**
Maternal characteristics and delivery experience			
Age category [a]			0.97
≤ 18 years (%)	21 (14.2-30.1)	20 (11.8-31.2)	
19-29 years (%)	58 (48.3-67.3)	59 (47.4-70.7)	
≥ 30 years (%)	21 (14.2-30.1)	20 (11.8-31.2)	
Weight (kg)	50.7 ± 7.2	51.6 ± 8.5	0.29
Height (cm)	145.9 ± 5.2	147.0 ± 4.9	0.12
Height < 145 cm (%)	39 (29.9-48.5)	42 (30.4-53.3)	0.71
BMI (kg/m^2)	23.8 ± 3.0	23.7 ± 3.3	0.80
Education			0.17
No education (%)	17 (10.4-25.0)	23 (14.4-34.4)	
Primary (%)	69 (59.6-77.3)	56 (44.0-67.1)	
Secondary or higher (%)	14 (8.3-21.9)	21 (12.3-31.5)	
Martial status			
Married/Union (%)	90 (83.3-95.0)	96 (89.0-99.1)	0.13
Occupation			

Housewife (%)	98 (93.7-99.7)	99 (92.9-99.9)	0.79
Language			
Spanish spoken (%)	54 (44.3-63.4)	57 (45.3-68.3)	0.66
Mam spoken (%)	99 (95.1-99.9)	91 (82.1-96.2)	0.01
Age at first pregnancy	18.1 ± 2.7	18.3 ± 2.8	0.49
Antenatal care (ANC)			
ANC during pregnancy (%)	98 (93.7-99.7)	99 (92.9-99.9)	0.79
ANC (times/pregnancy) [b]	5.5 ± 3.2	5.7 ± 3.3	0.72
Location of delivery			0.77
Home (%)	47 (37.4-56.5)	48 (36.5-59.7)	
Comadrona's house (%)	38 (29.0-47.6)	34 (23.3-45.4)	
Hospital/ Clinic (%)	15 (9.0-22.9)	18 (10.3-28.6)	
Birth attendant			
Comadrona (%)	82 (74.0-88.8)	79 (68.4-87.6)	0.59
Physician (%)	15 (9.0-22.9)	18 (10.3-28.6)	0.42
Parity			0.83
1st born (%)	34 (25.8-44.0)	37 (26.0-48.6)	
2nd-4th born (%)	48 (38.3-57.3)	43 (32.0-55.2)	
5th born or higher (%)	18 (11.1-26.0)	20 (11.4-30.4)	
Maternal breastfeeding experience			
Fed colostrum (%)	99 (95.1-99.9)	95 (87.0-98.5)	0.06
Initiation within 1 hr (%)	71 (61.4-79.1)	34 (23.3-45.4)	<0.001
Breastfeeding frequency [c]			

Total (#/24 hr)	11.7 ± 4.7	12.2 ± 5.5	0.50
Daytime (#)	6.7 ± 2.5	7.2 ± 3.5	0.36
Nighttime (#)	5.0 ± 2.5	5.0 ± 2.5	0.90
Breastfeeding support	86 (78.0-91.6)	87 (77.1-93.5)	0.84
From Health provider (%)	35 (25.8-44.0)	45 (34.0-57.2)	0.14
From *Comadrona* (%)	14 (8.3-21.9)	4 (0.8-10.9)	0.03
From Mother/ Mother-in-law (%)	39 (29.9-48.5)	36 (25.7-48.1)	0.72
Breastfeeding problems (%)	5 (1.9-11.3)	16 (8.3-25.6)	0.02
Cultural practices and beliefs during lactation			
Temascal (sauna) (%)	98 (93.7-99.7)	96 (89.0-99.1)	0.36
Frequency (times/week)	2.4 ± 0.7	2.5 ± 0.8	0.43
Duration (min./session)	30.0 ± 11.5	27.9 ± 9.1	0.19
Breast milk beliefs			
Susto (fright) in milk (%)	50 (40.8-59.9)	62 (50.5-73.1)	0.10
Enojo (anger) in milk (%)	42 (32.4-51.2)	57 (45.3-68.3)	0.03
Infant characteristics			
Sex (male) (%)	49 (39.1-58.2)	38 (26.8-49.4)	0.13
Age (days)	17.2 ± 9.1	21.5 ± 10.2	<0.001
HAZ score [d]	-1.69 ± 1.35	-1.63 ± 1.41	0.74
Stunted (< -2 SD) (%)	38 (29.3-48.0)	30 (20.2-41.8)	0.25
WAZ score [d]	-0.78 ± 1.20	-0.88 ± 1.18	0.58
Underweight (< -2 SD) (%)	14 (8.3-22.1)	17 (9.4-27.4)	0.59

WHZ score [d]	0.71 ± 1.2 2	0.51 ± 1.17	0.27
Wasted (< -2 SD) (%)	1 (0.1-4.8)	5 (1.4-12.7)	
HCZ score [d]	-0.57 ± 1.42	-0.56 ± 1.52	0.97
Microcephaly (< -2 SD) (%)	17 (10.5-25.2)	20 (11.4-30.4)	0.62

[a] maternal age (n=186)

[b] ANC frequency (n=174)

[c] feeding frequency (n=170)

[d] HAZ = height-for-age Z score; WAZ = weight-for-age Z score; WHZ = weight-for-height Z score; HCZ = head-circumference-for-age

Table 3-4: Comparison between *Mam*-Mayan maternal-infant dyads for breastfeeding frequency < 8 times (n=24), 8-12 times (n=83) and > 12 times (n=63) per day postpartum

	< 8 times/day Mean ± S.D or % (95% C.I.)	**8-12 times/day** Mean ± S.D or % (95% C.I.)	**>12 times/day** Mean ± S.D or % (95% C.I.)	**p-value**
Maternal characteristics and delivery experience				
Age category [a]				0.92
≤ 18 years (%)	26 (10.2-48.4)	20 (11.7-30.0)	19 (10.4-31.3)	
19-29 years (%)	57 (34.4-76.8)	62 (50.2-72.3)	58 (44.8-70.4)	
≥ 30 years (%)	17 (4.9-38.7)	16 (10.7-28.7)	23 (12.9-34.9)	
Weight (kg)	47.9 ± 4.4	51.4 ± 8.5	51.4 ± 8.1	0.14
Height (cm)	147.2 ± 5.4	145.8 ± 5.3	146.0 ± 4.9	0.51
Height < 145 cm (%)	33 (15.6-55.3)	43 (32.5-54.7)	41 (29.0-54.3)	0.67
BMI (kg/m^2) [b]	22.2 ± 2.2 [A]	24.1 ± 3.4 [B]	24.0 ± 3.2 [B]	0.04
Education				0.74
No education (%)	17 (4.7-37.3)	19 (11.4-29.4)	19 (10.2-30.9)	
Primary (%)	58 (36.6-77.8)	67 (56.3-77.3)	65 (52.0-76.6)	
Secondary or higher (%)	25 (9.7-46.7)	13 (6.8-22.4)	16 (7.8-27.2)	
Martial status				
Married/Union (%)	96 (78.8-99.8)	93 (84.9-97.3)	94 (84.5-98.2)	0.86
Occupation				

Housewife (%)	100 (85.7-100)	93 (84.9-97.3)	100 (94.3-100)	0.34
Language				
Spanish spoken (%)	42 (22.1-63.3)	53 (41.7-64.0)	60 (47.2-72.4)	0.28
Mam spoken (%)	92 (73.0-98.9)	95 (88.1-98.6)	97 (89.0-99.6)	0.59
Age at first pregnancy	17.9 ± 2.3	18.2 ± 2.6	18.0 ± 2.5	0.90
Antenatal care (ANC)				
ANC during pregnancy (%)	100 (85.7-100)	99 (93.4-99.9)	98 (91.4-99.9)	0.82
ANC (times/pregnancy) [c]	5.1 ± 2.9	5.2 ± 3.1	6.5 ± 3.5	0.06
Location of delivery				0.50
Home (%)	33 (15.6-55.3)	49 (38.2-60.6)	46 (33.3-59.0)	
Comadrona's house (%)	54 (32.8-74.4)	34 (23.7-44.9)	38 (26.1-51.2)	
Hospital/ Clinic (%)	13 (2.6-32.3)	17 (9.5-26.6)	16 (7.8-27.2)	
Birth attendant				
Comadrona (%)	88 (67.6-97.3)	82 (71.9-89.5)	79 (67.3-88.5)	0.67
Physician (%)	13 (2.6-32.3)	18 (10.4-28.0)	16 (7.8-27.2)	0.80
Parity				0.96
1^{st} born (%)	33 (15.6-55.3)	37 (26.2-47.9)	32 (20.5-44.6)	
2^{nd}-4^{th} born (%)	50 (29.1-70.8)	44 (32.9-55.3)	49 (36.3-62.1)	
5^{th} born or higher (%)	17 (4.7-37.3)	20 (11.5-29.7)	19 (10.2-30.9)	
Maternal breastfeeding experience				
Fed colostrum (%)	100 (85.7-100)	95 (87.9-98.6)	98 (91.4-99.9)	0.33
Initiation within 1 hr (%) [d]	42 (22.1-63.3)^{A}	48 (36.4-58.8)^{A}	68 (54.6-79.0)^{B}	0.02

Breastfeeding categories				0.81
Exclusive (%)	58 (36.6-77.8)	57 (45.2-67.4)	62 (48.8-73.8)	
Predominant (%)	42 (22.1-63.3)	43 (32.5-54.7)	38 (26.1-51.2)	
Breastfeeding support	88 (67.6-97.3)	89 (80.4-94.9)	86 (74.6-93.2)	0.82
From Health provider (%)	54 (32.8-74.4)	35 (24.8-46.1)	38 (26.1-51.2)	0.23
From *Comadrona* (%)	0 (0.0-14.2)	13 (6.8-22.4)	11 (4.5-21.5)	0.18
From Mother/ Mother-in-law (%)	25 (9.7-46.7)	43 (32.5-54.7)	38 (26.1-51.2)	0.26
Breastfeeding problems (%)	21 (7.1-42.1)	12 (5.9-21.0)	5 (0.9-13.2)	0.08
Cultural practices and beliefs during lactation				
Temascal (sauna) (%)	100 (85.7-100)	96 (89.8-99.2)	98 (91.4-99.9)	0.51
Frequency (times/week) [e]	2.2 ± 0.6 [A]	2.3 ± 0.6 [A]	2.7 ± 1.0 [B]	0.02
Duration (min./session)	26.0 ± 8.9	29.9 ± 11.1	29.0 ± 9.8	0.27
Breast milk beliefs				
Susto (fright) in milk (%)	50 (29.1-70.8)	57 (45.2-67.4)	63 (50.4-75.2)	0.47
Enojo (anger) in milk (%)	42 (22.1-63.3)	49 (38.2-60.6)	56 (42.4-68.0)	0.48
Agüitas (ritual fluids)				
Ever fed to infant (%)	42 (22.1-63.3)	43 (32.5-54.7)	38 (26.1-51.2)	0.81
Infant characteristics				
Sex (male) (%)	67 (44.6-84.3)	47 (35.9-58.2)	62 (48.8-73.8)	0.09
Age (days)	17.0 ± 10.0	19.8 ± 10.6	19.6 ± 8.8	0.45
HAZ score [f]	-1.65 ± 1.21	-1.72 ±1.35	-1.82 ± 1.49	0.85

Stunted (< -2 SD)(%)	38 (18.8-59.4)	38 (27.3-49.1)	37 (25.1-50.3)	0.99
WAZ score [f]	-1.05 ± 0.97	-1.00 ± 1.10	-0.72 ± 1.32	0.31
Underweight (< -2 SD) (%)	17 (4.7-37.3)	15 (7.8-24.1)	18 (9.2-29.5)	0.87
WHZ score [f, g]	0.28 ± 1.17 [A]	0.39 ± 1.09 [B]	1.10 ± 1.25 [C]	<0.001
Wasted (< -2 SD)(%)	4 (0.1-21.1)	4 (0.8-10.2)	2 (0.1-8.5)	0.72
HCZ score [f]	-0.49 ± 1.76	-0.66 ± 1.31	-0.59 ± 1.54	0.87
Microcephaly (< -2 SD)(%)	29 (12.6-51.0)	17 (9.6-26.9)	18 (9.2-29.5)	0.39

[a] maternal age (n=166)

[b] different uppercase letters indicate significant differences in breastfeeding frequency for BMI.

[c] ANC frequency (n=156)

[d] different uppercase letters indicate significant differences in breastfeeding frequency for initiation within 1 hr postpartum.

[e] different uppercase letters indicate significant differences in breastfeeding frequency for *temascal* frequency.

[f] HAZ = height-for-age Z score; WAZ = weight-for-age Z score; WHZ = weight-for-height Z score; HCZ = head-circumference-for-age

[g] different uppercase letters indicate significant differences in breastfeeding frequency for WHZ.

