

**CHARACTERIZATION OF HUMAN MILK PROTEOME AND
DETERMINATION OF FACTORS UNDERLYING GROWTH AND
NUTRITIONAL STATUS AMONG INFANTS HOSPITALIZED
WITH ACUTE ILLNESS**

BRENDA MUTHONI KAMAU

**A thesis submitted in partial fulfilment of the requirements for the Degree of Master
of Science in Bioinformatics of Pwani University.**


November, 2023

DECLARATION

This thesis is my original work and has not been presented in any other University or any other Award.

Brenda Muthoni Kamau

Registration Number: SG30/PU/36110/21

Signature: 

Date: 12/9/2024

Supervisors' Approval

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. James M. Njunge (PhD)

Signature: 

Date: 12/9/2024

The Biosciences Department, KEMRI-Wellcome Trust Research Programme

Dr. Abdirahman Abdi (PhD)

Signature: 

Date: 12/9/2024

The Biosciences Department, KEMRI-Wellcome Trust Research Programme

Dr. Amana Juma

Signature: 

Date: 12/9/2024

The Department of Biochemistry and Biotechnology, Pwani University

DEDICATION

I devote this work to my nephew Ace and Kamau's family who have been my pillar and for emotional support and encouragement.

ACKNOWLEDGMENT

I acknowledge my supervisors, Dr. James Njunge, Dr. Abdirahman Abdi and Dr. Amana Juma for their devotion to this project and nurturing me into research. Special thanks to my sponsors the Eastern Africa Network for Bioinformatics Training (EANBiT) and The Childhood Acute Illness and Nutrition Network (CHAIN) for their immense support in funding my master's project.

Many thanks to KEMRI-Wellcome Trust Research Programme (KWTRP) and Initiative to Develop Africa Research Leaders (IDeAL) for offering the necessary research infrastructure. I am grateful to the Breast Milk Composition (BMC) study members led by Prof. Martha Mwangome for developing the study and participants for participating in this study. Many thanks to Elisha Omer, Shaban Mwangi, and Justin Getonto for supporting the laboratory work and Evans Mudibo and Dr. Kioko Mwikali for their support in analyzing the proteomic data.

Finally, many thanks to my lecturers for equipping me with skills and knowledge in Bioinformatics.

ABSTRACT

There have been significant efforts to reduce childhood deaths such as increased access to health care, childhood vaccination, better hygiene and sanitation, and improved nutrition. Despite these efforts infants and young children with poor nutritional status from low- and middle-income countries are susceptible to infectious diseases and are at increased risk of mortality. Breastfeeding reduces the risk of infectious disease-related mortality and promotes infant growth and WHO recommends exclusive breastfeeding for the first six months of life. However, the composition of breast milk may have considerable individual variability and changes during lactation. It is not known whether the composition of breast milk varies among mothers with children of different nutritional status, which may impact recovery from acute illness and growth. This study aims to determine the association between breast milk proteome composition and infant nutritional status and growth following an acute illness. This study was nested within the Breast Milk Composition cohort study that aimed to determine the role of breast milk composition in recovery from infant illness and malnutrition. The study utilized breast milk samples (N=250) from Kenya and Pakistan collected from breastfeeding mothers with infants admitted to hospitals with acute illness and non-hospitalized infants from the same communities. Defatted and casein-depleted breast milk samples were analyzed using liquid chromatography-tandem mass spectrometry and protein identification and quantification were done using the MaxQuant software. Data was preprocessed through filtering, imputing missing values, normalization and correction of batch effects. Elastic net and Random Forest models were used to perform dimension reduction and feature selection. Biological pathway enrichment analysis of the differentially expressed proteins was conducted using ClusterProfiler and DAVID software. Crude linear regression and adjusted mixed-effect linear regression models were used to determine the association between identified proteins and infant's nutritional status and growth. Correction for multiple testing was carried out using the Benjamin Hochberg's false discovery rate method. Hospitalized undernourished infants were younger than non-hospitalized infants ($P < 0.001$). Differential protein expression analysis showed that mothers of hospitalized and non-hospitalized infants have different milk proteomic profiles. Mothers of hospitalized infants had upregulation of immune related biological processes and downregulation of body fluids regulation and lactation processes when compared with mothers of non-hospitalized infants. Beta-casein was positively while S1008A, lactadherin and transthyretin were negatively associated with nutritional status including MUAC, weight-for-age, and length-for-age Z scores among infants at hospital admission. Further, selenium-binding-protein1, chordin-like-protein2 and tenascin-c-hexabrachion were among the proteins positively associated with growth as depicted by change in infant's MUAC from admission to day 45 after hospital discharge. This study has demonstrated that breast milk proteome composition differs between mothers with hospitalized and non-hospitalized infants. Further, the study has shown that breast milk proteins are associated with infants' nutritional status and growth. While this study requires validation in larger cohorts, the study findings provide mechanistic understanding that can inform interventions to enhance convalescence and improve nutritional status and growth among acutely ill infants. Further studies should be undertaken to validate the findings from the current study.

TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGMENT	iv
ABSTRACT.....	v
TABLE OF CONTENTS	vi
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
LIST OF ABBREVIATIONS AND ACRONYMS	xi
CHAPTER ONE: INTRODUCTION.....	1
1.1 Background	1
1.2 Problem statement.....	4
1.3 Justification	4
1.4 Hypothesis.....	5
1.5 Objectives.....	5
1.5.1 Overall objective	5
1.5.2 Specific objectives.....	5
1.6 Scope of the study	5
CHAPTER TWO: LITERATURE REVIEW.....	7
2.1 The burden of malnutrition	7
2.2 The role of breast milk in infant’s health and protection from infectious diseases .	9
2.3 The role of breastfeeding in infants’ nutritional status and growth	12
2.4 Breast milk and its components	13
2.4.1 Micronutrients	14
2.4.2 Macronutrients.....	15

2.5 Factors affecting the breast milk composition	20
2.5.1 Influence of maternal diet on breast milk composition	20
2.5.2 The influence of maternal health on breast milk composition	21
2.5.3 The effect of geographical location and ethnicity on breast milk composition	22
2.5.4 Lactation stages	23
CHAPTER THREE: MATERIALS AND METHODS	26
3.1 Study design	26
3.2 Study Sites.....	26
3.3 Selection of study participants and samples size	26
3.4 Participants inclusion and exclusion criteria.....	27
3.5 Breast milk collection, preprocessing and storage	28
3.6 Ethics and consent to participate	28
3.7 Study Variables	28
3.7.1 Exposures and Outcomes	28
3.7.2 Potential confounders and variables affecting the relationship between milk proteome and nutritional status of the infants	29
3.8 Laboratory procedures	30
3.8.1 Samples preparation for Proteomics analysis using Liquid chromatography and mass spectrometry	30
3.8.2 Tandem Mass Tag Labelling (TMT).....	31
3.8.3 Liquid chromatography-tandem mass spectrometry analysis	31
3.8.4 Protein identification using MaxQuant software.....	32
3.9 Data preprocessing and Statistical analysis.....	32
3.9.1 Characteristics of study participants.....	32

3.9.2 Proteomics data preprocessing	32
3.9.3 Statistical and Bioinformatics analysis.....	37
3.9.4 Pathway enrichment analysis of differentially expressed proteins.....	38
3.9.5 Identification of breast milk proteins associated with infants’ nutritional status at admission	39
3.9.6 Identification of breast milk proteins associated with infants’ post-discharge growth.....	41
CHAPTER FOUR: RESULTS	42
4.1 Baseline characteristics of participants	42
4.2 Mothers of hospitalized and non-hospitalized infants have different milk proteomic profiles.....	45
4.3 Breast milk proteins associated with infants’ nutritional status at hospital admission	53
4.4 Breast milk proteins association with infants’ growth post-discharge.....	58
CHAPTER FIVE: DISCUSSION.....	62
5.1 Conclusion.....	68
5.2 Limitation.....	68
5.3 Recommendation.....	69
REFERENCES.....	70

LIST OF TABLES

Table 2.1: A summary of studies highlighting the role of breast milk in infant’s health	11
Table 2.2: Factors affecting breast milk composition.....	21
Table 2.3: A summary of the human milk proteome variation among different women	23
Table 3.1: Sample distribution across study sites	27
Table 3.2: Performance evaluation metrics on imputation methods	34
Table 3.3: Performance metrics for Boruta and Elastic net models	40
Table 4.1: Baseline characteristics of enrolled Infants	43
Table 4.2: Baseline characteristics of hospitalized Infants.....	44
Table 4.3: Upregulated protein network interactions and their functions	51
Table 4.4: Downregulated Protein network interactions and their functions	52

LIST OF FIGURES

Figure 2.1: A map showing the global burden of malnutrition in 2020	8
Figure 2.2: Estimated proportions of macro and micro components in breast milk.....	14
Figure 3.1: Proteomic data preprocessing workflow	33
Figure 3.2: Principle Component Analysis.....	36
Figure 4.1: Differential milk proteomic profiles between mothers of hospitalized and non-hospitalized infants	46
Figure 4.2: Boxplots showing differentially expressed proteins	47
Figure 4.3: Enriched Biological Processes in milk from mothers of hospitalized infants	48
Figure 4.4: Protein-protein interaction network map of differentially expressed proteins	50
Figure 4.5: Forest plot showing association between breast milk proteins and infants' MUAC at hospital admission.....	55
Figure 4.6: Forest plot showing association between breast milk proteins and infants' weight-for-age Z-scores at hospital admission	56
Figure 4.7: Forest plot showing association between breast milk proteins and infants' length-for-age Z-scores at hospital admission	57
Figure 4.8: Forest plot showing association between breast milk proteins and infants' change in MUAC 45 days post-discharge	59
Figure 4.9: Forest plot showing association between breast milk proteins and infants' change in weight-for-age Z-scores 45 days post-discharge.....	60
Figure 4.10: Forest plot showing association between breast milk proteins and infants' change in length-for-age Z-scores 45 days post-discharge.....	61

LIST OF ABBREVIATIONS AND ACRONYMS

BM	Breast Milk
CHAIN	Childhood Acute Illness and Nutrition Network
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DTT	Dithiothreitol
FDR	False discovery rate
GDM	Gestational diabetes mellitus
HIV	Human Immunodeficiency Virus
HMOs	Human Milk Oligosaccharides
LAZ	Length-for-Age Z-score
LMICs	Low and middle-income countries
MAM	Moderate Acute Malnutrition
MUAC	Mid-Upper Arm Circumference
NAM	Non-malnourished
PCA	Principle Component Analysis
RF	Random Forest
SAM	Severe Acute Malnutrition
SDGs	Sustainable Development Goals
TEAB	Triethylammonium bicarbonate
WAZ	Weight-for-Age Z-score
WHO	World Health Organization
WHZ	Weight-for-Height Z-score

CHAPTER ONE: INTRODUCTION

1.1 Background

Malnutrition, characterized by inadequate or unbalanced intake of essential nutrients, remains a global health challenge with far-reaching consequences. It encompasses both undernutrition, where individuals lack vital nutrients, and overnutrition, marked by excessive intake of unhealthy foods. Undernutrition can result in stunted growth, wasting and being underweight. In a recent collaborative report by the UNICEF, WHO & World Bank (2023), about 148 million (22.3%) children below five years of age are stunted and 45 million (6%) are wasted. The prevalence of undernutrition is higher in low and middle-income countries (LMICs) where about 64% of children are stunted and approximately 76% are wasted (UNICEF, WHO & World Bank, 2023).

During the first six months of life, infants grow more rapidly and gain more weight than other life stages (Patwari et al., 2015). Nonetheless, some infants do not follow the anticipated weight gain trajectory due to factors such as genetic variations, preterm birth, or undernutrition, which can arise from diverse causes. Globally, approximately 8.5 million infants under 6 months are undernourished (Kerac et al., 2019). The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life. Thereafter, to meet their evolving nutritional requirements, infants should receive complementary foods, while continuing to breastfeed for up to two years or longer (WHO, 2003). For HIV-positive mothers, WHO recommends exclusive breastfeeding for 6 months, the introduction of complementary food, and postnatal or maternal antiretroviral prophylaxis to reduce the risk of HIV transmission during breastfeeding (WHO, 2003). Exclusively breastfed infants <6 months of age are at a lower risk of mortality, asthma, insulin resistance in adolescence, hypertension, obesity, and infectious diseases such as diarrhoea and respiratory infections compared to non-breastfed infants (Victora et al.,

2016; Sankar et al., 2015; Mwiru et al., 2013). In situations where the mother cannot produce enough milk for the child, infant formula can be introduced (Richardson & Walters, 2014). Breastfeeding has been reported to significantly influence growth at the early stages of life up to 6 months (Kramer et al., 2002). A study in East Azerbaijan province reported that the growth index of exclusively breastfed infants was higher than that of formula-fed infants at 6 months (Beyrami & Samadi-Afshar, 2018).

Undernourished infants are more prone to suffer from acute illnesses such as respiratory tract infections, malaria and diarrhoea (Caulfield et al., 2004) and it is during hospitalization from these illnesses that the infants are pronounced to be undernourished. Acute illnesses can impact the growth of children through various ways, including reduced food consumption due to loss of appetite, higher energy requirements, impaired nutrient absorption, increased catabolism, and the depletion or retention of essential elements needed for tissue development and growth (Jones et al., 2015; Brown, 2003). In a prospective study in South Africa investigating clinical and growth outcomes of severely malnourished (SAM) children, acute illnesses such as diarrhoea, tuberculosis, and pneumonia were identified as the major causes of relapse and hospital readmission (Grimbeek & Saloojee, 2022).

Undernutrition weakens the immune system, making the infant more susceptible to infection and disease. Additionally, the presence of comorbidities makes it more difficult for the infant to absorb and utilize the nutrients they receive, which can prolong the recovery process or even lead to death. However, the biological factors associated with poor nutritional recovery and growth after hospitalization with an acute illness are not well described, especially among infants below 6 months.

Human milk is exceptionally well-suited for infants below 6 months because of its nutritional composition and the non-nutritional bioactive elements that promote both optimal growth and development (Ballard & Morrow, 2013). Breast milk comprises macronutrients, micronutrients and bioactive molecules which play a crucial role in infant growth and development (Huang & Hu, 2020; Eriksen et al., 2018). These nutrients also have antimicrobial, anti-inflammatory, and immune-regulatory activities that aid in developing the infants' immune system (Meek & Noble, 2022). The macronutrients include carbohydrates, proteins and fats while the micronutrients include vitamins and minerals.