Table 3-5: Multiple logistic regression models for exclusive breastfeeding (n=190) and for initiation of breastfeeding within 1 hr postpartum (n=187) for *Mam*-Mayan mother-infant dyads

Characteristic	**Odds Ratio (OR)**	**95% C.I.**	**p-value**
Breastfeeding within 1 hr postpartum [a]			
Delivery at *comadrona*'s house (0=no, 1=yes)	2.52	1.31 - 4.86	0.01
Don't believe in *susto* transmission (0=do believe, 1=don't believe)	2.42	1.30 - 4.57	0.01
Exclusive breastfeeding [b]			
Infant age (days)	0.95	0.92 - 0.99	0.01
Breastfeeding within 1 hr postpartum (0=no, 1=yes)	6.18	3.07 - 12.43	<0.001

[a] Overall model χ^2 (5)= 17.38, p <0.001; Nagelkerke R^2 = 12.1%

[b] Overall model χ^2 (6)= 43.45, p <0.001; Nagelkerke R^2 = 28.5%

Table 3-6: Multiple linear regression models for breastfeeding frequency (n=170) and for infant WAZ score (n=170) for *Mam*-Mayan mother-infant dyads

Characteristic	**Unstandardized Regression Coefficient**	**Standard Error of the Coefficient**	**Standardized Coefficient**	**p-value**
Breastfeeding frequency [a]				
Temascal duration (total minutes/week)	0.05	0.01	0.26	<0.001
Infant WHZ score	1.27	0.31	0.30	<0.001
Infant WAZ score [b]				
Breastfeeding within 1 hr postpartum (0=no, 1=yes)	0.39	0.16	0.18	0.02
Maternal weight (kg)	0.05	0.01	0.34	<0.001

a Overall model: $F(2,158)=14.48$, $p <0.001$, adjusted R^2 = 14.4%.Variables with $p > 0.10$ based on backward elimination: ever fed *agüitas* (0=no, 1=yes); *Susto* transmission (0=do believe, 1=don't believe); *Temascal* frequency (times/week) ; Infant sex (0=female, 1=male); Infant age (days); Mother weight (kg); Breastfeeding within 1 hr postpartum (0=no, 1=yes).

b Overall model: $F(2,158)=14.61$, $p <0.001$, adjusted R^2 = 14.5%. Variables with $p > 0.10$ based on backward elimination: Ever fed *agüitas* (0=no, 1=yes); Breastfeeding frequency (total times per 24 hours); *Temascal* duration (total minutes/ week); Infant sex (0=female, 1=male); Infant age (days).

Connecting Statement I

In Chapter III, I demonstrated that cultural practices used by Indigenous *Mam*-Mayan mothers in the Western Highlands of Guatemala were associated with initiation of breastfeeding and increased breastfeeding frequency. Specifically, I showed that early initiation of breastfeeding was delayed by use of *agüitas,* by beliefs surrounding transmission of *susto* and was enhanced by delivery at the *comadrona's* house. I also showed that the cultural practice of using a *temascal* was associated with higher breastfeeding frequency. Given that my results indicated that cultural practices were associated with WHO infant feeding recommendations (WHO, 2003), I was interested in further understanding if these practices were associated with subclinical mastitis.

In Chapter IV, I sought to investigate subclinical mastitis using a cross sectional design. My approach was to: (1) compare mothers with subclinical mastitis (breast milk Na/K ratio >0.6) and without subclinical mastitis for cultural practices identified in Chapter III. In addition, I sought to compare mothers with and without subclinical mastitis for other selected factors and practices including socio-demographics, delivery experience, breastfeeding practices, activities of daily living and health status. (2) evaluate if subclinical mastitis increased the likelihood of infant stunting (LAZ<-2SD), underweight (WAZ<-2SD) and microcephaly (HCZ<-2SD). The latter objective was grounded in previous research, which suggested that the effects of subclinical mastitis are not confined to the maternal system but may be evident in infants through reduced weight and length (Kasonka et al., 2006).

CHAPTER IV

Subclinical Mastitis is Associated with Impaired Anthropometric Indicators among Indigenous *Mam*-Mayan Mothers in The Western Highlands of Guatemala

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WELL ESTABLISHED

An elevated breast milk Na/K ratio is indicative of subclinical mastitis (SCM), an asymptomatic inflammatory condition of the lactating breast. In the dairy industry, cows with SCM produce less milk and have calves with reduced weight.

NEWLY EXPRESSED

Among indigenous *Mam*-Mayan mother-infant dyads living in the Western Highlands of Guatemala, a breast milk Na/K ratio >0.6 was associated with a higher odds of infant stunting, underweight and microcephaly.

Abstract

Background: Among indigenous *Mam*-Mayan mothers living in the Western Highlands of Guatemala, the prevalence of subclinical mastitis (SCM) and its association with infant anthropometry is unknown.

Objectives: This cross sectional study compared mothers with SCM (breast milk Na/K ratio >0.6) and without SCM for selected factors and evaluated if SCM was associated with a higher odds of infant stunting (LAZ<-2SD), underweight (WAZ<-2SD) and microcephaly (HCZ<-2SD).

Methods: A unilateral breast milk sample was collected from mothers at 0-6 weeks (n=52) or 4-6 months (n=53) postpartum. A structured questionnaire on cultural and breastfeeding practices, physical activity, maternal and infant health status and sanitation was administered. Maternal and infant anthropometry and maternal fecal and urine samples were collected.

Results: Fourteen percent of all mothers (n=105) had a milk Na/K ratio >0.6, which was more prevalent at 0-6 weeks (19%) than 4-6 months (9%) postpartum. Older maternal age, higher parity, higher breastfeeding frequency, and increased hours of maternal daily walking characterized mothers with a milk Na/K ratio >0.6. Importantly, SCM was associated with a higher odds of infant stunting (odds ratio [OR]=4.28, C.I.=1.15, 15.84), underweight (OR=6.91, C.I.=1.48, 32.23) and microcephaly (OR=7.73, C.I.=1.68, 35.53). In addition, the presence of maternal stool *Entamoeba coli* was also associated with a higher odds of microcephaly

(OR=3.29, C.I.=1.11, 11.00) whereas increasing maternal height (OR=0.89, 95% CI=0.79-0.96) was associated with a lower odds of stunting.

Conclusion: Given the demonstrated associations between SCM and anthropometric indicators of poor infant growth, improving maternal breast health may be required to improve infant growth.

BACKGROUND

Subclinical mastitis (SCM) is an asymptomatic inflammatory condition of the lactating breast and has been associated with adverse outcomes including increased risk of mother-to-child transmission of HIV.[1-4] During SCM, tight epithelial junctions become "leaky", allowing movement of plasma constituents, including sodium ions, into milk.[5.6] An elevated breast milk sodium/potassium ratio (Na/K) is considered indicative of SCM and both ratios of Na/K >0.6 [7-10] and Na/K >1.0[11-14] have been used to indicate SCM. Maternal factors positively associated with SCM include younger age [13,14] and primiparity. [4,11,14] Non-exclusive breastfeeding is also positively associated with SCM.[1, 11] Low to moderate grades of maternal undernutrition[13] and micronutrient deficiency [9,15,16] have been previously implicated but not clearly established. One study found that mothers who received lactation counseling had significantly lower rates of SCM than those without counseling.[17]

Less explored is whether maternal activities of daily living and cultural practices differ between mothers with and without SCM. In developing countries, it is generally believed that activities of daily living demand greater time and energy expenditure than comparable pursuits in developed countries.[18,19] Physical activity research in Guatemala has focused on primary domains of activity including walking, farming, domestic activities, and leisure-time activity.[20] It is not known whether these maternal activities of daily living are associated with SCM. Likewise, it is unknown if common cultural practices, deeply embedded in Guatemalan culture, are associated with SCM. In this population, several cultural practices have previously been linked to breastfeeding practices including the feeding of *agüitas* (ritual fluids), a practice shown to delay early initiation of breastfeeding and to disrupt exclusive breastfeeding in infants less than 6 months of age.[21] In addition, higher breastfeeding frequency was observed among mothers who spent more time in the *temascal* (sauna).[21] However, it is not known whether such practices are associated with SCM.

Moreover, in human lactation literature, there is some evidence that SCM may be associated with poor weight gain in breastfeeding infants [7,15,22] and negatively correlated with infant length at 6 weeks postpartum among both HIV-infected and uninfected mothers.[11,12] It is not known whether SCM is associated with infant anthropometry but given the high rates of growth faltering observed in this Guatemalan population[23], and given the limited information on SCM and its potential association with infant anthropometry, further investigation is warranted.

The goals of this study were to compare indigenous *Mam*-Mayan mothers with SCM (breast milk Na/K ratio >0.6) and without SCM for selected factors and practices and to explore if SCM was associated with a higher odds of infant stunting (LAZ<-2SD), underweight (WAZ<-2SD) and microcephaly (HCZ<-2SD) in the Western Highlands of Guatemala.

METHODS

Study Design

This cross-sectional study of breastfeeding mother-infant dyads at 0-6 weeks (n=52) or 4-6 months (n=53) postpartum was conducted in eight rural *Mam*-Mayan communities in the Western Highlands of Guatemala. An in-depth structured questionnaire was administered and a single breast milk sample was collected and later analyzed for Na and K. Mothers with breast milk Na/K ratio >0.6 were classified as having SCM.[7-10] Anthropometry of both mother and infant was measured and maternal fecal and urine samples were collected at a local health centre or at the participants' home.

Study Recruitment and Ethical Considerations

From June 2012 - January 2013, *comadronas* (traditional midwives) and community health care workers recruited breastfeeding mother-infant dyads at 0-6 weeks or 4-6 months postpartum. Of the 124 mothers initially enrolled, 117 (94%) provided breast milk samples. Mothers with an inability to provide at least 5 ml of milk (n=6) and not being the birth mother (n=1) were excluded. To avoid collection of milk before the reported closing of mammary tight junctions[24,25], mothers with infants less than 4 days (n=4) and mothers with breast milk volumes insufficient for triplicate analysis (n=8) were removed. Complete data (questionnaire and breast milk samples) were available for 105 breastfeeding mother-infant dyads with almost an equal number of infants at 0-6 weeks (n=52) and 4-6 months (n=53) postpartum. Ethical approval was obtained from McGill University Institutional Review Board and CeSSIAM (Center for Studies of Sensory Impairment, Aging and Metabolism), a Guatemala–based research organization. All mothers provided fully informed consent.

Questionnaire

The study questionnaire was developed in June 2011 and pilot tested for cultural appropriateness in 50 non-participant mothers with similar characteristics to the intended study population. Trained community health care workers administered the questionnaire orally in either Spanish or *Mam* to participants during a 30-40 min interview. In-depth details of the questionnaire have been described previously.[21] In brief, the questionnaire addressed the following topics: socio-demographics (household assets), hygiene and sanitation (water source, toilet), maternal characteristics (age, martial status, highest level of education), maternal and infant health within the last week (fever, headache, stomach ache, cough, flu, diarrhea), maternal delivery experience (age at first pregnancy, frequency of antenatal care, delivery location, parity), and maternal activities of daily living (time and type of activity were categorized as domestic chores in home, agriculture/outside chores, walking, and social activities). In addition, the questionnaire addressed cultural concepts of maternal and infant health (*susto* [fright], *enojo* [anger], *pujo* [air in stomach]), and cultural practices (milk transmission beliefs for *susto* and *enojo*, feeding of *agüitas* [ritual fluids], frequency and duration of *temascal* [sauna] use). Questions on breastfeeding practices (early initiation within 1 hr postpartum, frequency per day and night, exclusive, predominant and mixed feeding as defined by WHO[26] and breastfeeding support (provider, problems) were also included.

Maternal Fecal and Urine Collection and Analysis

Methods for collection and analysis of maternal fecal and urine samples have been previously described.[23] Briefly, a single stool sample was analyzed for protozoa using direct smear by an experienced laboratory technician trained in parasitology. Non-pathogenic protozoa (*Blastocystis hominis, Entamoeba coli*, *Iodamoeba butschli* and *Endolimax nana*) were used in this study as a measure of exposure to potentially contaminated food and water sources.[27] Maternal urine samples were analyzed for pyuria using dipstick analysis and microscopy.

Anthropometry

Two trained Guatemalan nutritionists measured maternal weight (kg) in triplicate (± 100 grams) using a digital scale (Seca 803) and height in duplicate (± 0.5 cm) using a wall stadiometer. Infant age was calculated based on date of birth recorded on the mother's health card or in the absence of a card, reported date of birth. The majority of births (79%) were attended by a *comadrona* and occurred at either the home of the mother or the *comadrona's* home. Recumbent length (cm) was measured in triplicate (± 0.5 cm) using an infantometer (Seca 210). Weight (kg) was measured using a digital infant scale (Seca 354) to the nearest 100 g. Head circumference was measured with a baby band (Seca 212). Infants were classified as stunted (length-for-age Z score (LAZ) < -2SD), underweight (weight-for-age Z score (WAZ) < -2SD) or wasted (weight-for-height Z score (WHZ) < -2SD) according to WHO Growth Reference Standards using WHO Anthro Software (3.1). Head circumference-for-age (HCZ score) was used to identify infants with microcephaly (< - 2SD).

Breast Milk Collection, Storage and Analysis

The protocol for breast milk collection was developed in June 2011 and pilot tested for feasibility and to ensure acceptability within the indigenous *Mam*-Mayan population. Mothers in this population consider breast milk collection to be non-invasive. Care was taken to minimize behavioral disruption between mother and infant.[28] Under the supervision of a trained *comadrona,* a single unilateral milk sample was collected via manual expression after the breast was cleaned with 70% ethyl alcohol. Mothers were asked to provide full milk expression from the breast not recently used for breastfeeding and all samples were collected in the morning. Time since last feeding was not recorded. Milk was collected in 60 ml plastic vials, immediately stored in a cold chest and subsequently aliquotted under sterile conditions and stored at -30º C. Milk samples were shipped on dry ice to McGill University where samples remained frozen at -80º C until analyzed for Na and K; mothers with breast milk Na/K ratio >0.6 were classified as having SCM. The ratio permits the use of spot milk samples without consideration of time of sampling or time since the infant was last fed[15,16], controls for assay variations that may occur due to the unequal distributions of the ions in the aqueous and lipid phases of milk[13] and accounts for the normal and almost parallel decline in both Na and K ions during lactation.[10,29]

Analysis of Sodium and Potassium Concentrations in Breast Milk

Inductively-coupled plasma mass spectrometry (ICP-MS) [Bruker ICP-820MS] was used to measure Na and K. Briefly, breast milk samples (0.5 ml) were digested in triplicate 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. Six quality controls including 0.16 g of commercial milk powder and 0.12 g of peach leaves (Standard Reference Material, National Institute of Standards and Technology, Gaithersburg, MD) and six reagent blanks were included in each analytical run. Digested samples, reference materials and 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% and the obtained solutions were measured.

Statistical Analysis

Data were analyzed using IBM SPSS statistical software version 22.0 (SPSS Inc./IBM Chicago IL, USA). Descriptive statistics (mean ± S.D, percentages ± 95% C.I.) were computed and chi-squares (x^2) and Fisher's exact test (categorical) and Student t-tests were used to determine whether selected factors and practices differed between mothers with and without SCM (see Table 1). Individual multiple logistic regression models were created for infant stunting (Model 1), underweight (Model 2) and microcephaly (Model 3). To build each anthropometric model, all variables from each univariate analyses (stunted vs. non, underweight vs. non, microcephaly vs. non) with $p \leq 0.25$ were considered for inclusion. Variables were further grouped into common themes as follows: maternal delivery experience (age at first pregnancy, frequency of antenatal care, delivery location, parity), maternal characteristics (age, weight, height, BMI, martial status, highest level of education), maternal health (*susto, enojo,* fever, headache, stomach ache, pyuria, cough, flu, diarrhea), maternal stool pathogens, maternal activities of daily living, cultural practices and beliefs, infant health (*susto, enojo, pujo,* fever, cough, flu, diarrhea), infant anthropometry, hygiene and sanitation (water source, toilet), socio-demographics (household assets), and breastfeeding practices (early initiation within 1 hr postpartum, frequency per day and night, exclusive, predominant and mixed feeding, breastfeeding support). Highly correlated variables ($p > 0.7$, VIF >10) within each theme were not tested in the same model to avoid multi-collinearity.

Combinations of non-correlated variables from each theme were entered into each anthropometric model. In addition, the variable "SCM" (SCM vs. Non SCM) and "Stage of lactation" (early vs. established) were included in all anthropometric regression models to test study objectives. For Model 1, variables with $p \leq 0.25$ from univariate analyses were: maternal characteristics (height, weight, BMI), maternal health (cough, stomach ache, *enojo*), maternal stool pathogens (*B.hominis*, *E.nana, E.coli*), maternal activities of daily living (activity hours in agriculture) and infant health (*susto*, cough, diarrhea). For Model 2, variables with $p \leq 0.25$ from univariate analyses were: maternal characteristics (age <34yrs), breastfeeding frequency (#/day), infant anthropometry (head circumference) and maternal activities of daily living (activity hours in agriculture). For Model 3, variables with $p \leq 0.25$ from univariate analyses were: maternal characteristics (height, weight, BMI >25), maternal health (headache), maternal delivery experience (age at first pregnancy), maternal stool pathogens (*E.coli),* maternal activities of daily living (activity hours in walking), breastfeeding practices (formal support) and infant health (*pujo*). For all multiple logistic regression models, the Nagelkerke R^2 and the Hosmer and Lemeshow statistical test for goodness of fit were reported [30] and variables with $p<0.05$ were considered significant."

RESULTS

Population Characteristics (Table 1)

Fourteen percent of all mothers (n=105) had SCM (Na/K ratio >0.6), which was more prevalent during 0-6 weeks (19%, n=53) compared to 4-6 months (9%, n=52) postpartum. As expected, the mean breast milk Na/K ratio was higher for mothers with SCM (0.75 ± 0.15, n=15) compared to mothers without SCM (0.41 ± 0.08, n=90). This indicator did not differ between transitional (4-17d) and early milk (18-46d) (data not shown).

Only 2% of mothers had pathogenic protozoa in their stool (data not shown) with prevalence of non-pathogenic protozoa as follows: *Entamoeba coli* (30%), *Endolimax nana* (19%), *Blastocystis hominis* (15%), and *Iodamoeba butschli* (9%). The prevalence of infant stunting, underweight and microcephaly among mothers with SCM was 60%, 33% and 40%, respectively (Figure 1).

Univariate Comparisons Between Mothers with SCM (Na/K ratio >0.6) and without SCM

Mothers with SCM were older (28.8 ± 6.4 vs. 23.9 ± 6.2 yrs, p=0.006) and had higher parity (4.2 ± 3.0 vs. 2.5 ± 2.0, p=0.005) compared to mothers without SCM (Table 1). There was no difference between SCM and non-SCM mothers for other maternal characteristics and socio-demographic factors, although a non-significant trend was observed for mothers with SCM who were less likely to have a home faucet for a water source compared to mothers without SCM (67% vs. 86%, p=0.072) (Table 1).

Breastfeeding frequency/ 24 hours was higher for mothers with SCM (15.0 ± 6.0) compared to mothers without SCM (12.0 ± 4.2) where specifically, frequency during the day was higher for mothers with SCM (9.4 ± 3.7 vs. 7.1 ± 9.4, p=0.008). Mothers with SCM walked more hours daily (1.2 ± 1.4 vs. 0.3 ± 0.9 hrs, p=0.003) compared to mothers without SCM. None of the cultural practices (*temascal* frequency and/or duration, feeding of *agüitas*) and cultural milk transmission beliefs (*susto, enojo*) differed between mothers with and without SCM. Likewise maternal delivery experience and breastfeeding support did not differ between mothers with and without SCM (data not shown).

Infants of mothers with SCM had lower WAZ scores (-1.43 ± 1.38 vs. -0.87 ± 0.90, p=0.043) and a similar, but non-significant, trend was observed for HAZ score (-2.47 ± 1.92 vs. -1.82 ± 1.21, p=0.083). SCM mothers had a higher percentage of underweight infants (WAZ < -2SD) (33% vs. 11%, p=0.023) and infants with microcephaly (HCZ <-2SD) (40% vs. 16%, p=0.027) (Figure 1). There was no difference between mothers with and without SCM for any other measures of maternal and/or infant health and anthropometry (data not shown).

Regression Models (Table 2)

Model 1: Infant Stunting (HAZ < - 2SD)

Subclinical mastitis (OR=4.28, 95% CI=1.15-15.84) was associated with a higher odds of infant stunting, whereas increasing maternal height (OR=0.89, 95% CI=0.79-0.96) was associated with a lower odds. Variables that emerged in the regression model but were not significant were infant age group (0-6 weeks vs. 4-6 months), infant diarrhea and maternal stool *Entamoeba coli* with the overall Nagelkerke R^2 =0.208 (p=0.005) and Hosmer and Lemeshow goodness of fit x^2 (8)=6.81 (p=0.557).

Model 2: Infant Underweight (WAZ < - 2SD)

Subclinical mastitis (OR=6.91, 95% CI=1.48-32.23) was the only variable that was associated with a higher odds of infant underweight. Variables that emerged in the regression model but were not significant were infant age group, infant head circumference, and feeding frequency with the overall Nagelkerke R^2 =0.161 (p=0.05) and Hosmer and Lemeshow goodness of fit x^2 (8)=9.73, p=0.287.

Model 3: Infant Microcephaly (HCZ < - 2SD)

Subclinical mastitis (OR=7.73, 95% CI-1.68-35.53) and maternal stool *Entamoeba coli* (OR=3.49, 95% CI=1.11-11.00) were associated with a higher odds of infant microcephaly. Variables that that emerged in the regression model but were not significant were infant age group, maternal height, and age at first pregnancy with the overall Nagelkerke R^2 = 0.256 (p=0.004) and Hosmer and Lemeshow goodness of fit x^2 (8)=4.51, p=0.808.

DISCUSSION

Our study in Guatemala provides the first report on the prevalence of SCM (Na/K ratio >0.6) and its association with infant anthropometry. Mothers with SCM were characterized by older maternal age, higher parity, higher breastfeeding frequency, and higher hours of maternal daily walking compared to mothers without SCM. Cultural practices, deeply embedded in Guatemalan culture, including the feeding of *agüitas* (ritual fluids) and frequency and duration of *temascal* (sauna) use did not differ between mothers with and without SCM. Importantly, SCM was associated with a higher odds of infant stunting, whereas increasing maternal height was associated with a lower odds of stunting. SCM also emerged as the only factor to increase the likelihood of infant underweight and interestingly, SCM and non-pathogenic *Entamoeba coli* were associated with a higher odds of infant microcephaly. Taken together, SCM, as measured by a breast milk Na/K ratio >0.6, emerged as a previously overlooked determinant of infant underweight, stunting, and microcephaly in the Western Highlands of Guatemala.

In our study, SCM was associated with a higher odds of infant underweight. This association compliments previous research, which also showed that maternal SCM was associated with lower infant weight.[7,15,22] How SCM negatively is associated with infant weight is unknown although several suggestions have been proposed. It has been shown that a "salty" taste of milk, due to increased milk sodium from mastitic breasts, may decrease milk acceptability and milk intake by the infant.[31] A recent study upheld this notion demonstrating that an increase in milk saltiness, using a taste sensing model, was associated with inflamed breasts.[32] It has also been proposed that mothers with SCM may produce less milk, which occurs in lactating cattle with

SCM.[33-35] However, a study in humans showed that maternal SCM did not effect breast milk intake among 3-6 month old infants, suggesting that quality of milk composition rather than quantity of milk production may be a determining factor in the association between SCM and lower infant weight.[14]

Additionally, our data showed that SCM was associated with a higher odds of infant stunting. This finding supports a previous study of infants born to HIV-infected and uninfected Zambian mothers, which demonstrated a negative association between breast milk Na/K ratio and infant length-for-age at 6 weeks postpartum.[11,12] A more recent study also found an association between SCM and lower infant length in infants less than 3 months[14] although, to the best of our knowledge, this is the first study to demonstrate this relationship in Guatemala where our understanding of the etiology of infant stunting is incomplete.[36] SCM may therefore add to a list of emerging subclinical conditions, including environmental enteropathy[37,38] , that might contribute to stunting.

An intriguing finding was that infant microcephaly was associated with not only SCM but with the presence of non-pathogenic bacteria. Microcephalic infants could have a less developed suckle [39-41] which may be associated with milk stasis, which is a risk factor for SCM.[42] Our data also showed that *Entamoeba coli* in maternal stool was associated with a higher odds of infant microcephaly. Typically, *Entamoeba coli* are considered non-pathogenic protozoa.[43] However, it is not inconceivable that fecal contamination of the breast through poor sanitation could put the infant in direct contact with exposure to non-pathogenic protozoa during breastfeeding.[44.45] Literature has suggested a relationship between non-pathogenic protozoa and infant prematurity[46] and poor infant nutritional status.[47] However further research will be required to understand how non-pathogenic protozoa and SCM may be interrelated and contribute to microcephaly.

Lastly, several factors differed between mothers with and without SCM. Higher breastfeeding frequency, specifically during daytime hours, was observed in mothers with SCM. Mothers with SCM may have increased feeding frequency if breast milk volume was reduced, as observed in the dairy industry where cows with SCM produce less milk. [34,35] In comparison, the cultural feeding of *agüitas* (ritual fluids) and frequency and duration of *temascal* (sauna) use did not differ between mothers with and without SCM. Older maternal age and higher parity characterized mothers with SCM. Intriguingly, our results are similar to results from dairy literature that showed that higher parity and increased age are “cow level” risk factors associated with SCM.[35] Previous research has also shown that maternal stress [48,49] and fatigue [50,51] may contribute to *clinical* mastitis, although unclear, the inter-relationship between stress, fatigue and mastitis could possibly be explained by mother’s susceptibility to breast infection when these conditions co-exist, [52,53] however, this requires further investigation.

Strengths and Limitations

To the best of our knowledge, this is the first study to identify the contribution of SCM to infant growth faltering in a non-HIV population. However, as this was a cross sectional study, we could not infer causality. Because we lacked data for gestational age, we could not control for this variable in our statistical models. Only maternal and not infant stool samples were collected.

Finally, because all mothers received breastfeeding support we could not explore whether additional breastfeeding counseling may have limited SCM.