The composition of breast milk is thought to vary from one mother to another and can be influenced by factors such as maternal diet, health, nutritional status, and demographic factors (Bravi et al., 2016). The breast milk fat component varies significantly among mothers and was reported to be in higher concentration among mothers who partially breastfed their infants compared to exclusively breastfeeding mothers (Aumeistere et al., 2019). The macronutrient composition in breast milk was found to be similar among different ethnic groups in New Zealand with varying dietary intake (Aumeistere et al., 2019). A longitudinal study in Poland showed that maternal dietary intake was not associated with milk composition but there was a positive correlation between maternal body mass index (BMI) and milk fat content (Bzikowska-Jura et al., 2018).

Despite breast milk's health and growth benefits during infancy and childhood, its composition among mothers, especially those in low-resource settings, remains unclear. Characterizing breast milk proteome among mothers in Low and Middle-Income Countries (LMICs) will help identify proteins and biological processes associated with infants' nutritional status and growth, thus improving our understanding of the health benefits of breastfeeding during and after acute illness among infants. Therefore, this

study may inform targets for interventions that will help enhance convalescence and improve nutritional status and growth among acutely ill infants.

1.2 Problem statement

Despite the reduction in childhood mortality, undernourished neonates and infants especially in LMICs, remain at a higher risk of poor growth and neurodevelopment. Breast milk remains optimal for infant feeding, and exclusive breastfeeding has been attributed to reduced childhood mortality and enhanced growth (Sankar et al., 2015). The association between breast milk composition and infant nutritional status and growth especially in LMICs, remains unclear. Whether breast milk proteome varies between mothers with infants of differing nutritional status or during periods of acute illness is not well understood and requires investigation. Additionally, although breast milk proteome is diverse, the specific proteins, protein clusters, or associated biological pathways that influence an infant's growth and nutritional status remain unknown. It can be hypothesized that breast milk proteome varies between mothers of hospitalized infants and that of mothers of non-hospitalized infants.

1.3 Justification

Poor post-hospital convalescence including impaired nutritional recovery and growth among undernourished infants remains high despite implementing current guidelines. These guidelines include micronutrient supplementation, treatment of infections, management of hypoglycaemia, hypothermia and acute infections, re-establishment of breastfeeding where possible and post-discharge follow-up. Exclusive breastfeeding is recommended for infants below 6 months even after an acute illness episode. However, hospitalized undernourished Kenyan infants continued to have poor nutritional recovery and growth 6 weeks after discharge despite aggressive lactation support (Mwangome et al., 2020). This scenario has raised questions as to whether breast milk composition and

quantity may be an underlying factor contributing to impaired convalescence and growth among infants. Therefore, characterizing breast milk components will generate valuable information related to the proteomic content and its association with nutritional recovery and growth. Identifying proteomic profiles and biological processes may inform targets for interventions to improve infants' growth and nutritional status after acute illnesses.

1.4 Hypothesis

The breast milk proteome composition is not associated with infants' nutritional status and growth post-discharge.

1.5 Objectives

1.5.1 Overall objective

To characterize human milk proteome and determine factors underlying nutritional status and growth among acutely ill undernourished infants from Kenya and Pakistan.

1.5.2 Specific objectives

1. To determine differentially expressed proteins in human milk among lactating mothers of hospitalized and non-hospitalized infants.
2. To determine whether human milk proteome profiles are associated with infants' nutritional status at hospital admission.
3. To determine whether human milk proteome profiles are associated with infants' growth post-discharge.

1.6 Scope of the study

This is a retrospective study that used data and samples (n=250) from lactating Kenyan and Pakistan mothers and their infants aged 7 days – 6 months enrolled in the Breast Milk Composition study at hospital admission and followed up to day 45 post-discharge. The focus of this study was to investigate the relationship between human milk proteome

profiles, nutritional status and growth of infants aged 7 days – 6 months hospitalized with acute illnesses.

CHAPTER TWO: LITERATURE REVIEW

2.1 The burden of malnutrition

The Sustainable Development Goals (SDGs), particularly target 2.2, represent a global commitment to end all forms of malnutrition in all its forms by 2030 (United Nations, 2015). This target is of utmost importance given the alarming statistics associated with malnutrition, particularly among children under the age of five. Annually, malnutrition contributes to over 3 million child deaths globally, with a significant proportion attributed to infectious diseases (Fanzo et al., 2018). This grim reality shows the urgency of addressing malnutrition comprehensively. The global prevalence of malnutrition is notably high in regions such as Africa, Asia, and Oceania, as evidenced by the striking percentages in Figure 2.1 (UNICEF, WHO & World Bank, 2020). These regions are grappling with the profound challenges posed by malnutrition, affecting not only child survival but also the long-term health and development of communities.

In 2011, it was estimated that approximately 8.5 million infants in LMICs were wasted; of which 3.8 million were severely wasted, while 4.7 million were moderately wasted (Kerac et al., 2011). These statistics emphasize the urgent need for effective interventions, healthcare access, and nutritional support to safeguard the health, growth and future prospects of these vulnerable infants. The SDG target 2.2 serves as a vital mobilization point for global efforts to address this pressing challenge and save the lives of millions of children worldwide.

Anthropometry, which refers to the measurement and assessment of various physical characteristics and dimensions of an infant's body, is commonly used to indicate malnutrition. These measurements typically include weight, length, and head circumference. The anthropometric indices used to describe wasting are weight-for-height Z-score (WHZ), where infants <6 months with WHZ <-2 standard deviations or Mid-

Upper Arm Circumference (MUAC) <11cm are classified as being wasted. It is a manifestation of weight loss resulting from insufficient dietary intake and illness and is evidenced by the visible depletion of body fat and muscle in the thighs, ribs and arms. Stunting is characterized by a child being too short for their age. Infants <6 months with length-for-age Z-score (LAZ) less than 2 are classified as stunted. Underweight is a measure of a child's body weight in relation to their age weight-for-age Z-score (WAZ). A child is categorized as underweight if their WAZ falls below -2 standard deviations (WHO, 2003). In a systematic review investigating anthropometric indices used to assess the nutritional status of infants <6 months, Hoehn and colleagues reported that WAZ and MUAC were the most common (Hoehn et al., 2021). The WHZ was least common, linked to challenges in the length measurement within this age group (Hoehn et al., 2021).

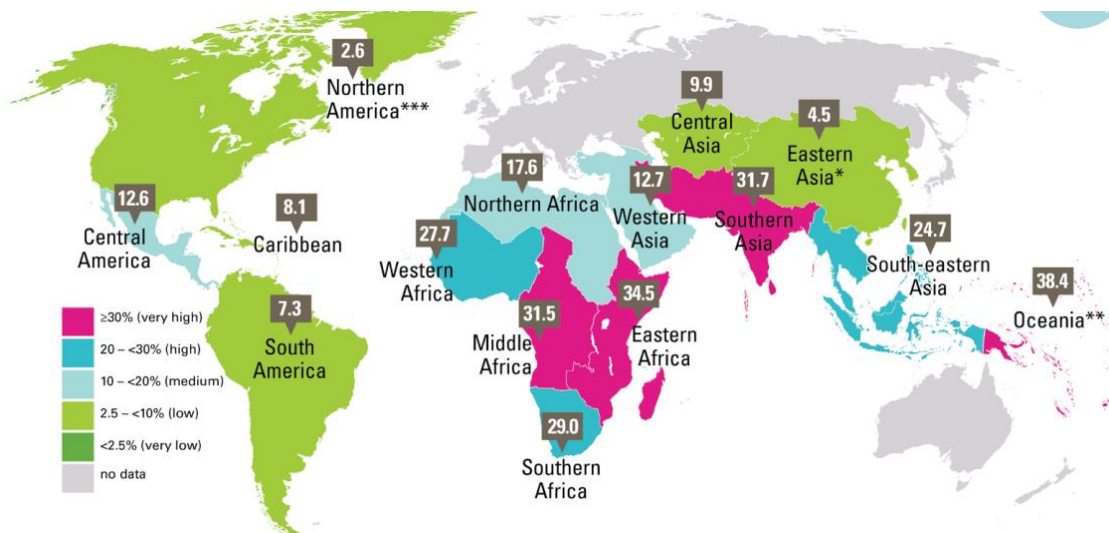


Figure 2.1: A map showing the global burden of malnutrition in 2020 (UNICEF, WHO & World Bank, 2020)

The drivers of undernutrition among infants <6 months are multi-factorial and are thought to include infectious diseases, maternal education level and poor breastfeeding practices, early introduction of complementary foods and poor sanitation and hygiene (Kerac et al.,

2019). Undernutrition in infants <6 months leads to stunted growth, development delays, and increased risk of morbidity and mortality (Partha, 2019). This emphasizes the critical importance of addressing these multifaceted determinants and implementing comprehensive interventions to ensure the health and well-being of this vulnerable age group. Undernutrition weakens the immune system making individuals more susceptible to infections and illnesses, which can lead to increased hospitalization and death. Clinical and growth outcomes post-discharge of severely malnourished children were investigated in a study that involved South African children (3-59 months) who were followed for 6 months (Grimbeek & Saloojee, 2022). It was reported that while 54% of severely malnourished children had recovered, 29% were still moderately malnourished, and 17% were severely malnourished with some of the children re-hospitalized after three months (Grimbeek & Saloojee, 2022). A cohort study among acutely ill malnourished Kenyan infants under 6 months reported poor early post-discharge nutritional recovery despite provision of breastfeeding peer supporters (Mwangome et al., 2020). Therefore, more investigations are required to understand the risk factors associated with malnutrition and improve the nutritional recovery among undernourished infants.

2.2 The role of breast milk in infant's health and protection from infectious diseases

Breast milk is the ideal source of nutrition for infants as it provides all the necessary nutrients in the right amounts (Lessen & Kavanagh, 2015). Among its numerous constituents, human milk plays a crucial role in supporting the development of the infant's immune system (Hosea et al., 2008). Over the years, research has focused on unravelling the intricate relationship between human milk components and immune system development, shedding light on the immunomodulatory properties of these components and their impact on the infant's health. The importance of breastfeeding is presented in Table 2.1 below.

In a systematic review to investigate breastfeeding and the risk of morbidity and mortality resulting from diarrhoea in infants, exclusive breastfeeding was associated with a lower risk of diarrhoea morbidity and mortality compared to non-breastfed infants (Lamberti et al., 2011; Horta, 2019). A meta-analysis study that compared breastfeeding practices reported that partially and non-breastfed infants had a higher risk of mortality and infections such as diarrhoea and pneumonia as compared to exclusively breastfed infants (Black et al., 2008).

Plenge-bönig and colleagues reported that breastfeeding protects against acute gastroenteritis due to rotavirus infection (Plenge-Bönig et al., 2010). Additionally, breastfeeding has been associated with reduced risk of infant mortality, respiratory syncytial virus infection, and hospitalization for diarrhoea and lower respiratory tract infections (Ware et al., 2019; Sankar et al., 2015; Nishimura et al., 2009; Quigley et al., 2007). This suggests that breast milk contains immunological factors that play a role in reducing the risk of infections thus lowering mortality.

Table 2.1: A summary of studies highlighting the role of breast milk in infant’s health

Categories compared	Outcome	Findings
Exclusively, partially and non-breastfeeding	Diarrhoea morbidity and mortality	Non-breastfed infants had a high risk of diarrhoea mortality and morbidity compared to exclusively and partially breastfed (Lamberti et al., 2011)
Breastfeeding and non-breastfeeding	Acute gastroenteritis	Breastfeeding protects against acute gastroenteritis in infants below 6 months of age in Germany, Switzerland and Austria (Lamberti et al., 2011)
Breastfed and not breastfed infants	Postnatal death	Breastfed infants had a lower risk of postnatal death compared to those not breastfed in the United States (Aimin & Rogan, 2004)
Breastfed and not breastfed infants	Infant mortality	In a study conducted in Shelby County, Tennessee breastfeeding was associated with reduced infant mortality (Ware et al., 2019)
Exclusively, partially and non-breastfeeding	Hospitalization for diarrhoea and LRTI	Exclusive breastfeeding was associated with a low risk of hospitalization for diarrhoea and LRTI in United Kingdom (Quigley et al., 2007)
Full, partially, and token breastfeeding	Risk of respiratory syncytial virus	Breastfeeding reduces the severity of the respiratory syncytial virus as shown by a study in Japan (Nishimura et al., 2009)
Exclusively, partially and non-breastfeeding	Mortality resulting from infections	In a multicultural study partially and non-breastfed infants (0-5months) had a higher risk of infection-related mortality compared to exclusively breastfed infants (Sankar et al., 2015)
Exclusively and non-breastfeeding	Risk of mortality from diarrhoea	Exclusively breastfeeding infants (0-5months) had a lower risk of diarrhoea-related mortality (Horta, 2019)

2.3 The role of breastfeeding in infants' nutritional status and growth

Infants' nutritional status and growth during the first year of life are critical for their overall health and development. Adequate nutrition is essential for optimal growth, brain development, and the establishment of a strong immune system. Human milk, the most complete and nutritionally balanced food for infants, plays a vital role in promoting their growth and development.

Moradi and colleagues investigated the effect of breastfeeding on infants' growth indices up to 6 months and reported that breastfed infants gained more weight and height and had a larger head circumference compared to infants fed with milk formula and a combination of breast milk and milk formula (Moradi et al., 2023). These findings are consistent with those reported by other studies, which also showed that exclusively breastfed infants were heavier and taller than formula-fed infants (Kramer et al., 2011; Kramer et al., 2004; Kramer et al., 2002). Breastfeeding has also been associated with cognitive development as reported by Wallenborn and colleagues, where exclusively breastfeeding for at least 6 months and continued breastfeeding with introduction of complementary food for 2 years was associated with improved cognitive development (Wallenborn et al., 2021). An observational study in Karachi, Pakistan, compared weight gain patterns for exclusively and non-breastfed infants from birth to 6 months and observed that non-breastfed infants gained more weight than exclusively breastfed ones (Bai et al., 2022).

A cross-sectional study conducted in Malawi involving infants aged 0-6 months investigated the effect of exclusively breastfeeding on infant growth (Kuchenbecker et al., 2015). Exclusively breastfed infants had more weight, were taller and were less likely to be stunted compared to non-breastfed infants (Kuchenbecker et al., 2015). Additionally, introduction of complementary food before the age of 4 months was associated with gastro-intestinal problems, which were likely to result in micronutrient deficiency,

retarded growth and increased risk of infections in the first 2 years of life (Kuchenbecker et al., 2015). Overall, it is evident that breastfeeding is associated with better growth outcomes. However, the specific components in breast milk that are associated with better growth outcomes are not known. It is therefore important to characterize breast milk components and identify these components.

2.4 Breast milk and its components

Breastfeeding reduces the risk of infant malnutrition by providing the necessary nutrients for growth and development (Scherbaum & Srour, 2016). Human milk is composed of water, macronutrients (carbohydrates, proteins, and fats), micronutrients (vitamins and minerals), and bioactive components such as Immunoglobulins and lactoferrin (Figure 2.2) (Martin et al., 2016). Water is the most abundant breast milk component followed by carbohydrates and fats. Proteins account for about 1% while vitamins and minerals constitute less than 1%. Mature milk (defined as milk produced 16days post-partum) comprises about 0.9 - 1.2 g/dL protein, 3.2 - 3.6 g/dL fat, and 6.7 - 7.8 g/dL lactose (Ballard & Morrow, 2013).

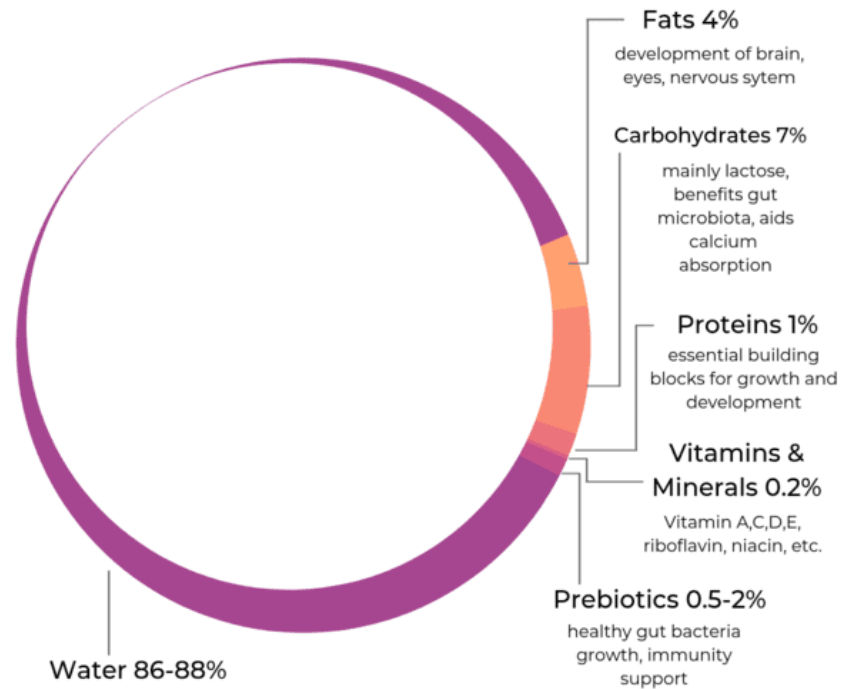


Figure 2.2: Estimated proportions of macro and micro components in breast milk

Source (<https://michaeljloomis.com/human-breast-milk-composition-as-guidepost/>)

2.4.1 Micronutrients

The micronutrient components of breast milk include among other vitamins B1, 2, 6, 12, A, selenium, zinc and iodine. They play important roles such as the infant brain development, differentiation of neural cells and maturation of the central nervous system (Lockyer et al., 2021; Michael et al., 2018). Vitamin B6 plays a role in amino acid metabolism, glycolysis and gluconeogenesis. Its deficiency results in neurological and behavioural disabilities. Neonatal iodine deficiency has been associated with a compromised mental ability (Skeaff, 2011). Additionally, inadequate iron intake during early development can irreversibly impair behavioural outcomes (Georgieff, 2011). Therefore, micronutrient deficiencies in infants can lead to later neurocognitive dysfunction. Micronutrient deficiency predominantly occurs in LMICs (Georgieff, 2011) and is increasing being linked to susceptibility to infectious and chronic diseases. The

m micronutrient composition of human milk is highly varied across populations and may be influenced by the mother's dietary intake (Gibson et al., 2020). Infants born to micronutrient-deficient mothers also tend to have a high risk of this deficiency (Bellows et al., 2017). Lack of micronutrients can lead to various health problems, including anaemia, stunted growth, and developmental delays.

2.4.2 Macronutrients

2.4.2.1 Carbohydrates

Carbohydrates are the most abundant component of human milk. They are important in developing and maintaining the composition of the infant's gut microbiome and infant cognitive development (Berger et al., 2020; Yi & Kim, 2021). Carbohydrates have shown a positive association with infant weight change and adiposity in infants aged 3-12 months (Prentice et al., 2016). There are various types of carbohydrates in breast milk including lactose, galactose, and oligosaccharides (Andreas et al., 2015).

Lactose is the most abundant carbohydrate accounting for about 7% of breast milk and it is readily digested in infants. It is important in maintaining the osmotic pressure of breast milk (Yi & Kim, 2021). Lactose is broken down into glucose and galactose by the enzyme lactase found in the small intestines of infants; these simple sugars are then absorbed and used by the infant's body as a source of energy (Kalyanasundaram et al., 2021). It also plays a role in developing the infant's immune system by stimulating the production of secretory immunoglobulin A (IgA), an important antibody that protects infants from infection (Enrique et al., 2019). However, in some cases, infants are lactose intolerant indicating that they are unable to digest lactose properly which often presents with symptoms such as gas, bloating and diarrhoea (Heyman, 2006). These lactose-intolerant infants may be given a lactose-free or low-lactose formula, or breastfeeding mothers may need to change their diet to reduce the amount of lactose in their milk.

Galactose is a component of lactose and is important for an infant's growth and development, as it is an essential component for the production of brain cells and the formation of the central nervous system (Amissah et al., 2020). It is important for the development of the eyes, as it helps form the retina, lens, and cornea (Enrique et al., 2019). In addition, it plays a role in the development of the immune system, as it helps in the production of white blood cells that fight infections (Amissah et al., 2020).

Human milk oligosaccharides (HMOs) are third most abundant component, with an average of 12g/L in mature milk and 20.9g/L in colostrum milk (Gila-diaz et al., 2019). The HMOs are not digested in the infants' gastrointestinal tract but instead serve as a prebiotic, promoting the growth of beneficial bacteria in the gut (Musilova et al., 2014). The HMOs play a central role in neonatal immune system development by promoting a healthy gut microbiome, preventing pathogen attachment, and modulating immune cells (Walsh et al., 2020). The gut microbiome helps the infant in metabolic and immunological maturation and is vital in growth during early life (Dogra et al., 2021). The gastrointestinal tract of well-nourished breastfed children has lactic acid-producing bacteria (LAB), including *Bifidobacterium* sp and *Lactobacillus* sp (Lyons et al., 2020). There are different HMO profiles, which differ based on whether the mother is a secretor or a non-secretor of Lewis and Secretor blood group genes (Nuzhat et al., 2022). The breast milk of secretor mothers contains a significantly higher amount of fucosylated oligosaccharides than non-secretor mothers (Charbonneau et al., 2017). A study conducted in Malawi showed that fucosylated and sialylated oligosaccharides were associated with malnutrition in that mothers of well-nourished infants had significant levels of fucosylated and sialylated oligosaccharides than mothers of SAM infants (Jorgensen et al., 2021). The study was extended to compare the levels of the two oligosaccharides from non-secretor mothers of healthy and stunted infants and reported that the levels were higher in non-secretor

mothers of healthy infants than in non-secretor mothers of stunted infants (Jorgensen et al., 2021). The HMOs are an important component as they play a key role in the development of the immune system of infants.

2.4.2.2 Lipids

Human milk lipids are an important energy source for infants and provide essential nutrients for growth and development (Duale et al., 2022). Lipids are the second most abundant macronutrient and play an important role in the nutrient supply in an infant. Some important biological functions in infants associated with milk lipids include gastrointestinal function, lipid and lipoprotein metabolism, membrane composition and function, infant growth, neurodevelopment, and immune function (Hahn-Holbrook et al., 2019).

The lipid fraction consists of triglycerides which contain three fatty acids. The fatty acids in breast milk include saturated, mono-unsaturated and polyunsaturated fatty acids. Triglycerides account for about 98% of the total fat component in human milk and are an important source of an infant's energy. Triglycerides are easily digested by the infant and they provide a readily available energy source. They also aid in the absorption of fat-soluble vitamins such as A, D, E, and K. It also contains other fats such as cholesterol and phospholipids (Delplanque et al., 2015).

Human milk fatty acid profiles have been shown to be associated with infant growth. In a study by Prentice and colleagues, higher human milk fat concentration was associated with BMI gain and lower infancy weight for infants aged 3-12 months (Prentice et al., 2016). A six-month birth cohort study involving exclusively breastfeeding Western Australian mothers showed that specific breast milk fatty acids, including Pentadecanoic acid (C15:0), oleic acid (C18:1), linoleic acid (C18:2) and eicosatrienoic acid (C20:3) were positively associated with infant growth (George et al., 2021). The findings are

consistent with those from a Norwegian birth cohort, which reported that triglyceride composition was associated with infant growth (Criswell et al., 2022). The human milk fat composition can be influenced by several factors such as maternal diet, BMI, genetic factors, and time of the day (Miliku et al., 2019).

2.4.2.3 Proteins

Human breast milk consists of different proteins that perform various functions, including providing essential nutrients, enhancing nutrient absorption, and aiding in immunomodulatory and maturation of the gut microbiome (Lønnerdal, 2016). Human milk proteins are classified into whey, and casein depending on their solubility (Zhu, 2019). The whey fraction consists of soluble proteins including antibodies, lactoferrin, α -lactalbumin, secretory Immunoglobulin A (sIgA), albumin, and lysozyme, which helps the infant to fight diseases and infections. Casein proteins are present suspended in micelles and include α -S1-, β -, and κ -casein (Zhu, 2019). Lactocytes synthesize some breast milk proteins while others are absorbed from maternal circulation through the lumen or by transcytosis.

Breast milk proteins provide protective immunity

One of the key groups of proteins found in human milk is immunoglobulins, also known as antibodies. These proteins are transferred from the mother's bloodstream to the breast milk and serve as the first line of defense for the infant against infections (Hanson & Korotkova, 2002). Secretory Immunoglobulin A is the predominant immunoglobulin in human milk and plays a vital role in protecting the infant's mucosal surfaces, such as the gastrointestinal and respiratory tracts, from pathogens (Brandtzaeg, 2010). In addition to immunoglobulins, human milk contains diverse antimicrobial proteins, including lactoferrin and lysozyme. Lactoferrin exhibits both antimicrobial and immunomodulatory functions, preventing bacterial growth and promoting the maturation of immune cells

(Ashraf et al., 2024). Lactoferrin protein has multiple biological functions, including immunomodulatory and anti-inflammatory properties (Czosnykowska-Łukacka et al., 2019) and promotes cellular proliferation and differentiation (Yang et al., 2018). Lysozyme, conversely, contributes to the innate immune defense by disrupting the cell walls of certain bacteria (Ferraboschi et al., 2021). Human milk cytokines, such as interleukins and interferons, modulate immune responses and inflammation, promoting immune homeostasis in the infant (Newburg & Walker, 2007).

Hormones and growth factors found in breast milk play a crucial role in supporting infant's optimal growth and development. The hormones include parathyroid hormone, insulin, nesfatin-1, leptin, ghrelin, obestatin, apelin, and adiponectin (Yi & Kim, 2021). Leptin and adiponectin help regulate appetite and metabolism, aiding in establishing healthy eating patterns and weight regulation in infants (Mazzocchi et al., 2019). These bioactive components are carefully tailored by the mother's body to meet the specific needs of her infant. Transforming Growth Factor-beta (TGF- β), for instance, contributes to the maturation of regulatory T cells, helping to establish immune tolerance and reduce the risk of allergic reactions (Hanson & Korotkova, 2002). Additionally, hormones like Insulin-like growth factors (IGFs), for example, stimulate cell growth and proliferation, contributing to healthy tissue development (Galante et al., 2020). Furthermore, breast milk contains a myriad of immunological and anti-inflammatory factors that help protect the infant from infections and support the maturation of their immune system. Collectively, these hormones and growth factors in breast milk form a remarkable biological blend that nourishes, safeguards, and fosters the growth and well-being of the breastfeeding infant.

2.5 Factors affecting the breast milk composition

2.5.1 Influence of maternal diet on breast milk composition

A mother's diet plays a pivotal role in shaping the composition of her breast milk, an essential source of nutrition for her infant as presented in Table 2.2. Nutrients consumed by mothers directly impact the quality and quantity of vital components in breast milk. The consumption of nuts by mothers and their dietary intake of zinc, iron, and B group vitamins were reported to be highly associated with increased levels of unsaturated fatty acid composition in breast milk (Calvo-Lerma et al., 2022). Maternal fish intake was associated with a high abundance of docosahexaenoic acid in milk (Dingess et al., 2017). Additionally, Mexitalia and colleagues showed that the concentration of human milk proteins was affected by maternal diet (Mexitalia et al., 2022). However, a study involving Caucasian breastfeeding women followed during the first, third and sixth months of lactation (Bzikowska-Jura et al., 2018) and another by Aumeistere and colleagues (Bzikowska-Jura et al., 2018) did not show an association between protein concentration and diet. In a systematic review investigating the effects of nutritional supplements on breast milk composition, maternal diet was reported to influence the concentration of micronutrients such as thiamin, riboflavin, vitamin B-6, vitamin B-12, and choline (Keikha et al., 2021). Therefore, it is recommended that these micronutrients are taken as supplements during pregnancy and lactation (Allen, 2012).