CONCLUSION

Subclinical mastitis was associated with poor infant anthropometry within the first 6 months postpartum in indigenous *Mam* Mayan mothers in the Western Highlands of Guatemala. Given that the first 1000 days of life have been recognized as a "window of opportunity" to improve maternal and child health[54], a greater focus on maternal breast health may be required to improve anthropometric indicators of poor infant growth. Health professionals need to consider asymptomatic SCM as a factor that is associated with infant anthropometry. Given that our study also found an association between maternal stool *Entamoeba coli* and infant microcephaly, integrated interventions that address measures of hygiene and sanitation, maternal breast health and infant feeding may be required to improve infant growth.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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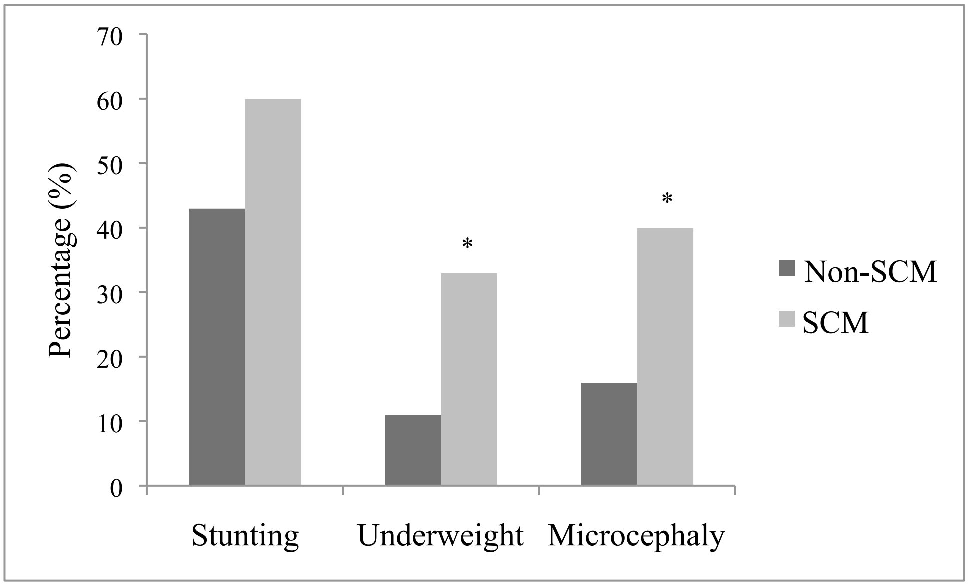
Figure 4-1: Prevalence of infant stunting, underweight and microcephaly among mothers with and without SCM



* Statistical significance established at $p < 0.05$

Table 4.1. A comparison of factors between breast milk Na/K ratio ≤0.6 and Na/K ratio >0.6 [a]

	Non SCM **Na/K ratio ≤0.6** (n=90) Mean ± S.D or % (95%C.I.)	**SCM** **Na/K ratio >0.6** (n=15) Mean ± S.D or % (95%C.I.)	**P-value**
Maternal Characteristics			
Age (years)	23.9 ± 6.2	28.8 ± 6.4	* 0.006
Weight (kg)	51.4 ± 7.9	52.4 ± 8.1	0.656
Height (cm)	146.6 ± 5.1	148.2 ± 3.9	0.264
BMI (kg/m^2)	23.9 ± 3.2	23.8 ± 3.3	0.949
Parity (#)	2.5 ± 2.0	4.2 ± 3.0	* 0.005
Maternal Health			
Susto (fright) (yes)	44 (33.9-55.3)	60 (32.2-83.6)	0.264
Enojo (anger) (yes)	21 (13.2-30.9)	27 (7.7-55.1)	0.736
Headache (yes)	28 (18.8-38.2)	40 (16.3-67.7)	0.337
Stomach ache (yes)	14 (7.9-23.4)	20 (4.3-48.0)	0.697
Pyuria (yes) [b]	22 (14.3-32.5)	29 (8.3-58.1)	0.734
Blastocystis hominis (yes) [b]	17 (9.4-26.3)	36 (12.7-64.8)	0.138
Entamoeba coli (yes) [b]	39 (28.8-50.5)	36 (12.7-64.8)	0.800
Endolimax nana (yes) [b]	27 (18.2-38.2)	7 (0.1-33.8)	0.177
Infant Health			
Diarrhea (yes)	22 (14.3-32.5)	53 (26.5-78.7)	0.434

Cultural Practices & Beliefs			
Temascal (sauna) use (yes)	97 (90.5-99.3)	93 (68.0-99.8)	0.532
Temascal frequency (times/week)	2.1 ± 0.8	2.5 ± 1.6	0.260
Temascal duration (minutes/week)	68.4 ± 31.1	75.7 ± 51.6	0.465
Susto (fright) through milk (yes)	50 (39.2-60.7)	60 (32.2-83.6)	0.473
Enojo (anger) through milk (yes)	34 (24.7-45.2)	40 (16.3-67.7)	0.677
Agüitas (ritual fluids) (yes)	40 (29.8-50.8)	40 (16.3-67.7)	1.000
Maternal Activities of Daily Living			
Domestic chore in home (hr)	2.9 ± 2.7	1.8 ± 2.2	0.167
Agriculture/Outside chore (hr)	1.3 ± 1.9	1.1 ± 1.8	0.812
Walking (hr)	0.3 ± 0.9	1.2 ± 1.4	* 0.003
Social activities (hr)	0.4 ± 1.1	0.7 ± 1.6	0.355
Hygiene and Sanitation			
Own faucet for water source (yes)	86 (76.5-92.0)	67 (38.3-88.1)	+ 0.072
Pit Latrine (yes)	86 (76.5-92.0)	73 (44.9-92.2)	0.234
Breastfeeding Practices			
Initiation within 1 hr (yes)	62 (51.3-72.2)	47 (21.2-73.4)	0.255
Frequency (#/24 hr) [b]	12.0 ± 4.2	15.0 ± 6.0	* 0.021
Frequency (#/day)	7.1 ± 9.4	9.4 ± 3.7	* 0.008
Frequency (#/night)	4.8 ± 2.3	5.6 ± 3.3	0.284
Exclusive breastfeeding (yes)	47 (36.0-57.4)	60 (32.2-83.6)	0.339
Infant Anthropometry			
Sex (male)	50 (39.2-60.7)	53 (26.5-78.7)	0.811

Age (days)	88.3 ± 64.1	64.7 ± 71.4	0.197
HAZ score	-1.82±1.21	-2.47 ± 1.92	+ 0.083
Stunted (%)	43 (32.9-54.2)	60 (32.2-83.6)	0.230
WAZ score	-0.87 ± 0.90	-1.43 ± 1.38	* 0.043
Underweight (%)	11 (5.4-19.4)	33 (11.8-61.6)	* 0.023
WHZ score [b]	0.70 ± 1.26	0.40 ± 0.55	0.410
HCZ score [b]	-0.41 ± 4.27	-0.89 ± 1.49	0.670
Microcephalay (%) [b]	16 (8.7-24.7)	40 (16.3-67.7)	* 0.027

Abbreviations: CI, confidence interval; HAZ, height-for-age z score; HCZ, head-circumference for age z score; OR, odds ratio; SCM, subclinical mastitis; WAZ, weight-for-age z score; WHZ, weight-for-height z score

* Significance established at P <0.05; + Non-significant trend at P <0.10

a The following additional factors were not significantly different between Non SCM and SCM (data not shown): Socio Demographics (household assets including vehicle, bike, radio, television, telephone), Maternal characteristics (education, marital status, occupation, language), Maternal delivery experience (age at first pregnancy, antenatal care, antenatal care frequency, location of delivery, birth attendant), Infant health (*susto, enojo, pujo*, fever), Breastfeeding practices (support, problems)

b Sample sizes were as follows: Urine Samples (n=103), Stool pathogens (n=98), Feeding frequency (n=100), HCZ and Microcephaly (n=104)

Table 4.2: Multiple logistic regression models for infant stunting (HAZ < -2SD), underweight (WAZ < -2SD) and for microcephaly (HCZ < -2SD)

Characteristic	Odds Ratio (OR)	95% C.I.	P-value
Model (1): Stunting [a]			
Subclinical mastitis (Na/K ratio >0.6) (yes)	4.28	1.15, 15.84	* 0.029
Stage of lactation (early lactation)	0.43	0.17, 1.05	0.065
Maternal height (cm)	0.88	0.79, 0.96	* 0.007
Infant diarrhea (yes)	7.07	0.64, 37.25	0.108
Maternal stool *Entamoeba coli* (yes).	0.89	0.36, 2.18	0.799
Model (2): Underweight [b]			
Subclinical mastitis (Na/K ratio >0.6) (yes)	6.91	1.48, 32.23	* 0.014
Stage of lactation (early lactation)	0.28	0.03, 2.45	0.251
Feeding frequency (#/Day)	0.81	0.62, 1.05	0.124
Infant head circumference (cm)	0.79	0.57, 1.11	0.188
Model (3): Microcephaly [c]			
Subclinical mastitis (Na/K ratio >0.6) (yes)	7.73	1.68, 35.53	* 0.009
Stage of lactation (early lactation)	0.52	0.16, 1.64	0.262
Maternal age at first pregnancy (yr)	0.83	0.66, 1.05	0.120
Maternal height (cm)	0.89	0.78, 1.02	0.085
Maternal stool *Entamoeba coli* (yes)	3.49	1.11, 11.00	* 0.032

[a] Overall model x^2 (5)=16.54,Nagelkerke R^2 =0.208, p=0.005. The Hosmer and Lemeshow x^2 (8)=6.81, p=0.557.

[b] Overall model x^2 (4)=9.31, Nagelkerke R^2 =0.161, p=0.05. The Hosmer and Lemeshow x^2 (8)=9.73, p=0.285.

[c] Overall model x^2 (5)=17.16, Nagelkerke R^2 = 0.256, p=0.004. The Hosmer and Lemeshow x^2 (8)=4.51, p=0.808.

* Denotes statistical significance $p < 0.05$

Connecting Statement II

In Chapter IV, I demonstrated that subclinical mastitis increased the likelihood of infant stunting (LAZ<-2SD), underweight (WAZ<-2SD) and microcephaly (HCZ<-2SD), respectively in a cross sectional study. I also showed that the presence of maternal stool *Entamoeba coli* independently increased the odds of infant microcephaly using multiple logistic regression. Given that my results indicated that subclinical mastitis and non-pathogenic protozoa were independently associated with poor infant anthropometry, I was interested in further exploring breast milk at different stages of lactation as a possible pathway for the vertical transmission of growth faltering.

In Chapter IV, I sought to investigate subclinical mastitis and infant growth using a longitudinal design. The framework for this study was based on previous literature that suggested SCM was associated with changes in breast milk composition including an association with markers of inflammation (Filteau et al., 1999). My approach was to: (1) describe the prevalence of subclinical mastitis (Na/K ratio >0.6) during early (0-6 weeks) and established (4-6 months) lactation among Indigenous *Mam*-Mayan mothers in The Western Highlands of Guatemala; (2) explore the determinants of subclinical mastitis during early and established lactation and (3) investigate the association of maternal stool non-pathogenic protozoa, breast inflammation and growth factors present in milk with infant weight-for-age (WAZ), height-for-age (LAZ), and head-circumference-for-age (HCZ) at both stages of lactation.

CHAPTER V

Interrelationships between maternal subclinical mastitis (SCM) and infant anthropometry in Guatemala: Role of infection, inflammation, hygiene and lactation practices

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Abstract

Background: Subclinical mastitis (SCM) is an asymptomatic inflammatory condition of the lactating breast and has been associated with adverse outcomes including poor infant growth. During SCM, tight epithelial junctions become "leaky", allowing movement of plasma constituents, including sodium ions, into milk. A milk sodium/potassium ratio (Na/K) > 0.6 is considered indicative of SCM. The goals of this longitudinal study were to: (1) describe the prevalence of SCM as defined by Na/K ratio >0.6 during early (0-6 wk) and established (4-6 mo) lactation among Indigenous *Mam*-Mayan mothers in The Western Highlands of Guatemala; (2) explore the determinants of SCM during early and established lactation using multiple logistic regression and (3) investigate the association of SCM (Na/K ratio), inflammation (milk pro-inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α), growth factors (milk VEGF, EGF) and maternal stool non-pathogenic protozoa (*Blastocystis hominis, Entamoeba coli*, *Iodamoeba butschlii* and *Endolimax nana*) with infant weight-for-age (WAZ), height-for-age (LAZ), and head-circumference-for-age (HCZ) at both stages of lactation.

Methods: Breast milk samples were collected from a cohort of lactating *Mam*-Mayan mothers during early (n=134) and established (n=120) lactation. Inductively Coupled Plasma Mass Spectrometry measured Na and K, and Luminex measured pro-inflammatory cytokines and growth factors in milk. Analysis for presence of urine leukocytes and direct smear of maternal stool samples for non-pathogenic protozoa were measured. A structured questionnaire describing lactation practices (breastfeeding category, initiation, frequency) and measures of hygiene (water source, sanitation) was administered. Anthropometry of both mother and infant was measured.

Results: Prevalence of SCM was 27% in early and 9% in established lactation; only 5 mothers (4%) had SCM during stages of lactation. Determinants of SCM at 0-6 wks were lack of home

faucet and parity and at 4-6 mo were lack of home faucet, lower feeding frequency, and higher early milk Na/K ratio. In early lactation, LAZ was positively associated with maternal height and presence of leukocytes in urine. WAZ was positively associated with maternal weight but negatively with milk Na/K ratio and maternal stool *Blastocystis hominis.* HCZ was also negatively associated with milk Na/K ratio and maternal stool *Blastocystis hominis* but positively with the pro-inflammatory cytokine IL-6 in milk. In established lactation, LAZ was associated positively with maternal height but negatively with maternal stool *Entamoeba coli* and EGF in milk. WAZ was also associated negatively with maternal stool *Entamoeba coli* but positively with maternal weight and pro-inflammatory cytokine TFN-□ in milk. HCZ was associated positively with maternal height and the pro-inflammatory cytokine IL-6 in milk but negatively with EGF in milk.

Conclusion: Compromised infant anthropometry was associated with SCM, milk EGF and with non-pathogenic protozoa in maternal stool, particularly *Blastocystis hominis* and *Entamoeba coli. In comparison,* milk pro-inflammatory cytokines were positively associated with infant anthropometry Given that lack of a home faucet was the only determinant of SCM at both periods of lactation, interventions may need to focus on improved hygiene practices to minimize fecal contamination, lower SCM and improve infant anthropometry.

BACKGROUND

Asymptomatic subclinical mastitis (SCM), common in veterinary medicine (Contreras & Rodríguez, 2011), is emerging in human lactation literature due to its implication as a risk factor for mother-to-child transmission of HIV (Gomo et al., 2003; Kasonka et al., 2006; Kantarci et al., 2007) and association with infant growth faltering (Morton, 1994; Filteau et al., 1999; Gomo et al., 2003; Aryeetey et al., 2009). An elevated breast milk sodium/potassium ratio (Na/K) is considered indicative of SCM and both ratios of Na/K >0.6 (Filteau, 1999; Gomo, 2003; Kantarci, 2007; Rasmussen et al., 2008; Lunney, 2010, Richards, 2010, Thurnham, 2010) and Na/K >1.0 (Willumsen, 2003; Kasonka, 2006; Aryeetey, 2008; Aryeetey, 2009) have been used to identify SCM. Previous research indicates that SCM, using an Na/K ratio >0.6, is most prevalent duirng early lactation (Rasmussen et al., 2008) although longitudinal research beyond the early postpartum period is limited. One recent study (Lunney et al., 2010) showed that the prevalencce of SCM at 6 weeks and at 3 and 6 months postpartum was 29%, 18% and 13%, respectively.

SCM can occur due to milk stasis and/or infection in the mammary gland (WHO, 2000). Poor lactation practices, which likely underscore milk stasis, have also been associated with SCM and one study demonstrated that non-exclusive breastfeeding was associated with an elevated Na/K ratio in milk (Kasonka et al., 2006). Older studies have also found that junctional complexes start to open when milk volume falls below 400 mL/day (Neville et al., 1991) and that both a reduction in feeding frequency (Garza et al., 1983) and low milk volumes (Dewey et al., 1984) are associated with increased permeability. Other maternal factors positively associated with SCM include younger age (Filteau et al., 1999) and primiparity (Arsenault et al., 2010). The role of poor hygiene and sanitation has previously been implicated in relation to *clinical* mastitis

(Fetherston, 1998; Michie et al., 2003; Peters et al., 1992) and emerging evidence indicates that poor sanitation will increase opportunity for infection, including potentially growth-suppressing subclinical infections caused by fecal-oral contamination during breastfeeding (Dewy & Mayers, 2011; Prendergast & Humphrey, 2014; Mbuya & Humphrey, 2015).

Little is known regarding the relationship between SCM and changes in breast milk composition although it can be extrapolated from our understanding in the dairy literature that SCM will lead to compromised quality of milk (Contreras & Rodríguez, 2011; Delgado et al., 2011). In humans, a single study has shown a positive association between breast milk Na/K ratio with milk enzymes (Rasmussen et al., 2008) and an older study found that increased mammary permeability was associated with higher concentrations of milk immune factors including secretory immunoglobulin A (sIgA) and lactoferrin (Filteau et al., 1999). It has also been shown that SCM is accompanied by the presence of the pro-inflammatory cytokine IL-8 (Semba et al., 1999; Willumsen et al., 2003; Rasmussen et al., 2008) suggesting that inflammation of the mammary gland causes tight junctions to become “leaky” allowing for plasma constituents to cross into milk (Thurnham, 2010).

There is also evidence to show that inflammation-induced permeability of the mammary epithelium may also underscore poor infant growth (Filteau et al., 1999). Chronic overproduction of pro-inflammatory cytokines may cause growth impairment that is mediated by a decrease in insulin-like growth factor (IGF-1) (De Benedetti et al., 1997; Jones et al., 2015) or may directly impede growth by inhibiting the process of bone remodeling (Skerry, 1994; Stephensen, 1999). Elevated concentrations of inflammatory cytokines can damage the infant gut and promote decreased nutrient absorption (Goldman, 1996; Garofalo, 2010; Ganeshan & Chawla, 2014). It is also plausible that other bioactive factors in milk, including growth factors EGF and VEGF critical for maintenance and the repair of the gastrointestinal epithelial barrier in infants (Dvorak, 2010; Ballard & Morrow, 2013), will be altered as a result of inflammatory SCM. Inflammatory cytokines have been implicated in impaired linear growth in mice models (Odiere et al., 2010) and a recent study found that inflammatory markers in plasma were higher in stunted children than in non-stunted children (Prendergast et al., 2014).

The rationale for this study is based on the assumption that SCM-mediated changes in breast milk composition can be vertically transmitted to the infant via breastfeeding, and that these compositional changes are associated with infant anthropometry. The goals of this longitudinal study were to: (1) describe the prevalence of SCM as defined by Na/K ratio >0.6 during early (0-6 weeks, n=134) and established (4-6 months, n=120) lactation among *Mam*-Mayan mothers in the Western Highlands of Guatemala, (2) explore the determinants of SCM during early and established lactation and (3) investigate the association of SCM (Na/K ratio), inflammation (milk pro-inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α), growth factors (milk VEGF, EGF) and maternal stool non-pathogenic protozoa (*Blastocystis hominis, Entamoeba coli*, *Iodamoeba butschlii* and *Endolimax nana*) with infant weight-for-age (WAZ), height-for-age (LAZ), and head-circumference-for-age (HCZ) at both stages of lactation.

METHODS

Study Design

This longitudinal study of breastfeeding mother-infant dyads at 0-6 weeks (4-41d) and 4-6 months (112-186d) postpartum was conducted in eight rural Indigenous *Mam*-Mayan communities in The Western Highlands of Guatemala. A structured questionnaire describing lactation practices (breastfeeding category, initiation, frequency) and measures of hygiene (water source, sanitation) was administered as previously described (Chomat et al., 2015). A single breast milk sample was collected from the breast not recently used for feeding at both stages of lactation and later analyzed for milk minerals (Na, K), pro-inflammatory cytokines (IL-1□, IL-6, IL-8, TNF-□) and growth factors (EGF, VEGF). Breast milk Na/K ratio was used to classify mothers as follows: Non-SCM (Na/K ratio ≤0.6) and SCM (Na/K ratio >0.6). Mothers with SCM were further classified for descriptive data as: Moderate SCM (0.6 ≥ Na/K ratio <1.0) and Severe SCM (Na/K ratio ≥ 1.0). Analysis for presence of urine leukocytes and direct smear of maternal stool samples for non-pathogenic protozoa (*Blastocystis hominis, Entamoeba coli*, *Iodamoeba butschlii* and *Endolimax nana*) were measured. Anthropometry of both mother and infant was measured at a local health centre or at the participants' home.

Study Recruitment and Ethical Considerations

From July 2012 - October 2013, *comadronas* (traditional midwives) and community health care workers recruited breastfeeding mother-infant dyads in early lactation. All mothers had been previously visited once during pregnancy (6-9 mo postpartum) as part of a larger study with sampling framework previously described (Chomat et al., 2015). Of the 144 mothers initially enrolled, 136 (94.4%) provided a breast milk sample at 0-6 weeks postpartum. Two infants, with infant age <4d, were excluded to avoid inclusion of milk samples before tight junctions in the mammary gland closed (Neville, 2001) for a total of 134 mothers at 0-6 weeks postpartum. Moreover, of the 144 mothers initially enrolled, 120 (83.3%) provided a breast milk sample at 4-6 months with reasons for not providing a sample including an unwillingness to participate and loss to follow up. Complete data (questionnaire and breast milk samples at both periods of lactation) were available for 114 breastfeeding mother-infant dyads with almost an equal number of male (n=61) and female (n=53) infants. Ethical approval was obtained from

McGill University Institutional Review Board and CeSSIAM (Center for Studies of Sensory Impairment, Aging and Metabolism), a Guatemala–based research organization. All mothers provided fully informed consent.

Questionnaire

The study questionnaire was developed in June 2011 and pilot tested for cultural appropriateness in 50 non-participant mothers with similar characteristics to the intended study population. Trained community health care workers administered the questionnaire orally in either Spanish or *Mam* to participants during a 30-40 min interview. In-depth details of the questionnaire have been described previously (Chomat et al., 2015). In brief, the questionnaire addressed the following topics: socio-demographics (household assets), hygiene and sanitation (water source, toilet), maternal characteristics (age, martial status, highest level of education), maternal and infant health (fever, headache, stomachache, cough, flu, diarrhea), and maternal delivery experience (age at first pregnancy, frequency of antenatal care, delivery location, parity. Questions on breastfeeding practices (early initiation within 1 hr postpartum, frequency per day and night, exclusive, predominant and mixed feeding as defined by WHO [WHO, 2003]) and breastfeeding support (provider, problems) were also included.

Maternal Fecal and Urine Collection and Analysis

Methods for collection and analysis of maternal fecal and urine samples have been previously described (Chomat et al., 2015). Briefly, a single stool sample was analyzed for protozoa using direct smear by an experienced laboratory technician trained in parasitology. Specifically, non-pathogenic protozoa (*Blastocystis hominis, Entamoeba coli*, *Iodamoeba* *butschlii* and *Endolimax nana*) were used in this study as exposure to feces contamination (CDC, 2012). Maternal urine samples were analyzed for pyuria and presence of leukocytes using dipstick analysis and microscopy.

Anthropometry

Two trained Guatemalan nutritionists measured maternal weight (kg) in triplicate (± 100 grams) using a digital scale (Seca 803) and height in duplicate (± 0.5 cm) using a wall stadiometer. Infant age was calculated based on date of birth recorded on the mother's health card or in the absence of a card, reported date of birth. The majority of births were attended by a *comadrona* and occurred at either the home of the mother or the *comadrona's* home (Chomat et al., 2015). Recumbent length (cm) was measured in triplicate (± 0.5 cm) using an infantometer (Seca 210). Weight (kg) was measured using a digital infant scale (Seca 354) to the nearest 100 g. Head circumference was measured with a baby band (Seca 212). Infants were classified as stunted (length-for-age Z score (LAZ) < -2SD), underweight (weight-for-age Z score (WAZ) < -2SD) or wasted (weight-for-height Z score (WHZ) < -2SD) according to WHO Growth Reference Standards using WHO Anthro Software (3.1). Head circumference-for-age (HCZ score) was used to identify infants with microcephaly (< - 2SD).

Breast Milk Collection, Storage and Analysis

The protocol for breast milk collection was developed in June 2011 and pilot tested for feasibility and to ensure acceptability within the Indigenous *Mam*-Mayan population. Mothers in this population consider breast milk collection to be non-invasive and care was taken to minimize behavioral disruption between mother and infant. Under the supervision of a trained *comadrona,* a single unilateral milk sample was collected via manual expression after the breast was cleaned with 70% ethyl alcohol. Mothers were asked to provide full milk expression from the breast not recently used for breastfeeding and all samples were collected in the morning. Time since last feeding was not recorded. Milk was collected in 60 ml plastic vials, immediately stored in a cold chest and subsequently aliquotted under sterile conditions and stored at -30º C. Milk samples were shipped on dry ice to McGill University where samples remained frozen at -80º C until analyzed for Na and K. The ratio permits the use of spot milk samples without consideration of time of sampling or time since the infant was last fed (Filteau et al., 1999), controls for assay variations that may occur due to the unequal distributions of the ions in the aqueous and lipid phases of milk (Allen et al., 1991; Aryeetey et al., 2008) and accounts for the normal and almost parallel decline in both Na and K ions during lactation (Allen et al., 1991; Richards et al., 2010).

Analysis of Sodium and Potassium in Breast Milk

Inductively-coupled plasma mass spectrometry (ICP-MS) [Bruker ICP-820MS, Billerica, MA, USA] was used to measure Na and K. Briefly, 0.5 mL of milk was digested in 0.5 mL trace metal grade concentrated nitric acid overnight in 15 mL polypropylene tubes (VWR metal free centrifuge tubes #89049-170). Then, 0.5 mL of trace metal grade hydrogen peroxide was added and the solution was heated to 80° C for 3 hours. Six quality controls including 0.16 g of commercial milk powder and 0.12 g of peach leaves (Standard Reference Material, National Institute of Standards and Technology, Gaithersburg, MD) and six reagent blanks were included in each analytical run. Digested samples, reference materials and 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% and the obtained solutions were measured.

Analysis of Pro-Inflammatory Cytokines in Breast Milk

The EMD Millipore's MILLIPLEX® MAP Human High Sensitivity Cytokine panel was used to measure pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in fat-free milk samples in duplicate according to manufacturer's specifications (EMD Millipore, 2012). The anlaysis was conducted with Luminex® 200™ using xMAP® platform (Luminex Corp., Austin, TX) and a minimum of 50 bead events was collected for each cytokine and median fluorescence intensities (MFI) obtained. Data was processed using the MasterPlex® CT software version 1.2 (MiraiBio, Inc., Alameda, CA). Minimum detectable concentrations were based on calculated values by StatLIA® Immunoassay Analysis Software from Brendan Technologies (Luminex Corporation, 2010) with LOD (pg/mL) for IL-1β, IL-6, IL-8, TNF-α at 0.06, 0.20, 0.05 and 0.07, respectively.