Table 2.2: Factors affecting breast milk composition

Location	Population and follow-up	Findings and comments
Poland	Caucasian breastfeeding women followed for 6 months	Human milk protein composition was not affected by the maternal diet (Bzikowska-Jura et al., 2018)
Germany	Lactating mothers of preterm infants followed for the first 8 weeks of lactation	The milk composition of mothers who delivered preterm and term infants differed (Bauer & Gerss, 2011)
Western Australia	Lactating mothers of infants aged 1-6 months follow-up period was 24 hours	The average fat content was affected by the time of the day (Kent et al., 2006)
California	Breastfeeding mothers, 12 months postpartum	No relation between milk fat content and maternal diet (Lovelady, 1991)

2.5.2 The influence of maternal health on breast milk composition

Maternal health can affect the composition of the breast milk. Maternal metabolic profiles have been associated with differences in the composition of human milk. Mothers with Gestational diabetes Mellitus (GDM) were reported to have lower colostrum whey proteins (Grapov et al., 2015). Additionally, alpha-2-HS-glycoprotein, a liver-derived protein known to downregulate insulin signaling in peripheral tissues, was lower in mothers suffering from GDM (Grapov et al., 2015). Ley and colleagues reported that mothers with GDM exhibited elevated insulin levels in mature human milk compared to mothers with normal blood sugar levels (Ley et al., 2012). A study that compared the human milk composition of healthy and mothers with celiac disease reported that mothers with celiac disease had lower levels of TGF- β 1 and sIgA (Samuel et al., 2020). There is contradicting literature on whether the breast milk composition of mothers with an allergic history differs from those without. Higher levels of IL-4, IL-5, IL-13 and low levels of TGF- β 1 were reported in the breast milk of mothers with a history of an allergic disease

compared to mothers with no history of an allergic disease (Samuel et al., 2020). In addition, breast milk composition of mothers with mastitis had a higher level of minerals, and low levels of lactose, fat, and proteins compared to breast milk from healthy mothers (Samuel et al., 2020). A study involving Peruvian breastfeeding women, investigated the effect of maternal infection on the BM composition (Zavaleta et al., 1995) . Serum and zinc minerals and breast milk were reported to be significantly lower in breast milk of ill mothers while there was no significant difference in the concentration of total proteins, whey and casein proteins between ill and healthy mothers (Zavaleta et al., 1995). Hettinga and colleagues reported 19 breast milk proteins being differentially enriched between allergic and non-allergic mothers (Hettinga et al., 2015). Therefore, there is a need to investigate the effects of maternal health on the composition of other breast milk macronutrients.

2.5.3 The effect of geographical location and ethnicity on breast milk composition

Geographical location and ethnicity are considered to affect breast milk composition. A multi-ethnic study involving breastfeeding women from Asian, Pacific and European origin living in New Zealand reported that there was no significant difference in the concentration of carbohydrates, proteins, fats and moisture in breast milk (Butts et al., 2022). In addition, geographical location and ethnicity were shown to influence the composition of human milk in a study involving multi-ethnic lactating mothers from different locations in China (Zhang et al., 2019). Some milk serum proteins associated with promoting healthy development of infants were shown to be significantly different in the Chinese study (Zhang et al., 2019). However, a study among Chinese and Dutch lactating women reported that the concentrations of highly abundant breast milk serum proteins were similar (Elwakiel et al., 2020). The effects of geographical location and

ethnicity on the composition of breast milk could be due to the differences in diet and environmental exposures among lactating women as presented in Table 2.3.

Table 2.3: A summary of the human milk proteome variation among different women

Location	Population	Sample /Time	Findings
China	Chinese lactating women	30 - 300 days post-partum	The quantity and quality of human milk proteins were similar across ethnic groups (Zhang et al., 2019)
China and Amsterdam	Chinese and Dutch lactating mothers	1-20 weeks Chinese 1-24 weeks Dutch post-partum	Milk serum was similar in terms of abundant proteins for the two populations (Elwakiel et al., 2020)
Netherlands	Allergic and non-allergic lactating mothers	2-4 months post-partum	Some proteins were upregulated in allergic mothers (Hettinga et al., 2015)
United States	Lactating mothers with GDM and without	1–3 days post-partum	Gestational diabetes mellitus was associated with lower colostrum whey proteins (Grapov et al., 2015)

2.5.4 Lactation stages

There are three lactation stages, and the breast milk composition varies across these different stages (Ballard & Morrow, 2013). Colostrum is the first stage and lasts for 3 days postpartum. Colostrum milk contains high protein and low carbohydrate composition compared to mature milk. Colostrum protein ranges between (14–16 g/L) and is rich in immunoglobulins, cytokines which are key in developing the immune system of an infant as well as growth factors (Elwakiel et al., 2020). The second stage is the transition period, which occurs between 3 and 16 days postpartum. Protein degradation is inhibited and

protein synthesis is upregulated in this stage. Mature milk is present from 16 days onwards, where fatty acid synthesis is higher than protein synthesis in this stage. The protein concentration of mature milk ranges between 7-10g/L (Elwakiel et al., 2020). Additionally, protein composition may vary across the lactation stages as reported in a study carried out in China, where 2005 proteins were identified in the colostrum stage, 1952 proteins in transitional milk, and 1855 in mature milk (Zhang et al., 2022). The composition and concentration of breast milk changes across the lactation stages to meet the infant's growth and development requirements.

A higher abundance of immunoglobulins such as sIgA and IgM was reported in transition milk than in mature milk and a higher abundance of IgG in mature milk (Gao et al., 2012). These results are consistent with another study that showed that human serum proteins differed in concentration and composition in Chinese women over a 20-week lactation period (Elwakiel et al., 2020). A systematic review carried out by Italianer and others showed strong evidence that triacylglycerol, fats, cholesterol, iron, melatonin, cortisol, and cortisone composition in human milk could be affected by the circadian cycle (Italianer et al., 2020). In addition, the human milk fat content was reported to be lower in the morning and night than in the afternoon and evening when milk samples were analyzed for 24 hours (Kent et al., 2006). The concentration of fats in mature milk is higher (almost 40 g/L) than in colostrum milk (about 15–20 g/L) and it is also higher in hind milk than in fore milk (Saarela et al., 2005).

However, less information is available about the composition of breast milk of lactating women from LMICs, how the composition differs across individual women, and how it impacts infants' nutritional recovery and growth. Undernourished infants hospitalized with acute illness tend to have poor nutritional recovery and growth even after being discharged from the hospital. It can be hypothesized that breast milk components play a

role in infant nutritional recovery and growth after an acute illness. This study characterized breast milk proteome of lactating women from Kenya and Pakistan and determined proteome profiles associated with the nutritional status and growth of infants hospitalized with an acute illness.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

This was a retrospective study that utilized data and samples from the Breast Milk Composition study (BMC), which was a sub-study within The Childhood Acute Illness and Nutrition Network study (CHAIN, 2019). The CHAIN study was aimed at improving the understanding and identifying intervenable mechanisms leading to death among acutely ill children in Africa and South Asia. The observational study focused on acutely ill children under the age of 2 years admitted to hospitals in Africa (Kenya, Uganda, Malawi, and Burkina Faso) and South Asia (Bangladesh and Pakistan) who presented to hospitals with acute illnesses. The BMC study aimed at understanding the role of breast milk composition in recovery from infant illness and undernutrition.

3.2 Study Sites

Participants were recruited from Migori County Referral Hospital and Mbagathi County Hospital in Kenya as well as Civil and Kharadar hospitals in Pakistan. The selection of these sites was based on their elevated rates of hospitalizations among infants aged < 6 months, combined with their urban location characterized by a heightened risk of infant malnutrition.

3.3 Selection of study participants and samples size

The BMC sub-study recruited breastfeeding mothers and their infants aged 7 days to 6 months who were acutely ill and hospitalized. Infants' age, gender, anthropometric measurements, and information on exclusive breastfeeding status were taken within 48 hours of hospital admission and on day 45 following hospital discharge. In addition, mothers information about health, diet, breastfeeding practices, demographic and breast milk samples were also taken.

Breastfeeding mothers and their infants aged 7 days to 6 months who were non-hospitalized and living in the same communities as the hospitalized infants were also recruited to provide community norms. Infants' age, gender, anthropometric measurements and mothers' information on diet, health, demographics, breast milk samples and breastfeeding information were collected at a single time point when they were enrolled in the study.

This study utilized samples from the BMC study (n=250). The samples were distributed across the study sites as presented in Table 3.1.

Table 3.1: Sample distribution across study sites

Study Site	Hospitalized	Non-hospitalized	Total
Migori	76	32	108
Mbagathi	20	17	37
Kharadar	68	26	94
Karachi AHU	11	0	11
Total	175	75	250

3.4 Participants inclusion and exclusion criteria

The BMC study included mothers who were producing milk and breastfeeding their infants admitted to the hospitals and were planning to continue breastfeeding their infants for an additional two (2) months following discharge from the hospitals. Additionally, these mothers were included if they were able to provide informed consent, planned to remain within the hospital catchment areas, and were willing to avail themselves for a 45-day follow-up after infants' discharge from the hospitals. Mothers of non-hospitalized residing within the same community as the hospitalized infants were included if they were breastfeeding, had not participated in the CHAIN cohort study before and could give informed consent. For the hospitalized cohort, a mother was excluded if she was not producing milk, was not planning to breastfeed her infant for an additional 2 months

following discharge, and if the mother and infant were previously enrolled in the CHAIN Cohort. For non-hospitalized participants, a mother was excluded if she was not breastfeeding or had been previously enrolled in the CHAIN cohort study and an infant was excluded if he or she was currently or previously enrolled in the CHAIN cohort study.

3.5 Breast milk collection, preprocessing and storage

Infants feed approximately every 3 hours; therefore, breast milk was collected about 2 hours after the infant's last feed which was approximately 1 hour before the next feed. At the time of breast milk collection, the breastfeeding peer supporter accompanied the mother to an identified quiet and private room and guided the mother through the collection process. The collection was done in the morning preferably from the breast that was not last used to feed the infant. A volume of 30 ml was collected and placed in a covered cool box out of direct light and taken to the study laboratory where a portion of whole milk was aliquoted into sterile cryopreservation vials. The remaining sample was centrifuged and the milk serum was divided into aliquots and stored at -80°C until further laboratory analysis.

3.6 Ethics and consent to participate

The Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Committee reviewed and approved the study (KEMRI/SERU/CGMR-C/143/3763). Mothers and infants were enrolled after the mother provided written informed consent.

3.7 Study Variables

3.7.1 Exposures and Outcomes

The study exposures were human milk proteome measured at admission. The study outcomes were the infant's nutritional status at admission and growth post-discharge. The nutritional status was defined by the MUAC, WAZ and LAZ measurements at admission. The WHO set standard MUAC measure for well-nourished infants below 6 months is

>11cm, WAZ > 2 SD and LAZ > 2 SD. Growth indices were defined as a child's measured value of weight or height for their age and gender relative to the reference population according to WHO growth charts expressed in terms of z-scores (WHO, 2003) and MUAC measurement. Growth referred here as delta (Δ) was defined as the difference between the value of the z scores at admission (A0) and the value at day 45 (D45) follow-up time point.

$$WAZ = \frac{\text{infant's weight} - \text{median weight of a reference population}}{SD}$$

$$LAZ = \frac{\text{infant's length} - \text{median length of a reference population}}{SD}$$

$$\Delta WAZ = WAZ (D45) - WAZ (A0)$$

$$\Delta LAZ = LAZ (D45) - LAZ (A0)$$

Where, ΔWAZ = Delta WAZ, WAZ = Weight for Age Z score, LAZ = Length for Age Z score

SD = standard deviation,

A0 = value at admission, D45 = value at day 45 after hospital discharge.

3.7.2 Potential confounders and variables affecting the relationship between milk proteome and nutritional status of the infants

The study's potential confounders include the infant's age, gender, and site. Infant's gender has been reported to affect the composition of mother's milk, where a high fat content (Hosseini et al., 2020). Therefore, the current analysis included gender as a covariant affecting the relationship between breast milk proteome and infant's nutritional status. Studies have reported that human milk proteomic composition varies in the different lactation stages (Ballard & Morrow, 2013), and since this study involves infants aged 7 days to 6 months, the infant's age can be a confounding factor for this study. The effect of these variables on the relationship between human milk proteins and infants' nutritional status were determined by comparing the regression coefficient of the model

before and after adding these variables individually to qualify them as confounders. An increase or decrease of the coefficient estimate by more than 10% indicated a confounding effect and the respective variable was adjusted for in the model (VanderWeele, 2019).

3.8 Laboratory procedures

3.8.1 Samples preparation for Proteomics analysis using Liquid chromatography and mass spectrometry

Breast milk serum samples were retrieved from freezers and then centrifuged at 3,000×g for 30 minutes at 4°C to defat and remove casein, large debris, and cells. Casein was removed because the protein is very abundant in breast milk and its presence in this analysis would mask the other less abundant proteins. However, the method used to deplete casein was less effective and some casein was still detected. The aqueous portion beneath the fat layer was aspirated into new Eppendorf tubes. The protein concentrations of the samples were determined using the Qubit assay according to the manufacturer's instructions. Protein concentrations were calculated and an equivalent of 20µg was picked from all the samples and adjusted to 100µL using 100mM Triethylammonium bicarbonate (TEAB). The samples were reduced with 4µL of 1M dithiothreitol (DTT) at 70°C for 1 hour followed by alkylation with 8µL of 1M iodoacetamide (IAA) at room temperature in the dark with continuous shaking for 1 hour and subsequently quenched with 8µL of dithiothreitol (DTT) for 30 minutes at room temperature. The samples were precipitated using four times the sample volume with cold acetone (pre-chilled at -20°C) and then incubated for 1 hour at -20°C followed by centrifugation at 15 000g for 10 minutes at 4°C. The supernatant was discarded, and the pellets were re-suspended in 100µL of 100mM TEAB buffer after slight drying. This was followed by thorough vortexing to ensure complete pellet dissolution. Trypsin was used to digest the protein for 16 hours, at 37°C with shaking.

3.8.2 Tandem Mass Tag Labelling (TMT)

Briefly, the samples were randomly grouped into 28 batches each comprising 9 samples, and the last 28th batch with 7 samples. A control sample (common pool) was prepared by obtaining 5 μ L from all samples. Lyophilized TMT reagents were equilibrated at room temperature and centrifuged at 15 000g for 1 minute. Each vial of 10-plex TMT isobaric tags containing 0.8mg per vial was dissolved in 45 μ L of acetonitrile and thoroughly vortexed for 5 minutes and then centrifuged. Each peptide sample was labeled with 15 μ L of TMT tags and incubated for one hour at room temperature. Samples were quenched using 5 μ L of 5% hydroxylamine. Samples from each batch were pooled into one tube to give 28 batches and the tagged common pool was added to all the batches equally. The labeled batches were purified using P10 C18 pipette ZipTips according to the manufacturer's instructions and purified peptides were quantified using a qubit assay to determine the concentration for the LC-MS/MS analysis.

3.8.3 Liquid chromatography-tandem mass spectrometry analysis

Purified peptides (2 μ g) were loaded in Dionex Ultimate 3000 nano-flow ultra-high-pressure liquid chromatography system (Thermo Scientific) with a 75 μ M \times 2 cm C18 trap column and separated on a 75 μ M \times 2 cm C18 reverse-phase analytical column at 40°C. Using a mobile phase gradient, elution was done for 360 minutes at a flow rate of 0.3 μ L/min. Peptides were measured using a Q Exactive Orbitrap mass spectrometer coupled to the chromatography system via a nano-spray electrospray ion source. The MS1 scanning was done at a resolution of 70000; Automatic gain control (AGC) target, 3e6; maximum injection time, 120ms; scan range, 400-2000m/z; while MS2 settings were done at a resolution of 17500; AGC target, 5e4; maximum injection time, 128 ms; isolation window, 1.6 m/z and obtained a chromatogram ready for MaxQuant protein searches.

3.8.4 Protein identification using MaxQuant software

Protein identification and quantification were done using the MaxQuant software version 2.1.3.0 using the human proteome database from UniProt. A text file of protein groups with corresponding intensities was obtained and ready for bioinformatics analysis.

3.9 Data preprocessing and Statistical analysis

3.9.1 Characteristics of study participants

Continuous variables including anthropometry (MUAC, WAZ, LAZ) and age, were summarized using descriptive statistics including mean or median interquartile range (IQR), depending on whether their distribution was normal or skewed. The type of distributions were determined using histograms, boxplots, and Shapiro–Wilk normality tests and decisions were made based on the p-values with the level of significance set at p-value ($p \leq 0.05$) (Khatun, 2021). Categorical variables including gender and site were summarized using frequencies or percentages and the categories were compared using a chi-squared test. Only samples with information about infants for example age, gender, and anthropometric measurements at A0 and D45 were included in this analysis.

3.9.2 Proteomics data preprocessing

3.9.2.1 Data cleaning

The proteomic data preprocessing followed the steps illustrated in Figure 3.1. First, protein identifiers (protein IDs), protein names, gene names, and reporter intensity columns from the MaxQuant software protein database searches were selected and used in this analysis. Proteins flagged by MaxQuant as contaminants and reverse sequences were filtered out. Proteins detected in less than 20% of the samples were filtered out; therefore, only proteins detected in more than 80% of the samples were included in this study.

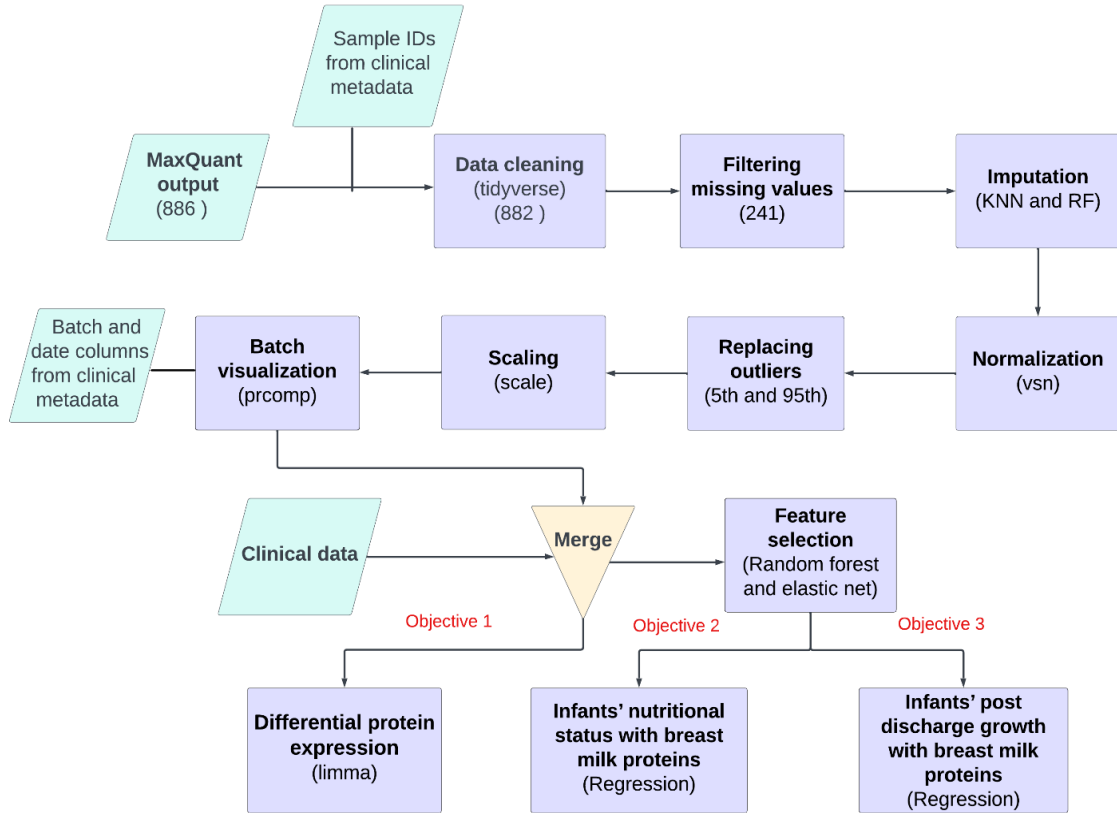


Figure 3.1: Proteomic data preprocessing workflow

3.9.2.2 Imputation

The K-Nearest Neighbors (KNN) and Random Forest (RF) methods were used to impute values for proteins with up to 20% missingness. The KNN imputation method uses the KNN algorithm to search for K nearest neighbours of a protein whose value is missing using the Euclidean distance, and imputing the missing value with the average of the non-missing neighbours (Troyanskaya et al., 2001). The KNN impute function in impute package was used. The random forest imputation technique involves constructing multiple decision trees, each using a random subset of samples and features from the original data. Missing values were handled during tree construction, with only available features being considered at each node. The imputation process took place as the algorithm traverse the trees, utilizing the values of available features in predicting missing values. The imputed values from individual trees were then combined through ensemble averaging, ensuring a

more robust and accurate imputation. The missForest package in R was used to perform the random forest imputation (Stekhoven & Bühlmann, 2012).

The performance of the two imputation methods was evaluated using the Root Mean Squared Error (RMSE) and Mean Absolute Error (MAE) metrics. The RMSE computed the difference between the predicted values and the actual values. Its computation involves squaring the differences, calculating their mean, and then taking the square root of this mean. A lower RMSE signifies that the imputations closely align with the actual values, indicating a higher level of accuracy and a better-performing imputation model. Conversely, a higher RMSE suggests larger discrepancies between imputed and actual values. The MAE metric measured the average magnitude errors between predicted and actual values. The absolute differences between imputed and actual values were calculated and then averaged. The resulting value offers a direct interpretation: a lower MAE indicates that imputed values align more closely with actual values, indicating better performance of the imputation model. The KNN outperformed RF imputation method (Table 3.2) and was therefore selected for this study.

Table 3.2: Performance evaluation metrics on imputation methods

Algorithm	MAE	RMSE
KNN	0.24	0.26
RF	0.26	0.28

3.9.2.3 Normalization

Data normalization was done using variance stabilization normalization (VSN), a function in the R Bioconductor package (Huber et al., 2002). The VSN works by transforming the data so that the variance remains nearly constant over the entire intensity spectrum (Välikangas et al., 2018). The data was first transformed using the natural logarithm where

the estimates of location and scale parameters for each protein were derived. This was followed by variance stabilization which adjusted the variances of the data across the samples, making them more similar and easier to compare. Finally, normalization was performed to ensure that the data assumes a normal distribution (Huber et al., 2002).

3.9.2.4 Outliers and scaling

Boxplots and histograms were plotted to check for outliers in the data (Spratt & Ju, 2016). The data range was defined with the lower limit as $Q1 - 1.5 * IQR$ and the upper limit as $Q3 + 1.5 * IQR$ (Langenbacher et al., 2023). Any data point that was outside the range was referred to as an outlier and was replaced using the interquartile range method of replacing outliers where values beyond the data range were replaced with either the 5th value for those below the fifth percentile or with the 95th value for those above the ninety-fifth percentile in the data (Spratt & Ju, 2016). The data was subsequently centered and autoscaled using R statistical language.

3.9.2.5 Batch effects

The data was examined for batch effects that could have arisen from samples being analyzed in different tandem mass tags (TMT) multiplexed batches and on different days using the Prcomp package in R. The batch effects were visualized using principle component analysis (PCA) plots. The PCA plots showed no distinct clustering based on the different batches or analysis dates for the samples and therefore batch correction was not performed (Figure 3.2); this is because, during protein identification with MaxQuant, there is a normalization step that removes the batch effects.

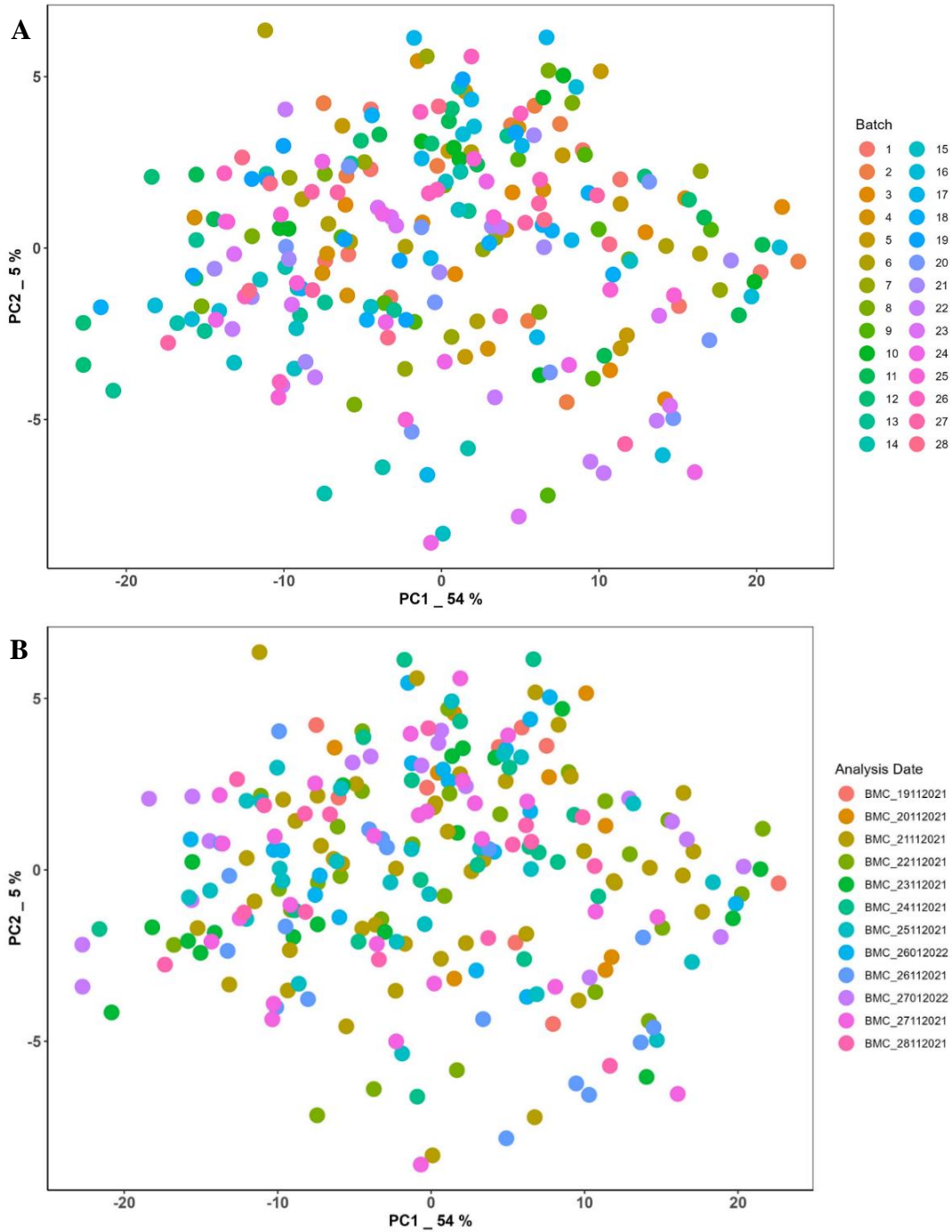


Figure 3.2: Principle Component Analysis

(A) PCA plot showing no batch effect as a result of samples being analyzed in different TMT multiplexed batches. The colors indicate an individual batch. (B) PCA plot showing no batch effect due to the batched analysis of samples on different dates. Colors represent sample analysis dates. There is no distinct clustering indicating no batch effect.

3.9.3 Statistical and Bioinformatics analysis

3.9.3.1 Baseline characteristics of study infants

The `gtsummary` package in R was used to generate the baseline characteristics table for infants' age, gender, site, and anthropometric indices. The test statistics used for the continuous normally distributed variables was T-test and for non-normally distributed was the Wilcoxon rank sum test. In addition, differences in categorical variables between well-nourished and undernourished hospitalized infants were investigated using the Chi-square test.

3.9.3.2 Differential protein expression

To identify breast milk proteins that exhibit significant changes in abundance between mothers of non-hospitalized infants and those hospitalized, crude and adjusted analysis were performed. A design matrix was created to fit a linear model that captures the relationship between individual protein expression and the outcome of interest. The study used the Linear models for microarray data (Limma, version 3.54.2) algorithm in R. This algorithm employs empirical Bayes methods which involves estimating the variances of protein expression within each condition and combining them using shrinkage estimation techniques. The moderated t-statistic is computed for each protein, representing the evidence for differential expression. To account for multiple testing Benjamini-Hochberg method was used. The design matrix for the crude analysis is shown in Equation 1 below.