Analysis of Growth Factors in Breast Milk

The R&D Systems Human Magnetic Luminex Screening Assay (R&D Systems, Inc., MN, USA) was used to measure EGF and VEGF in fat-free milk in duplicate according to manufacturer's specifications (R&D, 2014). The anlaysis was conducted with Luminex® 200™ using xMAP® platform (Luminex Corp., Austin, TX) and a minimum of 50 bead events was collected for each factor and median fluorescence intensities (MFI) obtained. Data was processed using the MasterPlex® CT software version 1.2 (MiraiBio, Inc., Alameda, CA). For EGF, the sensitivity of the test is 0.7pg/ml with a minimum detectable level of 3.9pg/ml. The sensitivity for VEGF is 9pg/ml with a minimum detectable level of 31.2 pg/mL.

Statistical Analysis

Data were analyzed using IBM SPSS statistical software version 22.0 (SPSS Inc./IBM Chicago IL, USA). Descriptive statistics (mean ± S.D, percentages ± 95% C.I.) were computed and chi-squares (x^2) and Fisher's exact test (categorical) and Student t-tests were used to determine whether selected factors and practices differed between mothers with SCM (Na/K ratio >0.6) and without SCM. Multiple logistic regression models for the determinants of SCM during early (Table1a) and established lactation (Table1b) were created. To build models, all variables from univariate analyses with p≤0.25 were considered for inclusion. Variables were further grouped by common themes (socio-demographics, maternal characteristics, breastfeeding practices, measures of hygiene and sanitation). Highly correlated variables (p>0.7, VIF >10) within each theme were noted to avoid multi-collinearity and combinations of non-correlated variables were entered into each model. Breast milk biomarkers and stool non-pathogenic protozoa were tested but not included in final models to avoid circular logic. For all logistic regression models, the Hosmer and Lemeshow statistical test for goodness of fit was provided. Spearman rank correlations were also computed between bioactive factors in breast milk. Multiple linear regression analyses were used to evaluate infant anthropometric indicators HAZ, WAZ, and HCZ during both early and established lactation. All biomarkers were natural log transformed [Ln(y)] to achieve normality. To order to evaluate study objectives, final models were chosen based on study objectives and the themes of SCM, inflammation, infection and growth where combinations of non-correlated variables were entered into each model. Significance was set at p <0.05 for all models.

RESULTS

Population Characteristics

At 0-6 weeks postpartum (n=134), 23 mothers (17%) were classified as Moderate SCM and 13 mothers (10%) were classified as Severe SCM (Na/K ratio ≥ 1.0). At 4-6 months postpartum (n=120), 8 mothers (7%) were classified as Moderate SCM and 3 mothers were classified as Severe SCM. Overall, 27% of mothers had a Na/K ratio >0.6 at 0-6 weeks and 9% at 4-6 months postpartum. Interestingly, only 5 mothers had a Na/K ratio >0.6 at both periods of lactation (n=114). Prevalence of infant underweight, stunting and microcephaly was 12%, 32% and 14%, respectively at 0-6 weeks (n=136) and 15%, 43% and 12% at 4-6 months (n=120).

Spearman Rank Correlations

Differences existed by stage of lactation. During 0-6 wks, the milk Na/K ratio was positively correlated with all pro-inflammatory cytokines including with IL-1□ (r=235, p=0.010), IL-6 (r=0.481, p<0.01), IL-8 (r=0.506, p<0.001) and TNF-□ (r=0.459, p<0.001). During 4-6 mo, the milk Na/K ratio was only positively correlated with pro-inflammatory cytokine IL-6 (r=0.245, p=0.009). The Na/K ratio was not correlated with growth factors at either stage of lactation.

Multiple Logistic Regression Models for the Determinants of SCM (Na/K ratio >0.6) by Stage of Lactation

Model 1a: SCM in Early Lactation (Table 5-1)

Maternal parity (OR=0.66, 95% CI=0.49-0.91) decreased the odds of SCM during early lactation, whereas no home faucet (OR=4.69, 95% CI=1.43-15.43) increased the odds. The overall Hosmer and Lemeshow goodness of fit x^2 (8)=9.51, p =0.301.

Model 1b: SCM in Established Lactation (Table 5-1)

Breastfeeding frequency (#/daytime) (OR=0.53, 95% CI=0.31-0.91) decreased the odds of SCM during established lactation, whereas, no home faucet (OR=6.48, 95% CI=1.09-38.38) and breast milk Na/K ratio (early lactation) (OR=61.13, 95% CI=1.36-27.69) increased the odds. The overall Hosmer and Lemeshow goodness of fit x^2 (8)=9.93, p =0.270.

Multiple Linear Regression Models for Anthropometric Indicators by Stage of Lactation

Early Lactation (0-6 weeks postpartum)

Infant WAZ (Table 5-2): Multiple linear regression showed that infant WAZ was negatively associated with milk LN Na/K ratio (B=-0.60, p=0.039) and maternal stool B.hominis (B=-0.52,p=0.048) and positively associated with maternal weight (B=0.03, p=0.041) in a model that captured 11% of the variance.

Infant HAZ (Table 5-3): Multiple linear regression showed that infant HAZ was positively associated with maternal height (B=0.05, p=0.028) and maternal urine leukocytes (B=0.065, p=0.014) in a model that captured 14% of the variance.

Infant HCZ (Table 5-4): Multiple linear regression showed that infant HCZ was negatively associated with milk LN Na/K ratio (B=-1.33, p=0.001) and maternal stool B.hominis (B=-0.84,p=0.012) and positively associated with pro-inflammatory cytokine IL-6 (B=0.53, p=0.006) in a model that captured 14% of the variance.

Established Lactation (4-6 months postpartum)

Infant WAZ (Table 5-5): Multiple linear regression showed that infant WAZ was negatively associated with maternal stool E.coli (B=-0.68, p=0.003) and positively associated with maternal weight (B=0.04, p=0.005) and pro-inflammatory cytokine TNF-α (B=0.38, p=0.014) in a model that captured 16% of the variance.

Infant HAZ (Table 5-6): Multiple linear regression showed that infant HAZ was negatively associated with maternal stool E.coli (B=-0.52, p=0.033) and milk EGF (B=-0.76, p=0.044) and positively associated with maternal height (B=0.07, p=0.005) in a model that captured 12% of the variance.

Infant HCZ (Table 5-7): Multiple linear regression showed that infant HCZ was negatively associated with milk EGF (B=-1.31, p=0.001) and positively associated with maternal height (B=0.05, p=0.042) and pro-inflammatory cytokine IL-6 (B=0.49, p=0.041) in a model that captured 14% of the variance.

DISCUSSION

This longitudinal study in The Western Highlands of Guatemala provides the first report on subclinical mastitis at two stages of lactation and the association between breast milk composition and maternal stool non-pathogenic protozoa with infant anthropometry. Research showed that the prevalence of SCM (Na/K ratio >0.6) and the determinants of SCM differed between early (0-6 wks) and established (4-6 mo) lactation in a cohort of Indigenous *Mam*-Mayan mothers. Determinants of SCM at 0-6 wks were lack of home faucet and parity whereas at 4-6 mo were lack of home faucet, lower breastfeeding frequency, and higher early milk Na/K ratio. Most notably, predictors of anthropometric measurements also differed by stage of lactation. Impaired anthropometric measurements were associated with SCM and maternal stool *B.hominis* at 0-6 wks and with breast milk EGF and maternal stool *E.coli* at 4-6 mo. Interestingly, milk pro-inflammatory cytokines IL-6 and TNF-α were positively associated with infant anthropometry. Specifically, IL-6 was positively associated with HCZ during both early and established lactation, whereas, TNF-α was positively associated with WAZ only during established lactation. These findings suggest that an inflammatory response, which results in higher concentrations of pro-inflammatory cytokines in milk, may provide a measure of protection to the infant. In contrast, maternal SCM and maternal stool non-pathogenic protozoa were shown to compromise infant anthropometry indicating that breast milk may be a pathway through which non-genetic transmission of growth faltering can be passed.

SCM did not persist throughout lactation in this study indicating a more transient nature of tight epithelial junctions than previously understood (Aryeetey et al., 2009). Under normal conditions, tight junctions of the alveolar epithelial cells in the mammary gland are closed (Neville et al., 2001), however, junctions can be regulated by certain external stimuli including milk stasis resulting in breast “leakiness” (McManaman & Neville, 2003). In this study, lower breastfeeding frequency was associated with higher odds of SCM during established lactation. Ineffective milk removal for which milk stasis can be a consequence is more likely to occur in established lactation when complementary foods are introduced (WHO, 2000; WHO, 2003; Matias et al., 2010). However, unlike previous research that showed that non-exclusive breastfeeding was associated with a raised milk Na/K ratio (Kasonka et al., 2006), this study did not show an assocation between exclusivity and SCM suggesting that frequency of feeds rather than exclusivity may be underscoring milk stasis and breast leakiness in our population.

Increased permeability of the mammary epithelium has previously been associated with inflammation in the breast (Filteau et al., 1999). Inflammation-induced permeability can alter milk composition and previous research has shown an increase in milk cytokines IL-8 (Semba et al., 1999; Willumsen et al., 2000) and TGF-β (Filteau et al., 1999). Results from this study demonstrated that the breast milk Na/K ratio was positively correlated with all pro-inflammatory cytokines during early and with pro-inflammatory cytokine IL-6 during established lactation suggesting a “true” inflammatory condition of the breast and not merely leakiness. Similarly, a recent study showed that pro-inflammatory cytokine IL-6 was increased in milk of mothers with *clinical* mastitis with symptoms of fever and malaise, compared to milk from healthy mothers (Mizuno et al., 2012). In breast milk, it is generally understood that cytokine IL-6 is produced predominantly by macrophages and monocytes, is the major pro-inflammatory cytokine needed to stimulate synthesis of the acute phase response, and acts as a potent pyrogen (Rudloff et al., 1993; Agarwal et al., 2011). An inflammatory response in the mammary gland, and corresponding elevated cytokines in milk, may result from either a local response (Goldman, 1996; Ballard, 2011) or from systemic inflammation (Srivastav et l.,1996; Meki et al., 2003) which cannot be clarified in this study.

Contrary to previous literature, which showed a negative association between infant anthropometry and pro-inflammatory cytokines in blood (Prendergast et al, 2014), in this study, infant anthropometry was positively associated with pro-inflammatory cytokines IL-6 and TNF-α in breast milk. In particular, milk IL-6 was positively associated with HCZ at both stages of lactation and milk TNF-α was positively associated with WAZ in established lactation. These findings compare to previous research in mice models, which found that infection during lactation elevated cytokine IL-6 in pup milk but was associated with reduced IGF-1 levels (Odiere et al., 2010). These findings also differ from the general understanding that inflammatory cytokines inhibit bone formation and stimulate bone resorption (Skerry, 1994; Stephensen, 1999) and underscore impaired growth in children (Prendergast et al., 2014). However, it is imperative to distinguish between pro-inflammatory cytokines in blood and cytokines in breast milk. To that extent, these study findings differ with a recent study which showed that higher breast milk TNF-α was associated with lower infant lean mass and that higher breast milk cytokine IL-6 was negatively associated with infant adiposity (Fields & Demerath, 2012). It is unclear what underscores these study findings although, on one hand, it may suggest that mothers who are able to effectively mount an immunological response required to eliminate infection, including higher concentrations of pro-inflammatory cytokines in breast milk, (Goldman, 1996; Agarwal et al., 2011) may have infants with "better" growth compared to mothers less able to fight infection. On the other hand, in addition to their roles in the maternal immune system, cytokines in milk may be equally important for an infant's mucosal defense development and protection (Srivastava et al., 1996). Cytokines in human milk may compensate for the developmental delay of the infant's immune system by providing immunomodulating effects to the mucosal or systemic sites (Garofalo, 2010). Taken together, study results indicate that the protective nature of an inflammatory response likely occurs on a continuum; at one end of the spectrum excess inflammation triggers physical damage to the mammary gland, opens tight junctions and distorts milk composition and on the other end, an inflammatory response may be associated with improved immune protection and infant growth.

An unexpected finding in this study was that milk EGF was negatively associated with LAZ and HCZ during established lactation. Previous research has found that milk EGF stimulates epithelial proliferation (Borellini & Oka, 1989) and supports ductual growth in the mammary gland (Silberstein, 2001). Emerging evidence has also demonstrated the protective role of milk EGF in the prevention of infant necrotizing enterocolitis (NEC) given its ability to contribute to epithelial protection from injury and post-injury mucosal repair in the intestinal epithelium of the infant (Nair et al., 2008; Dvorak, 2010). Therefore, if milk EGF is sequestered in the mammary gland and not reaching the infant, the lack of delivery of milk EGF to the infant could impact the development of the intestinal function (Bruder et al., 2008) and contribute to increased susceptibility to poor growth. Alternatively, in vitro studies have shown that milk EGF inhibits the expression of various milk proteins (Borellini & Oka, 1989), prolactin (Saji et al.,1990; Quijano et al., 1998) and an older study using mouse mammary epithelial cells found that EGF inhibited the synthesis of casein and alpha-lactalbumin by about 45% and 55%, respectively (Taketani et al., 1983). If higher concentrations of milk EGF inhibit alpha-lactalbumin and inhibit prolactin, the hormone in milk needed for milk synthesis (Lonnderdal, 2003), this may explain the negative association observed in this study. Likewise, the distinctive role of milk EGF may also explain why milk VEGF did not emerge in multiple linear regression models in this study. Taken together, these findings highlight the complexity in plausible interrelations between milk cytokines and milk growth factors, which may be associated with infant anthropometry although further research is needed.

In this study, lack of a home faucet emerged as a determinant of SCM at both stages of lactation suggesting that mothers without a home faucet may be more exposed to contaminated water sources and/or lack opportunity for hand hygiene, which is an essential factor recognized to prevent *clinical* mastitis (Fetherston,1998; Michie et al., 2003; Peters et al., 1992). It is well recognized that poor sanitation and hygiene contribute to fecal contamination, which may increase opportunity for infection (Solomons, 2003; Bhutta et al., 2008). Less clear is how maternal SCM and exposure to fecal contamination interact to adversely affect infant growth, however, it is not inconceivable that fecal contamination of the breast through poor hygiene will put the infant in direct contact with exposure to non-pathogenic protozoa through breastfeeding (Humphrey, 2009; Dewey & Mayers, 2011). Supporting the notion of fecal oral transmission, previous research has demonstrated the sharing of bacterial strains between breast milk and infant feces (Martin et al., 2012) and shown that maternal gut bacteria may be vertically transferred from mother to neonate via breastfeeding (Jost et al., 2014). Likewise, it has been suggested that high exposure to any fecal contamination may contribute to environmental enteric dysfunction (EED) as a modulator of growth (Mbuya & Humphrey, 2015) although further research is required to clarify associations found in this study.

In this study, infant anthropometry was negatively associated with non-pathogenic protozoa in maternal stool. Specifically, *B.hominis* was negatively associated with infant WAZ and HCZ during early, whereas, *E.coli* was negatively associated with WAZ and LAZ during established lactation. Typically, non-pathogenic protozoa exhibit little or no overt pathology (CDC, 2012) however literature has also emerged suggesting that non-pathogenic protozoa may be responsible for outbreaks of diarrheal disease (Thapar, 2004). More specifically, research has demonstrated that *B.hominis* is associated with increased gastrointestinal morbidity (El-Shazly et al., 2005; Tan, 2008) and a recent study showed an association between non-pathogenic *Entamoeba coli* and poor infant nutritional status including lower erythrocyte folate and higher incidence of fever (Boeke et al., 2010). In this study, non-pathogenic stool *E.nana* and *I.butschlii* were not associated with infant anthropometry, which may suggest that only certain non-pathogenic protozoa could elicit an overt response. Nonetheless, from this study, it is possible to suggest that non-pathogenic protozoa in maternal stool, specifically *E.coli* and *B.hominis*, may be added to a growing list of factors associated with poor infant growth (Dewey & Mayers, 2011, Khlangwiset et al., 2011; Bhutta et al., 2013; de Onis et al., 2013). Health practitioners should not underestimate maternal stool non-pathogenic protozoa and SCM as contributors to poor growth in developing countries such as Guatemala.

Limitations and Strengths

Reliance of self-reported data on infant feeding practices was a limitation. Urine leukocytes were only measured in early and not established lactation limiting our ability to comment. Caution is required when interpreting cross sectional data and thus this study has shown associations between SCM and infant growth. A major strength of this study was our precise measure of Na and K using ICP-MS to identify cases of SCM during two stages of lactation.

Conclusion

Our investigation in The Western Highlands of Guatemala has highlighted maternal subclinical mastitis, an inflammatory condition of the lactating breast, as a complex condition that is associated with changes in breast milk composition and associated with growth impairment. In this study, SCM did not persist throughout lactation suggesting a transient nature of the condition. This study also showed that determinants of SCM differ by stage of lactation but that poor hygiene/sanitation likely exacerbates the condition. Interventions such as "Baby-WASH" (Mbuya & Humphrey, 2015), aimed at reducing contamination during breastfeeding, may be crucial to reduction of SCM and impaired anthropometry. Interestingly, milk pro-inflammatory cytokines were positively associated with infant anthropometry whereas milk EGF was associated with impaired anthropometry suggesting that more attention needs to be paid to the inflammation-growth paradigm as it relates to milk composition. Maternal stool non-pathogenic protozoa and SCM compromised infant growth indicating that there is a definite need for prioritizing maternal health in Guatemala specifically during the period of lactation when altered breast milk composition may promote the vertical transmission of growth faltering.

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Table 5-1: Multiple logistic regression models for subclinical mastitis (Na/K ratio >0.6) during early lactation (4-41d, n=134) and established lactation (112-186d, n=120)

	Odds Ratio (OR)	**95% C.I.**	**p-value**
Model (1): SCM (0-6 weeks) [a]			
Infant age (days)	0.95	0.89-1.0	0.065
Maternal height (cm)	1.03	0.93-1.14	0.558
Parity (#)	0.66	0.49-0.91	* 0.011
Exclusive breastfeeding (yes)	0.44	0.14-1.38	0.157
Breastfeeding within 1 hr postpartum (no)	2.19	0.68-7.09	0.191
Breastfeeding frequency (#/day)	0.89	0.74-1.09	0.269
Water source – Own faucet (no)	4.69	1.43-15.43	* 0.011
Model (2): SCM (4-6 months) [b]			
Infant age (days)	1.02	0.99-1.07	0.326
Maternal weight (kg)	0.95	0.86-1.05	0.311
Breastfeeding within 1 hr postpartum (no)	0.38	0.07-1.91	0.239
Breast Milk Na/K ratio (0-6 weeks)	6.13	1.36-27.69	* 0.019
Breastfeeding frequency (#/day)	0.53	0.31-0.91	* 0.022
Breastfeeding frequency (#/night)	1.50	0.93-2.41	0.097
Water source – Own faucet (no)	6.48	1.09-38.38	* 0.040

a Overall model x^2 (7)=21.35, p =0.003; Hosmer and Lemeshow x^2 (8)=9.51, p =0.301.
b Overall model x^2 (7)=18.47, p=0.010; Hosmer and Lemeshow x^2 (8)=9.93, p =0.270.
* Denotes statistical significance P <0.05

Table 5-2: Multiple Linear Regression Model for Infant Weight-for-Age (WAZ) during early (4-41d) lactation

	Unstandardized Regression Coefficient	**Standard Error of the Coefficient**	**p-value**
Weight-for-age (WAZ)			
Infant age (days)	-0.01	0.01	0.607
Maternal weight (kg) (early)	0.03	0.01	* 0.041
Maternal Stool *Blastocystis hominis* (yes) (early)	-0.52	0.26	* 0.048
Maternal Urine Leukocytes (yes) (early)	0.26	0.23	0.248
Milk LN Na/K ratio (early)	-0.60	0.29	* 0.039
Milk LN IL-6 (early)	0.20	0.17	0.202
Milk LN TNF-α (early)	-0.20	0.15	0.180
Milk LN EGF (early)	0.02	0.10	0.865

Overall model: $F(8,95)=2.59$, $P=0.019$, adjusted $R^2=0.111$
* Denotes statistical significance $P<0.05$

Table 5-3: Multiple Linear Regression Model for Infant Height-for-Age (HAZ) during early (4-41d) lactation

	Unstandardized Regression Coefficient	**Standard Error of the Coefficient**	**p-value**
Height-for-age (HAZ)			
Infant age (days)	-0.03	0.01	0.061
Maternal height (cm)	0.05	0.02	* 0.028
Maternal Stool *Blastocystis hominis* (yes) (early)	-0.37	0.30	0.217
Maternal Urine Leukocytes (yes) (early)	0.65	0.26	* 0.014
Milk LN Na/K ratio (early)	-0.43	0.33	0.189
Milk LN TNF-α (early)	-0.14	0.74	0.413
Milk LN IL-6 (early)	0.23	0.19	0.252
Milk LN VEGF (early)	0.08	0.11	0.463

Overall model: F (8,94)=2.54, P=0.015, adjusted R^2 =0.142
* Denotes statistical significance P <0.05

Table 5-4: Multiple Linear Regression Model for Infant Head circumference-for-Age (HCZ) during early (4-41d) lactation

	Unstandardized Regression Coefficient	**Standard Error of the Coefficient**	**p-value**
Head circumference-for-age (HCZ)			
Infant age (days)	-0.01	0.01	0.882
Maternal height (cm)	0.05	0.03	0.072
Maternal Stool *Blastocystis hominis* (yes) (early)	-0.84	0.33	* 0.012
Maternal Urine Leukocytes (yes) (early)	0.51	0.29	0.083
Milk LN Na/K ratio (early)	-1.33	0.37	* 0.001
Milk LN IL-6 (early)	0.53	0.19	* 0.006
Milk LN IL-1 (early)	-0.22	0.16	0.179
Milk LN VEGF (early)	0.02	0.12	0.890

Overall model: $F(8,93)=2.99$, $P=0.005$, adjusted $R^2=0.136$
* Denotes statistical significance $P<0.05$

Table 5-5: Multiple Linear Regression Model for Infant Weight-for-Age (WAZ) during established lactation

	Unstandardized Regression Coefficient	**Standard Error of the Coefficient**	**p-value**
Weight-for-age (WAZ)			
Infant age (days)	0.01	0.01	0.462
Maternal weight (kg) (established)	0.04	0.01	* 0.005
Maternal Stool *Entamoeba coli* (yes) (established)	-0.68	0.22	* 0.003
Milk LN Na/K ratio (established)	0.48	0.33	0.154
Milk LN TNF-α (established)	0.38	0.15	* 0.014
Milk LN IL-8 (established)	-0.17	0.12	0.150
Milk LN EGF (established)	-0.66	0.34	0.058

Overall model: $F(7,83)=3.37$, $P=0.003$, adjusted $R^2=0.156$
* Denotes statistical significance $P<0.05$

Table 5-6: Multiple Linear Regression Model for Infant Height-for-Age (HAZ) during established (4-6mo) lactation

	Unstandardized Regression Coefficient	**Standard Error of the Coefficient**	**p-value**
Height-for-age (HAZ)			
Infant age (days)	0.00	0.01	0.779
Maternal height (cm)	0.07	0.02	* 0.005
Maternal Stool *Entamoeba coli* (yes) (established)	-0.52	0.24	* 0.033
Milk LN Na/K ratio (established)	0.41	0.36	0.253
Milk LN TNF-α (established)	0.15	0.12	0.191
Milk LN IL-1 (established)	0.24	0.15	0.098
Milk LN EGF (established)	-0.76	0.37	* 0.044

Overall model: $F(7,84)=2.75$, $P=0.013$, adjusted $R^2=0.119$
* Denotes statistical significance $P<0.05$

Table 5-7: Multiple Linear Regression Model for Infant Head circumference-for-Age (HCZ) during established (4-6mo) lactation

	Unstandardized Regression Coefficient	Standard Error of the Coefficient	p-value
Head circumference -for-age (HCZ)			
Infant age (days)	0.00	0.01	0.963
Maternal height (cm)	0.05	0.02	* 0.042
Milk LN Na/K ratio (established)	0.47	0.33	0.164
Milk LN IL-6 (established)	0.49	0.24	* 0.041
Milk LN TNF-α (established)	0.17	0.14	0.217
Milk LN EGF (established)	-1.31	0.38	* 0.001

Overall model: $F(6,101)=3.85$, $P=0.002$, adjusted $R^2=0.138$
* Denotes statistical significance $P<0.05$

CHAPTER VI

General Discussion

Novel Findings

This dissertation investigated subclinical mastitis (SCM), an asymptomatic inflammatory condition of the lactating breast, among Indigenous *Mam*-Mayan women in The Western Highlands of Guatemala. Three novel findings that emerged from this study included:

(1) Previous research had suggested a relationship between SCM and poor infant weight (Morton, 1994; Gomo et al., 2003) and length (Makasa et al., 2007; Aryeetey et al., 2009) in African countries. Our data demonstrated that SCM, measured by a breast milk Na/K ratio >0.6, independently increased the odds of infant stunting, underweight and microcephaly, respectively, using multiple logistic regression analyses (Chapter IV). This was the first study to show associations between SCM and WHO infant growth standards, including the specificity of recommended cut-off values at <-2SD, which was important given that there are known negative effects associated with infants below these cut-of values including increased infant morbidity and mortality (Victora et al., 2008; Martorell et al., 2010). These findings are especially relevant in Guatemala, a country with the highest prevalence of stunting in Latin America (MSPAS, 2010). The realization that most stunting cannot be explained by poor diet or by diarrhea (Mbuya & Humphrey, 2015) has led researchers to consider alternative hypotheses. To that extent, my findings imply that, in order to prevent suboptimum growth, public health practitioners should consider subclinical mastitis as a condition underscoring growth faltering in Guatemala.