Equation 1

$$Design < - model.matrix(\sim hospitalization\ status, data)$$

Where hospitalization was a binary variable depicting whether the infant was hospitalized or non-hospitalized.

For adjusted analysis, the infant's age at admission and gender were included in the model as confounders while site was treated as a random effect (Equation 2). The proteomic data

was further fitted in a linear model using `lmFit` in R for both crude and adjusted analysis (Equation 3).

Below is the design matrix for the adjusted model:

Equation 2

```
Design <- model.matrix(~hospitalization status + infant age at admission
                        + gender + (1 | site))
```

Equation 3

```
fit <- lmFit(object = proteins, design = Design)
```

Proteins were considered as differentially expressed if they had a false discovery rate (FDR) < 0.05 and an absolute log fold change (logFC) > 0. A positive logFC indicated that the protein was upregulated in the mothers of hospitalized infants and a negative logFC indicated that the protein was upregulated in the milk of mothers with non-hospitalized infants. The results were visualized using volcano plots, boxplots, and a heatmap.

3.9.4 Pathway enrichment analysis of differentially expressed proteins

The upregulated and downregulated proteins were separately used to determine enriched biological processes using ClusterProfiler version 4.6.2, a Bioconda package in R. Protein IDs were converted to Entrez gene IDs using the Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>) gene ID conversion Tool. The proteins were searched against illuminaHuman4.db (version 1.26.0) and the ontology term was set as “biological processes (BP)” and the adjustment method as described by (Benjamini & Hochberg, 1995) with a cutoff of <0.05.

Network analysis was performed using the STRING biological database and web resource (<https://string-db.org/>), a functional protein association network of known and predicted protein–protein interactions to predict the interactions of the differentially expressed

proteins. The protein lists were uploaded in the STRING interphase and the species was selected as *Homo sapiens*.

3.9.5 Identification of breast milk proteins associated with infants' nutritional status at admission

To determine human milk proteins that were associated with infants' nutritional status at admission, feature selection was performed followed by fitting selected features in linear regression models. Feature selection before fitting a regression model is essential to enhance model interpretability, reduce overfitting, and improve prediction accuracy by identifying and retaining features associated with the outcome. The Elastic Net and random forest regression models were explored to select breast milk proteins that were highly correlated with the infant's nutritional status as defined by MUAC measurement at admission. The elastic net algorithm combines both L1 (Lasso) and L2 (Ridge) regularization penalties to achieve a balance between variable selection and coefficient shrinkage. The lambda parameter controls the degree of regularization, with larger values leading to more shrinkage of coefficients. Additionally, the glmnet package allows for fine-tuning the elastic net method by specifying the alpha parameter. A value of 1 corresponds to the Lasso penalty, favouring sparsity by encouraging more coefficients to be exactly zero. A value of 0 represents the Ridge penalty, promoting shrinkage of coefficients towards zero without necessarily enforcing sparsity. Values between 0 and 1 provide a trade-off between L1 and L2 penalties, combining feature selection and coefficient shrinkage, for this study an alpha of 0.5. The features that had a coefficient greater than 0 were selected as important features.

The Boruta package in R was also used for feature selection, the boruta algorithm is a wrapper feature selection method that combines random forest with permutation-based importance measures to determine the importance of each feature to the target variable

(Nguyen et al., 2015). The algorithm creates shadow features that are random permutations of the original features. The algorithm compares the importance of each original feature with that of its shadow features through a random forest model. If a feature has significantly higher importance than its shadows, it is deemed important for prediction. The algorithm conducts multiple comparisons and eliminates features that are not statistically significant, narrowing down the set of important features. For this study, the selection process was run for 500 iterations and the selected features classified as “confirmed” or “rejected” respectively for features associated or not, with infant’s nutritional status. The performance of the two feature selection methods was compared using the RMSE and the MAE metrics. Boruta feature selection method yielded a lower MAE and RMSE score (Table 3.3) and was therefore used for this study.

Table 3.3: Performance metrics for Boruta and Elastic net models

Algorithm	MAE	RMSE
Boruta	0.8903077	1.104784
Elastic Net	1.062751	1.287577

Linear regression models were used to confirm the relationship between the proteins selected by the boruta algorithm and MUAC in the primary analysis as shown in the crude (Equation 4) and adjusted (Equation 5) analysis.

Equation 4

$$\text{MUAC} \sim \text{breast milk proteins}$$

The lm function and the lme4 package in R were used respectively for the crude and adjusted analysis. Crude models were adjusted for confounders including the infant age at admission and gender while the recruitment site was treated as a random effect as shown in Equation 5.

Equation 5

MUAC ~ breast milk proteins + infant age at admission + gender + (1|site)

The Benjamini-Hochberg's false discovery rate (FDR) correction method was used to correct for false discovery (Benjamini & Hochberg, 1995). The significance level was set at adjusted p-values less than 0.05 for any associations between MUAC and proteins at admission. Secondary analysis was performed using WAZ and LAZ anthropometric outcomes.

3.9.6 Identification of breast milk proteins associated with infants' post-discharge growth

Feature selection was performed using boruta and the selected were then fitted in a crude model (Equation 6) and mixed effect linear regression models (Equation 7) to identify proteins associated with infants' growth post-discharge. Change in MUAC was used as the outcome for primary analysis and change in WAZ and LAZ used in the secondary analysis.

Equation 6

Change in MUAC ~ breast milk proteins

For the adjusted model, infant age at admission and gender were adjusted for as confounders and recruitment site treated as a random effect as shown in Equation 7.

Equation 7

Δ Change in MUAC ~ breast milk proteins + age + gender + (1|site)

The Benjamini-Hochberg's FDR method was used to correct for multiple testing FDR<0.05. Forest plots were generated to visualize the association between infants' change in MUAC and breast milk. Similar analysis was repeated for change in WAZ and change in LAZ outcome variables.

CHAPTER FOUR: RESULTS

4.1 Baseline characteristics of participants

The baseline characteristics of the study infants at enrolment are summarized in Table 7. Of the 250 study infants, one infant did not have anthropometric information at admission and was dropped, hence a total of 249 was analysed at admission. The hospitalized infants were younger compared to non-hospitalized infants in the community ($p=0.001$), and there was no significant difference in the proportion of infants recruited in hospital or in community based on gender (0.079) (Table 4.1). There were no non-hospitalized participants recruited from Kharadar while a larger proportion of hospitalized infants were recruited from Migori ($p=0.024$). Further, anthropometric indices including underweight (WAZ), wasting (MUAC), and stunting (LAZ) indicated that hospitalized infants were undernourished compared to non-hospitalized ($p<0.01$).

The number of hospitalized infants recruited in the study was 175, some of them had missed follow-up at day 45 due to withdrawal from the study or mortality and therefore 140 participants were included for the third objective. Hospitalized study infants were further classified into well-nourished ($n=85$, $MUAC>11\text{cm}$) and undernourished ($n=55$, $MUAC \leq 11\text{cm}$) as described in Table 4.2. Among the hospitalized infants, age ($p=0.6$), gender ($p=0.6$) and recruitment sites ($p=0.4$) did not significantly differ between well-nourished and undernourished infants (Table 4.2). As expected, the undernourished infants were more wasted, underweight, and stunted based on median MUAC, WAZ, and LAZ, respectively.

Table 4.1: Baseline characteristics of enrolled Infants

Participants' characteristics	Community (n=75)	Hospitalized (n=174)	Total (n=249)	p-value
Demographic				
Age (months)				0.001
Median (IQR)	2.83 (1.61 - 4.22)	1.81 (0.50 - 3.51)	2.07 (0.66 - 3.94)	
Sex (%)				0.079
Female	43 (57%)	77 (44%)	120 (48%)	
Male	32 (43%)	97 (56%)	129 (52%)	
Site				0.024
Civil	26 (35%)	68 (39%)	94 (38%)	
Kharadar	0 (0%)	11 (6.3%)	11 (4.4%)	
Migori	32 (43%)	75 (43%)	107 (43%)	
Nairobi	17 (23%)	20 (11%)	37 (15%)	
Anthropometric indices				
Mid-upper arm circumference (cm)				<0.001
Median (IQR)	12.70 (12.10 - 13.60)	11.20 (9.50 - 12.50)	11.80 (10.00 - 12.90)	
Weight-for-age z-score				<0.001
Median (IQR)	-0.46 (-1.54 - 0.19)	-1.64 (-2.95 - -0.42)	-1.30 (-2.46 - -0.19)	
Length-for-age z-score				0.022
Median (IQR)	-1.31 (-1.90 - -0.26)	-1.58 (-2.92 - -0.44)	-1.43 (-2.47 - -0.34)	
Weight-for-height z-score				<0.001
Median (IQR)	0.38 (-0.65 - 0.99)	-0.67 (-1.55 - 0.30)	-0.38 (-1.29 - 0.64)	

¹ n (%)

² Wilcoxon rank sum test; Pearson's Chi-squared test

Table 4.2: Baseline characteristics of hospitalized Infants

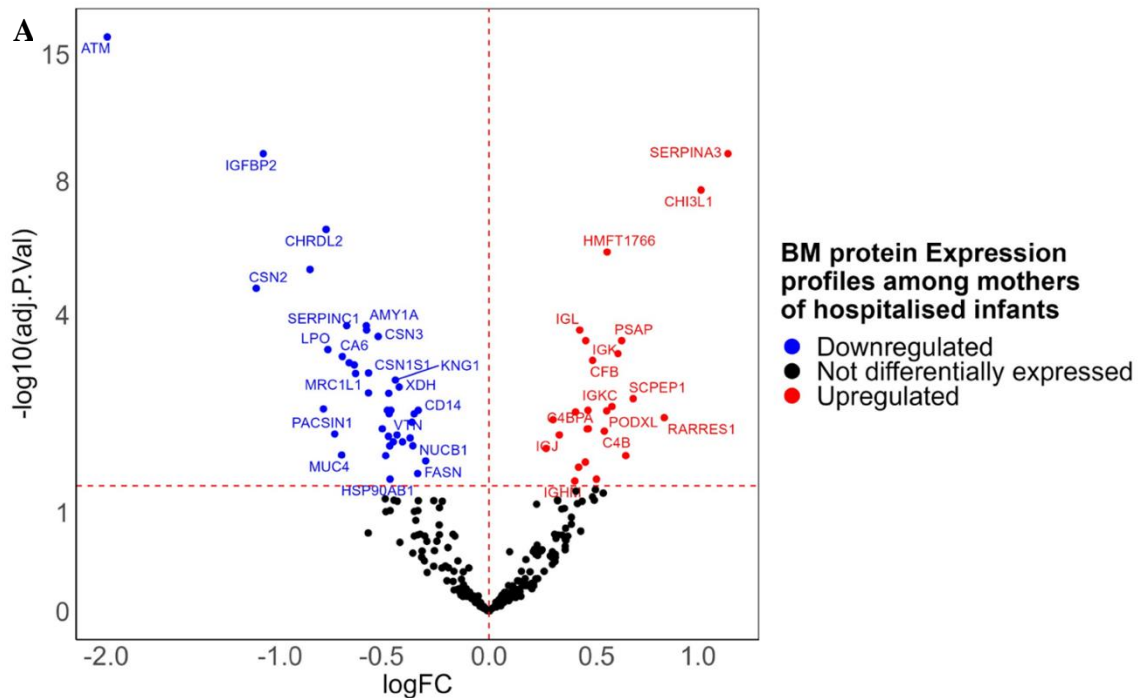
Participants' characteristics	Undernourished (n=55)	Well-nourished (n=85)	Total (n=140)	p-value
Demographic				
Sex (%)				0.6
Female	23 (42%)	41 (48%)	64 (46%)	
Male	32 (58%)	44 (52%)	76 (54%)	
Site				0.4
Civil	20 (36%)	41 (48%)	61 (44%)	
Kharadar	5 (9.1%)	3 (3.5%)	8 (5.7%)	
Migori	25 (45%)	34 (40%)	59 (42%)	
Nairobi	5 (9.1%)	7 (8.2%)	12 (8.6%)	
Age (months)				0.6
Median (IQR)	1.94 (0.51 - 3.32)	1.97 (0.59 - 3.78)	1.97 (0.53 - 3.61)	
Anthropometric indices				
Mid-upper arm circumference (cm)				<0.001
Median (IQR)	10.10 (8.70 - 11.10)	12.10 (10.90 - 12.90)	11.30 (9.78 - 12.60)	
Weight-for-age z-score				<0.001
Median (IQR)	-3.10 (-4.01 - -2.45)	-0.93 (-1.42 - 0.02)	-1.62 (-2.76 - -0.60)	
Length-for-age z-score				<0.001
Median (IQR)	-2.93 (-4.06 - -1.97)	-0.67 (-1.57 - -0.07)	-1.50 (-2.66 - -0.43)	
Weight-for-height z-score				<0.001
Median (IQR)	-1.53 (-2.47 - -0.41)	-0.39 (-1.01 - 0.65)	-0.68 (-1.51 - 0.19)	

¹ n (%)

² Pearson's Chi-squared test; Wilcoxon rank sum test

4.2 Mothers of hospitalized and non-hospitalized infants have different milk proteomic profiles

A total of 886 proteins were identified and quantified, of which 241 remained after preprocessing. Expression analysis identified 65 proteins as significantly different (p -value < 0.05 and FDR < 0.05) between milk from mothers of hospitalized and non-hospitalized infants. Of these, 25 were upregulated while 40 were downregulated in the milk from mothers with hospitalized infants (Figure 4.2A). The differential expression was also evident when only the differentially expressed proteins (DEPs) were clustered by hospitalization status (hospitalized versus non-hospitalized) and represented in a heat map (Figure 4.2B). Boxplots showing the expression profiles of some selected DEPs in the breast milk of mothers with hospitalized infants and those with non-hospitalized infants are shown in Figure 4.3 A and B respectively.