(2) Previous research in both mice (Odiere et al, 2010) and infants (Prendergast et al., 2014) has suggested that inflammation impairs growth through a complex interplay between undernutrition and infection,(Palmer, 2011; Salam et al., 2014). Research has indicated that growth impairment arises from an inflammatory process via a diversion of nutrients, a decrease in insulin-like growth factor (IGF-1) and that pro-inflammatory cytokines directly inhibit the process of bone remodeling (Prendergast et al., 2014; Jones et al., 2015; Mbuya & Humphrey, 2015). Contrary to previous understanding, my study challenged the current paradigm and indicated that inflammation produced during SCM may also serve as a protective mechanism and promote growth. My research showed that higher concentrations of pro-inflammatory cytokines in breast milk were associated with improved anthropometry (Chapter V). In particular, pro-inflammatory cytokine IL-6 was positively associated with HCZ at both stages of lactation whereas TNF-α was positively associated with WAZ in established lactation. Given that pro-inflammatory cytokines may be transferred to the infant via breast milk (Goldman, 1996; Garofalo, 2010), it is plausible that cytokines may play a role in infant gut development that remains undefined in the literature. From my study, future researchers should consider that milk pro-inflammatory cytokines might modulate the function and/or integrity of the infant gut and enhance protection. Cytokines in human milk may target the infant GI tract including the epithelium, enteric nervous system and/or mucosal immune system (Goldman, 2000). Pro-inflammatory cytokines transferred to the infant may in part, compensate for the developmental delay of the infant's immune system. Consistent with this notion is the fact that breast-fed infants are at less risk than non-breastfed infants from respiratory infections (Goldman et al., 1996). In that regard, my research suggests that cytokines in milk may compensate for developmental delays in the infant but also that elevated pro-inflammatory cytokines in milk, and the

subsequent inflammatory process, confer protection to the maternal mammary gland. However, given that the possible residual effects of inflammation on breast milk composition are largely unknown, particularly in view of Indigenous Guatemalan infants who may have specialized nutritional needs, further investigation is warranted.

(3) Recent evidence has suggested that the unhygienic environments in which infants live and grow, including exposure to *any* fecal contamination may contribute to stunting (Rah et al., 2015; Mbuya & Humphrey, 2015). Our data demonstrated that non-pathogenic protozoa in maternal stool were associated with compromised infant anthropometry. Specifically, maternal stool *B.hominis* was negatively associated with infant WAZ and HCZ during early postpartum, whereas *E.coli* was negatively associated with WAZ and LAZ during established lactation (Chapter V). Together, these findings indicated that non-pathogenic protozoa may play an under recognized role as contributors to infant growth faltering in Guatemala. Moreover, non-pathogenic *B.hominis* and *E.coli* in maternal stool are new contributions to the literature and should be added to a growing list of factors associated with poor infant growth (Dewey & Mayers, 2011; Khlangwiset et al., 2011; Bhutta et al., 2013; de Onis et al., 2013). My data also revealed that lack of a home faucet independently increased the odds of SCM during both 0-6 wk and 4-6 mo, respectively, using multiple logistic regression analyses (Chapter V). These findings suggested a more complex etiology of the causal determinants of SCM than previously understood and highlighted the complex plausible interrelations between non-pathogenic protozoa, inflammation and mammary gland permeability. Given that fecal contamination of the breast leading to fecal ingestion by infants via breastfeeding has been suggested as a possible pathway through which poor sanitation and hygiene may contribute to poor growth (Humphrey, 2009), my study findings compliment the notion of a vertical transmission of growth faltering

between mother and infant via breastfeeding. Together, my research implies that health practitioners should not underestimate fecal-oral transmission via breastfeeding as a contributor to poor growth in developing countries such as Guatemala.

My study has made several additional significant contributions to the literature. First, my data showed that cultural practices influenced compliance with WHO infant feeding recommendations (WHO, 2003) among Indigenous *Mam*-Mayan mother-infant dyads. Surprisingly, very little research exits on the association between cultural factors and breastfeeding practices in Guatemala. Yet, if cultural beliefs and practices displace necessary breast milk needed for favorable infant growth within the first 6 mo postpartum, my study findings make a noteworthy contribution to both public health researchers and mothers living within these communities. Our results showed that early initiation of breastfeeding was delayed by use of *agüitas* (ritual fluids) and by the belief in the transmission of *susto* (fright) through milk (Chapter III). Our results also showed that increased time in the *temascal* (sauna) increased breastfeed frequency, consistent with Indigenous mothers' perception that the *temascal* stimulates milk flow. This finding suggested that t*emascal* use might be protective against milk stasis and help to explain the generally low rates of SCM in this population. Together, my findings imply that public health practitioners, including practitioners complying with WHO infant feeding recommendations (WHO, 2003), need to understand how cultural practices and beliefs influence breastfeeding in order to promote infant growth.

Second, my data showed that SCM does not persist throughout lactation. This finding implied that breast leakiness and opening of tight junctions in the mammary gland was transient (Nguyen & Neville, 1998). Although research suggested that SCM is more common in early lactation (Filteau et al., 1999; Aryeetey et al., 2008) few studies have measured SCM using a longitudinal design. My data also showed that the determinants of SCM differ by stage of lactation. This suggested that leakiness in the mammary gland may be regulated by multiple stimuli, including milk stasis and infection, (Neville et al., 2001) but that these stimuli may have different impacts on the breast by stage of lactation, which has rarely been considered in human lactation literature. Future research should consider stage of lactation and the possible transient nature of SCM.

Lastly, contrary to our expectation that growth factors in milk would promote growth, in our study, milk EGF was negatively associated with LAZ and HCZ during established lactation. Recent research has demonstrated the protective role of milk EGF in the prevention of infant necrotizing enterocolitis (NEC) given its ability to contribute to epithelial protection from injury and post-injury mucosal repair in the intestinal epithelium of the infant (Dvorak, 2010; Nair et al., 2008). My results suggested that if milk EGF was sequestered in the mammary gland and not reaching the infant, the lack of delivery of milk EGF to the infant could decrease cell proliferation and impact the development of the intestinal function (Bruder et al., 2008) and contribute to increased susceptibility to poor growth. However, I am also cognizant of the notion that growth factors are sensitive to inflammation (Agarwal et al., 2011) and that previously research has demonstrated that individuals with chronic inflammatory conditions commonly have suppressed concentrations of IGF-1, which are inversely related to the level of circulating pro-inflammatory cytokines (Jones et al., 2015). A gap in understanding in the literature on the role of growth factors and cytokines in milk implies that researchers should consider that milk composition may change in the presence of other pro-inflammatory cytokines, be modified with increased/decreased permeability of the breast or be altered in the presence of other maternal conditions such as stress and infection. Therefore, the differences that I observed in breast milk highlight the complexity of the interrelations between inflammation and growth, which independently or together may be determinants of infant growth, and contribute an alternative paradigm for consideration of future researchers.

Limitations

Several limitations to this study need to be acknowledged. First, human milk is a complex biological fluid and studying it in its entirety extends beyond the scope of this dissertation. For that reason, it is possible that I may have omitted to analyze nutritional and/or bioactive factors in milk that are related to infant growth. To this extent, alternative hypotheses should be considered. For example, this study does not measure metabolic hormones adiponectin, leptin and ghrelin in human milk, which have known associations to infant BMI and appetite regulation (Martin et al., 2006; Woo et al, 2011; Fields & Demerath, 2012). The strongest evidence for this association is leptin, which has been positively associated with maternal BMI and negatively associated with infant weight (Miralles et al., 2006; Schuster et al., 2011). Second, our findings that SCM independently increased the odds of infant stunting, underweight and microcephaly, respectively, are limited by the use of a cross sectional design. As such, caution must be applied not to infer causal relationships. Instead, from our data, it is possible to state that associations between SCM and impaired infant anthropometry existed in The Western Highlands of Guatemala. Moreover, our longitudinal study design was limited by the use of only two stages of lactation and thus we were unable to comment on SCM in the time interval between 46 days and 4 mo postpartum. Given that *clinical* mastitis is often a debilitating condition which encourages mothers to limit or cease breastfeeding (WHO, 2000), additional information on SCM would help us to establish a greater degree of accuracy on how to differentiate between SCM cases that will resolve in comparison to those which will progress to *clinical* mastitis and possibly breast abscess. Moreover, prevalence of SCM was limited by the collection of unilateral breast milk samples, which may have underestimated the percentage of SCM in the population. Lastly, an issue that was not addressed in this study was whether infant prematurity could have affected the determinants of growth faltering. Recently it was reported that infant prematurity in this population, defined as infants born < 37 weeks postpartum, was 21% (n=82) based on maternal last menstrual period (LMP) (Chomat et al., 2015). My preliminary analyses exploring infant prematurity, within the confines of a smaller sample size (n=82), found that prematurity was associated with an increased likelihood of being stunted (unadjusted OR=4.62, p=0.007) during the first 41 days postpartum but that infant prematurity was not associated with being underweight or being classified as microcephalic, respectively. Prematurity remained a significant determinant of infant stunting during early lactation in multiple logistic regression analyses when controlling for infant sex, maternal height, maternal age, and pregnancy weight (data not shown). However, my preliminary data also showed that milk composition including milk pro-inflammatory cytokines, growth factors, and the Na/K ratio did not differ between mothers with premature infants and mothers with non-premature infants in univariate analyses suggesting that prematurity was not related to milk composition in this sub-population. However, the main weakness in this data is the reporting of LMP by maternal recall and the possibility of confounding factors. Therefore, if the consideration of infant prematurity is to be moved forward, a better understanding of the validity of LMP as an accurate measure of gestational age needs to be developed.

Recommendations for Future Research and Conclusion

Future research following my study, should consider the following insights for subsequent research.

First, previous research has recognized that one of the major changes in milk composition, as a result of increased permeability, is the decrease in the concentration of lactose in milk (Neville et al., 2001). Under normal conditions, high concentrations of lactose within the mammary gland leads to the osmotic influx of water, contributing to the fluidity of milk and making lactose a key determinant in osmotic pressure and milk volume (Neville, 1991; Neville et al., 2001; McManaman & Neville, 2003). It would therefore be intriguing to evaluate if the elevated sodium concentrations observed in milk from mothers with SCM occurs in order to offset the osmotic pressure caused by lower levels of lactose in milk. Interestingly, the measurement of lactose in urine has also been used as a measure of increased mammary epithelial permeability of the breast during inflammation, which may confer additional insights (Fetherston et al., 2006).

Second, there is a definite need to measure other bioactive factors in milk, including milk cortisol, which have been demonstrated to underscore the inflammation-growth paradigm in animal models (Odiere et al., 2010). This is of particular importance if inflammatory SCM drives activation of the HPA axis, resulting in elevated levels of cortisol in milk, which can be vertically transmitted to the infant with an unknown impact on development and growth. Drawing from our understanding in the cow literature (Contreras & Rodríguez, 2001) and general understanding from lactation physiology (Neville, 1991; Neville et al., 2001), it can be speculated that reduced infant feeding leading to milk stasis will place the engorged milk duct under severe mechanical stress. My preliminary data using a cross sectional sample (n=107), not presented in this dissertation, indicated that breast milk cortisol was positively associated with increased mammary gland permeability as indicative by an increasing milk Na/K ratio. This hints towards a relationship between maternal stress and SCM, however, it is unknown if the elevated cortisol observed in milk was a result of maternal psychological stress and/or mechanical stress in the mammary gland. Nonetheless, these preliminary findings are intriguing, especially if the effects of stress, in part, mediate the threshold between subclinical and *clinical* mastitis (WHO, 2000). Understanding the interactions between stress and SCM will be important in managing possible negative outcomes that may arise if milk cortisol is transferred to the infant. It is equally important to consider measuring a broad range of growth factors in milk, including IGF-1, to focus on the mechanism by which inflammatory cytokines, hormones and growth factors may interact to affect growth. Previous research has suggested that elevated pro-inflammatory cytokine levels are inversely related to IGF-1 levels, which may indirectly suppress bone growth (Jones et al., 2015). It will be important for future studies to distinguish between the impact of free and bound IGF-1 on infant growth. In addition, a considerable amount of research is needed in order to bridge the existing knowledge gap in human lactation literature on how growth factors in milk, including EGF and VEGF, contribute to mammary tissue growth and secretory function but also how they may further influence the maturation of the infant gut.

Third, in addition to measuring nutrient composition in human milk, it may be of interest to investigate nutrient absorption in the infant. It has been previously suggested that changes in laculose/ mannitol (L/M) ratio reflects absorption and intestinal permeability (van der Hulst et al., 1998; Lunn et al., 1991). Previous research in the Gambia demonstrated that an increase in L/M ratio predicted growth faltering, suggesting that both barrier and absorptive functions of the small intestine were compromised (Campbell et al., 2002; Campbell et al., 2003). With this knowledge, it may shed insight into how altered gut function contributes to nutrient malabsorption in the breastfeeding infant. Lastly, maternal nutritional status may affect the vertical transmission pathway between mother and infant observed during breastfeeding. Previous research has suggested that maternal malnutrition impacts the quality and quantity of bioactive factors available for transfer to the infant (Palmer, 2011). Nevertheless, it is likely that a complex combination of insults during the period of lactation – malnutrition, stress, inflammation, poor immunity and infection – contribute to the pathogenesis of growth faltering, providing ample justification for further research in this field.

In conclusion, this study investigated subclinical mastitis, an asymptomatic inflammatory condition of the lactating breast, and its link to breast milk composition and infant anthropometry in The Western Highlands of Guatemala. This research showed that SCM is associated with poor indicators of infant anthropometry but that inflammation of the mammary gland may be a protective mechanism of the breast and confer additional protection to the developing infant. Significant effects of non-pathogenic protozoa on poor infant growth during both stages of lactation suggested that fecal contamination may play an under recognized role in growth faltering. Given that vertical mother-infant transmission occurs via breastfeeding, these results highlight the complexity of SCM and its impact on not only milk composition but also infant development and growth. Public health practitioners need to scale up efforts to promote interventions such improved hand washing during infant feeding which may interrupt fecal-oral transmission and improve growth faltering in this population.

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