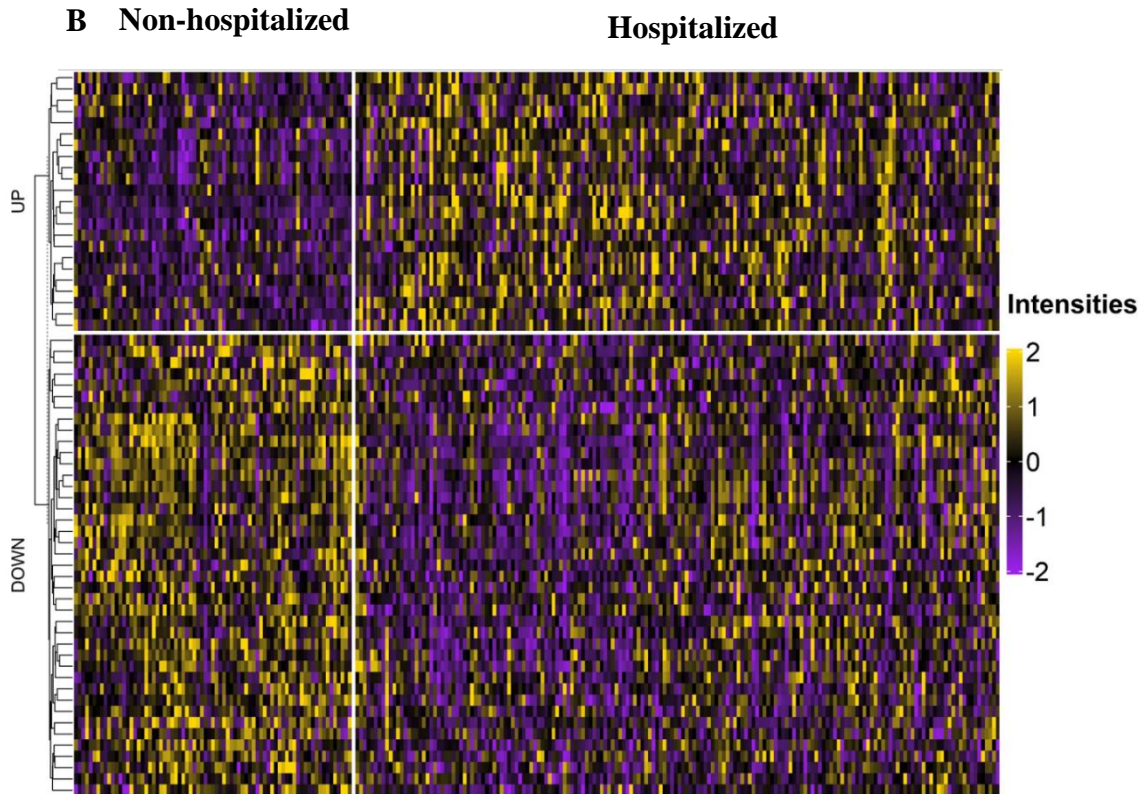


Figure 4.1: Differential milk proteomic profiles between mothers of hospitalized and non-hospitalized infants

(A) A volcano plot showing differentially expressed milk proteins from mothers of hospitalized versus non-hospitalized infants. Top significant proteins are above the horizontal dashed red line ($p\text{-value} < 0.05$ and $FDR < 0.05$). Blue color represents proteins upregulated in non-hospitalized and downregulated in the mothers of hospitalized participants. Red represents proteins upregulated in the hospitalized and downregulated in the non-hospitalized. Black color shows proteins that did not show significant change between the two groups. (B) A heatmap showing the clustering pattern of differentially expressed proteins. The yellow color represents upregulated proteins and purple represents downregulated proteins.

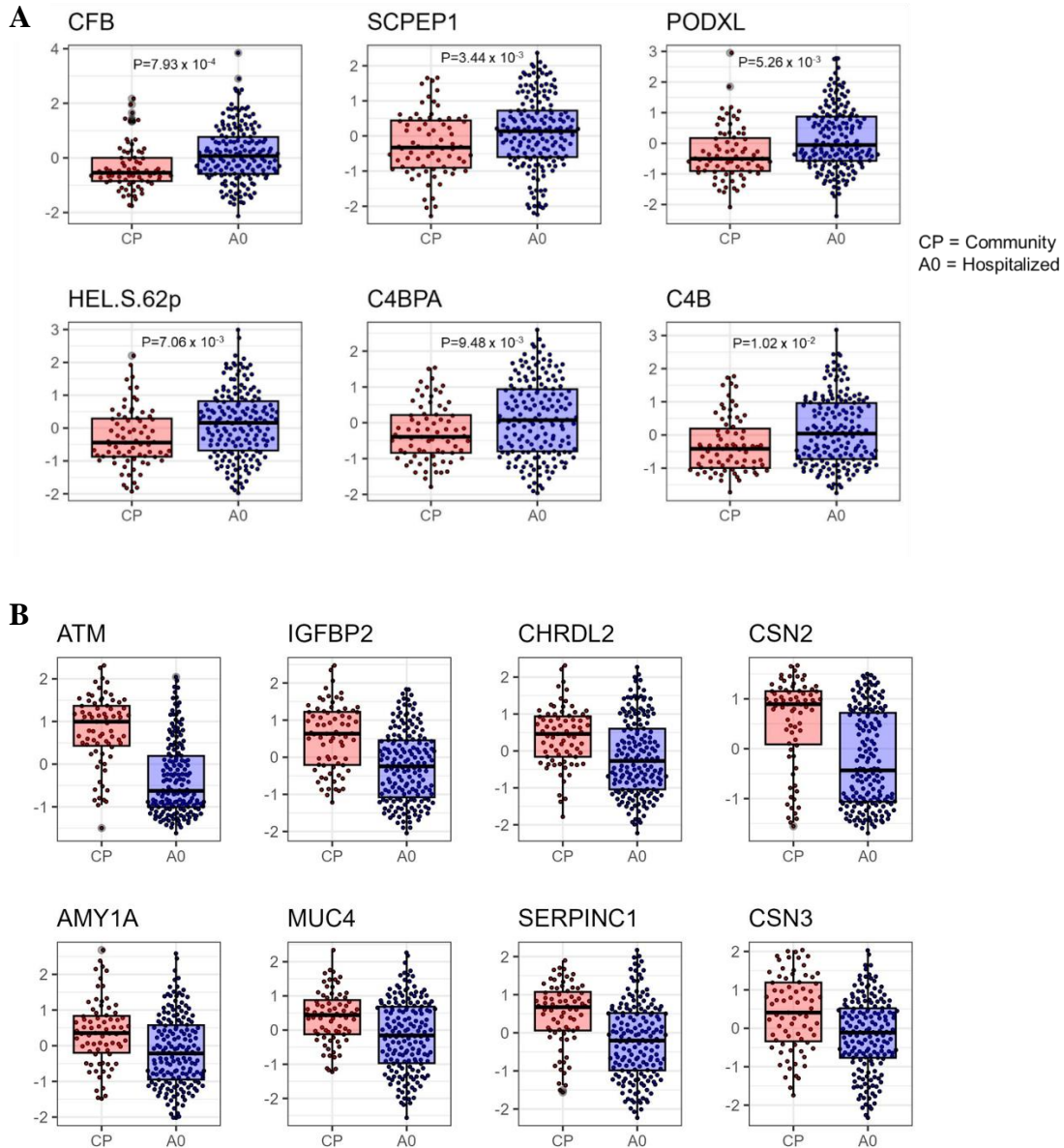


Figure 4.2: Boxplots showing differentially expressed proteins

(A) Upregulated proteins in the breast milk of mothers with hospitalized infants showed a higher expression profile. (B) Upregulated proteins in the breast milk of mothers with non-hospitalized infants showed a higher expression profile

Enrichment analysis suggested that mothers of hospitalized infants had higher levels of proteins associated with complement activation, regulation of apoptotic cell clearance, regulation of endopeptidase activity, and tissue homeostasis processes than mothers of non-hospitalized infants (Figure 4.4A). In addition, biological processes associated with

body fluids regulation, lactation, and cellular response were downregulated in the mothers whose infants were hospitalized and enriched in mothers of non-hospitalized infants (Figure 4.4B).

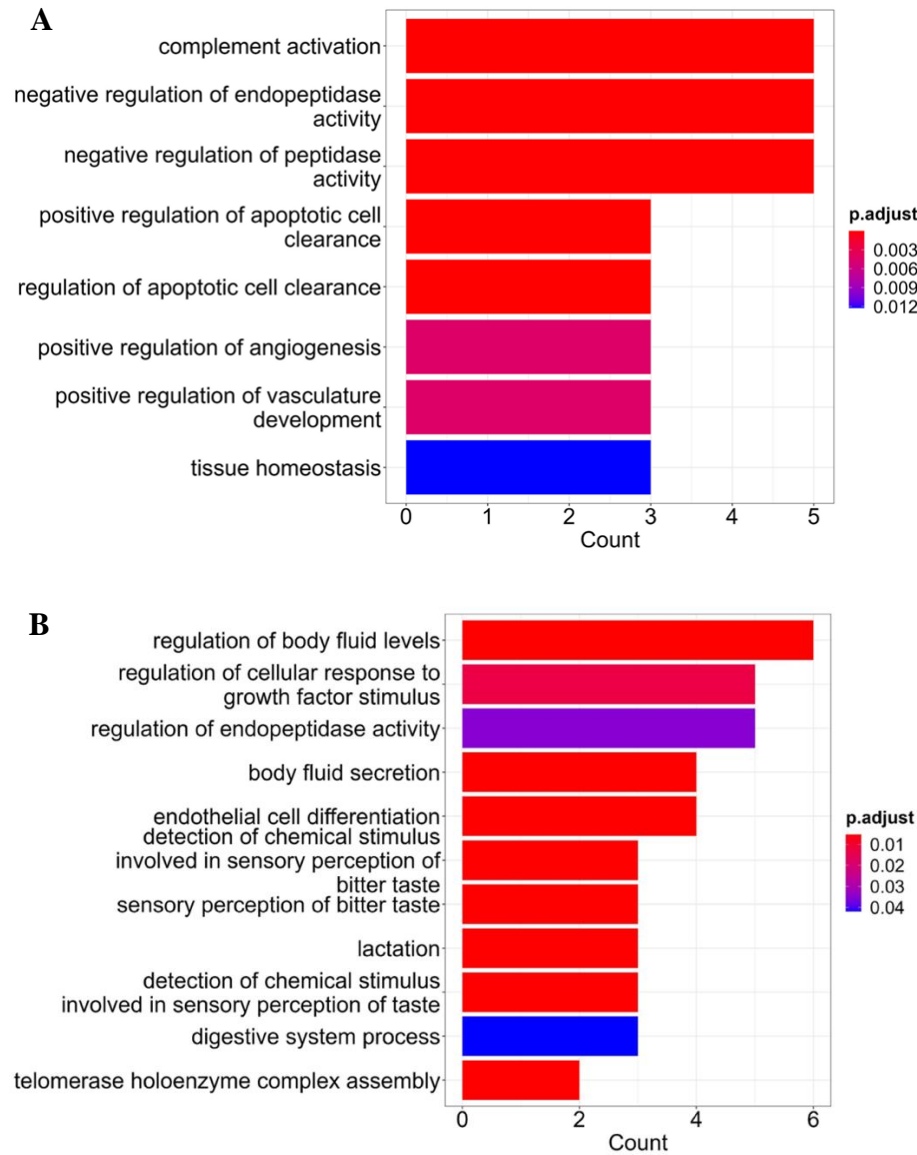
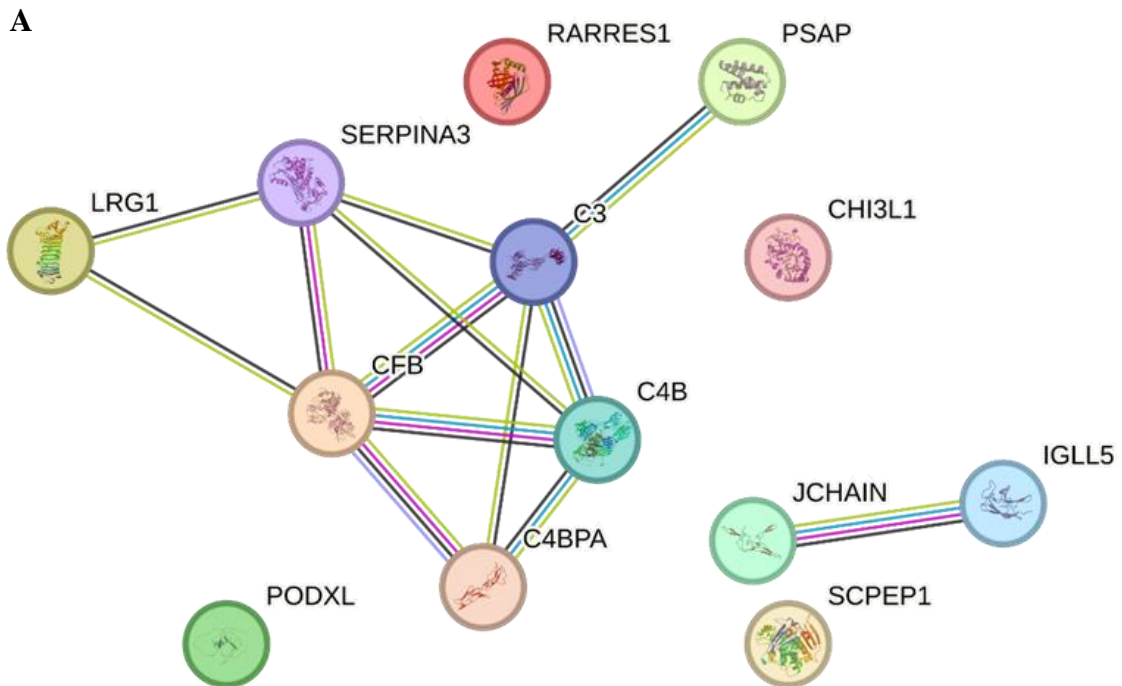


Figure 4.3: Enriched Biological Processes in milk from mothers of hospitalized infants

(A) Shows upregulated processes in milk from mothers of hospitalized infants. Most upregulated processes are involved in immune responses. (B) Shows downregulated processes in milk from mothers of hospitalized infants. Most downregulated processes are involved in body fluid regulation. For A and B, colors depicts the level of significance.

Network correlation analysis revealed that proteins that perform similar functions clustered together. Only proteins that had gene symbols were represented in the network analysis and this resulted in 13 out of 25 upregulated proteins and 37 out of 40 downregulated proteins being represented in Figure 4.5A and 4.5B respectively. Proteins CFB, C3 and C4B that play a role in the complement system were upregulated in mothers with hospitalized infants clustered together in the network analysis (Figure 4.5A). A similar observation was made where proteins ATM, EZR, ALDOA, FASN and HSP90AB1 which were upregulated in the non-hospitalized participants formed a cluster (Figure 4.5B). These proteins are involved in cell signaling, metabolism and homeostasis. The inter-correlated proteins and their functions are summarized in Table 4.3 and 4.4.



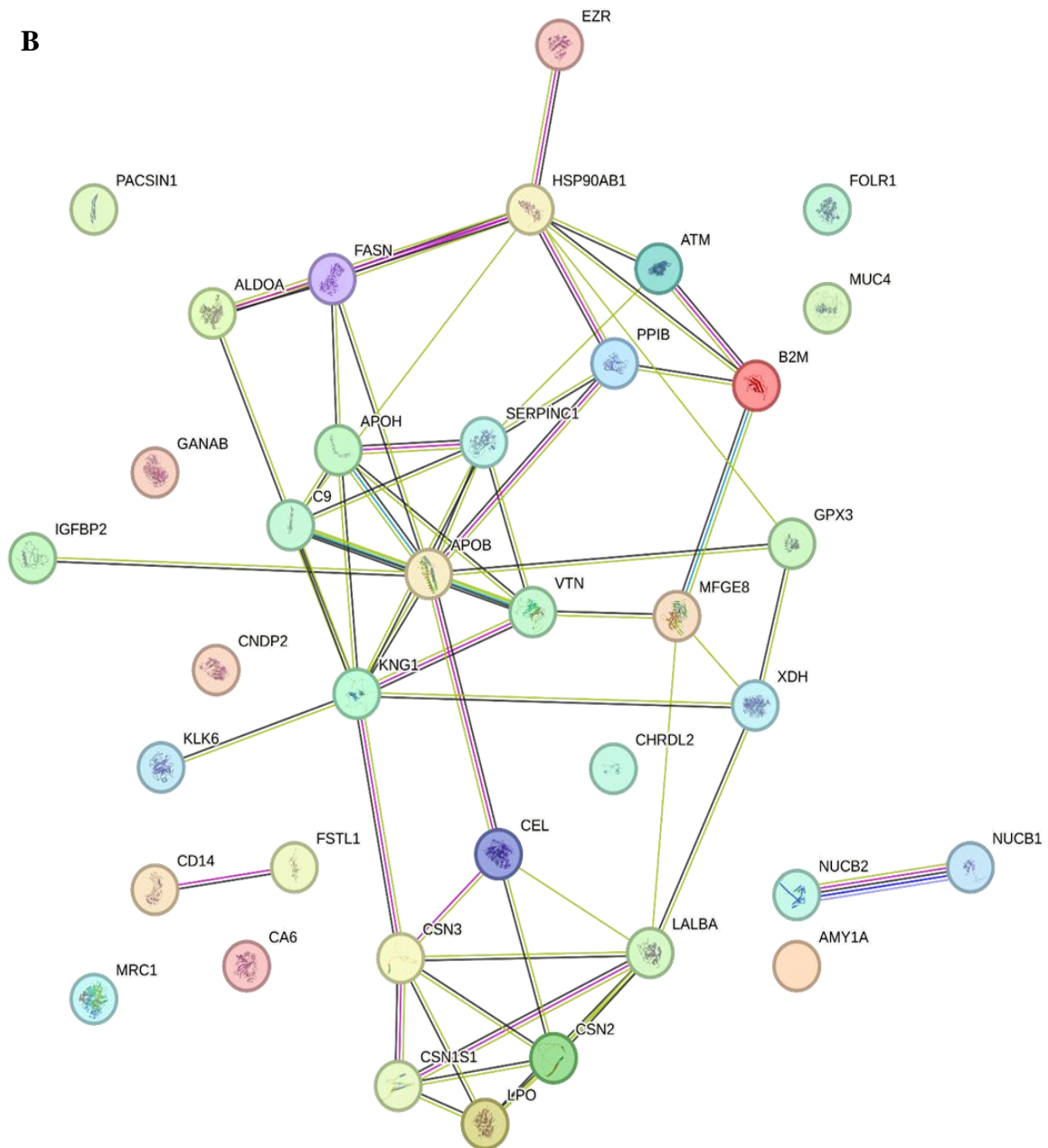


Figure 4.4: Protein-protein interaction network map of differentially expressed proteins

(A) Interaction network of upregulated proteins. (B) Protein interaction network for downregulated proteins. Each node represents a protein, and each edge represents the direct interaction between proteins.

Table 4.3: Upregulated protein network interactions and their functions

Gene	Function
Complement Component Factor B (CFB)	- It's a component of the alternative pathway of complement activation and plays a role in immune response regulation
Complement factor 4 (C4B)	- Plays a regulatory role in the host defense complement system and also Acts as an Innate Immune Effector Against the Influenza A Virus
Complement C3	- Plays a central role in the activation of the complement system and regulation of inflammatory responses
Serine proteinase inhibitor A3 (SERPINA3)	- Regulation of inflammatory responses
Immunoglobulin J Chain (IGJ)	- Play a role in the immune response
Serine carboxypeptidase 1 (SCPEP1)	- Regulation of proteolysis and blood pressure
Retinoic acid receptor responder 1(RARRES1)	- Promotes apoptosis (programmed cell death) in certain cells, thereby aiding in the removal of damaged cells
Chitinase-3 like-protein-1 (CHI3L1)	- Plays a role in inflammation and tissue repair

Table 4.4: Downregulated Protein network interactions and their functions

Gene	Function
Serine/threonine kinase (ATM)	<ul style="list-style-type: none"> - Involved in the normal development and activity of several body systems such as the immune system - Involved in the synthesis of a protein that controls cell growth and division
Ezrin (EZR) gene	Involved in signal transduction
Fatty acid synthase (FASN)	- Play a role in fatty acid synthesis and lipid metabolism
Fructose-bisphosphate aldolase A (ALDOA)	- Involved in glycolysis
Heat shock protein 90 alpha family class B member 1 (HSP90AB1)	- Maintaining cellular protein homeostasis and aiding in the degradation of proteins
Lactalbumin Alpha (LALBA)	<ul style="list-style-type: none"> - Provides essential amino acids crucial for growth and development - Immune regulation
Alpha-S1-casein (CSN1S1)	- Responsible for the transfer of calcium to the newborn
Casein beta (CSN2)	<ul style="list-style-type: none"> - It is the primary source of vital amino acids for a suckling infant - Has antimicrobial activity
Kappa-casein (CSN3)	- It is the major source of vital amino acids for neonates
Lactoperoxidase (LPO)	- Has antibacterial and antiviral properties
Kallikrein 6 (KLK6)	<ul style="list-style-type: none"> - Has antimicrobial properties - Involved in physiological processes, such as tissue remodeling, inflammation, and neural development
Nucleobindin 2 (NUCB2)	<ul style="list-style-type: none"> - Calcium level maintenance - Regulation of metabolism

Follistatin-like 1 (FSTL1)	<ul style="list-style-type: none"> - Influences processes such as cell proliferation, differentiation, and tissue remodeling - Regulation of inflammatory responses
Chordin-like protein 2 (CHRDL2)	<ul style="list-style-type: none"> - Involved in development and signaling processes
Insulin-like Growth Factor Binding Protein 2 (IGFBP2)	<ul style="list-style-type: none"> - Involved in signaling and cellular processes related to growth and development
Glutathione Peroxidase 3 (GPX3)	<ul style="list-style-type: none"> - Acts as an anti-oxidant and offers cell protection from oxidative stress and maintains cellular health - Role in modulating inflammation
Vitronectin (VTN)	<ul style="list-style-type: none"> - Involved in tissue repair, cell adhesion, and immune regulation
Xanthine dehydrogenase (XDH)	<ul style="list-style-type: none"> - Regulation of metabolism, inflammatory responses, and tissue repair
Mucin 4 (MUC4)	<ul style="list-style-type: none"> - Protects epithelial surfaces against pathogens - Cell signaling and adhesion
Glucosidase II alpha subunit (GANAB)	<ul style="list-style-type: none"> - Involved in maintaining cellular homeostasis
Cytosolic Non-specific Dipeptidase 2 (CNDP2)	<ul style="list-style-type: none"> - Regulates metabolism and cell homeostasis

4.3 Breast milk proteins associated with infants' nutritional status at hospital admission

The boruta feature selection algorithm identified a total of 36 milk proteins that were associated with infants' MUAC at admission in the primary analysis. Upon subjecting these proteins to crude linear regression analysis, 29 proteins were shown to be significantly associated with MUAC at admission (Figure 4.6A) which further reduced to 27 in the adjusted analysis (Figure 4.6B). Lysozyme and beta-casein were positively

correlated while lactadherin, ceruloplasmin, serotransferrin, S100A8, and immunoglobulins were negatively correlated with MUAC in crude analysis. Alpha-casein and lysozyme were no longer significantly associated with MUAC after adjusting for infant's gender, age at admission and site. Beta-casein a protein known to play a role in infant growth and development was positively associated with infants' nutritional status at admission. This implied that a one-unit increase in the concentration of beta-casein in breast milk was on average associated with a 0.25cm change in MUAC among hospitalized infants. A large number of proteins that were negatively correlated with MUAC play a role in immune-related functions including immunoglobulins, the S100A8 (a subunit of calprotectin), mucin4 and lactadherin. The negative correlation implies that as these immune-related proteins increase in breast milk, infants may experience improved immune system functionality, potentially leading to enhanced resistance to infections and a better nutritional status outcome.

In secondary analysis, 13 and 20 proteins showed an association with WAZ and LAZ respectively. In the crude regression analysis, 12 proteins were associated with WAZ (Figure 4.7A). Subsequently, upon adjusting for infant's age, gender and site, all 13 proteins (Figure 4.7B) were found to be associated with WAZ. Beta-casein was positively associated while actin, transthyretin and calprotectin were negatively associated with WAZ. In the crude regression analysis for LAZ, 9 proteins showed significant associations (Figure 4.8A). However, after adjusting for infant's age, gender and site, the number of proteins with significant associations decreased to 4 (Figure 4.8B). S1008A, actin and myosin reactive proteins were negatively associated with infant wasting, underweight and stunting (Figure 4.6, 4.7, 4.8).

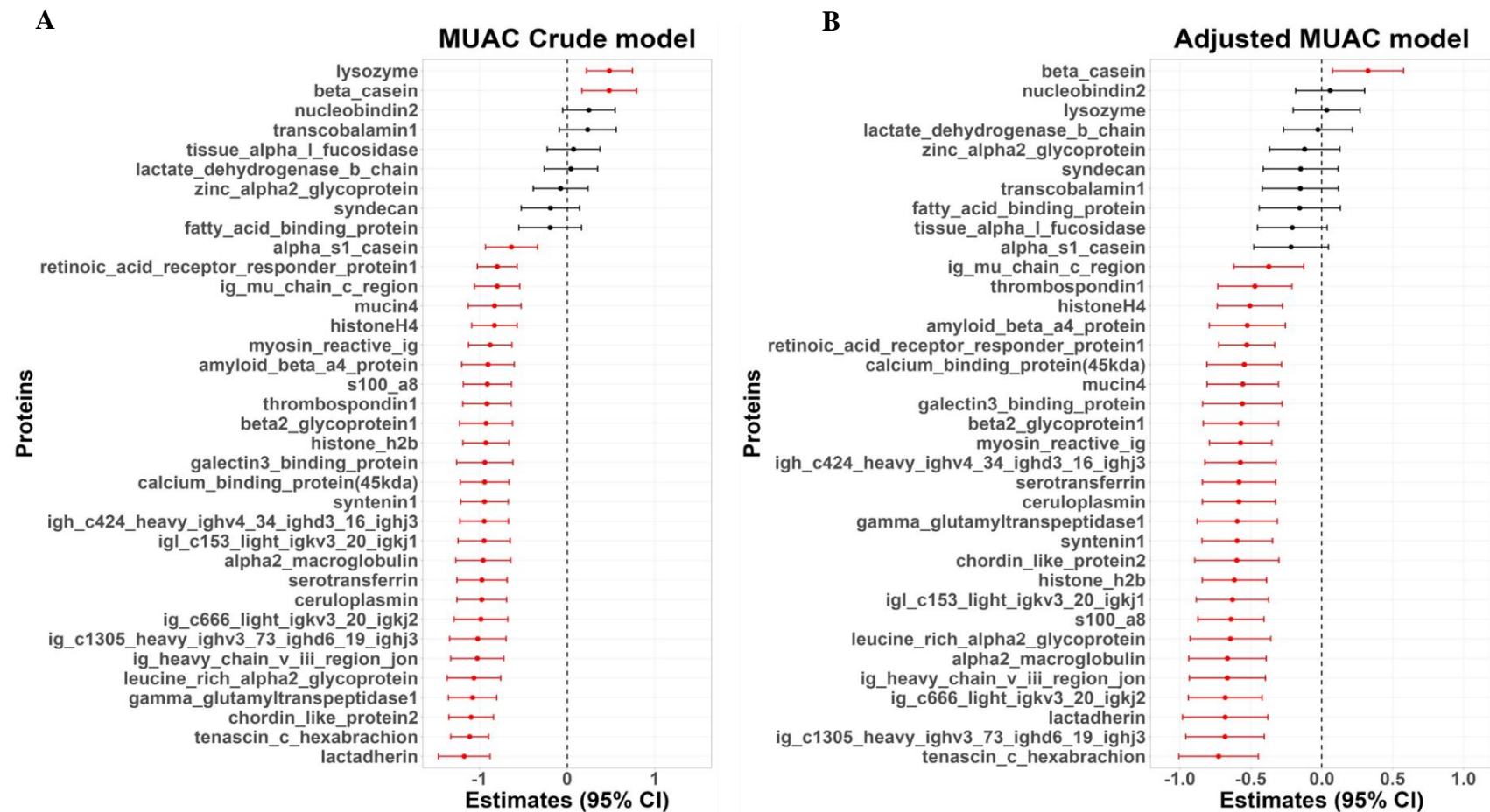


Figure 4.5: Forest plot showing association between breast milk proteins and infants' MUAC at hospital admission

(A) Crude model. (B) The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

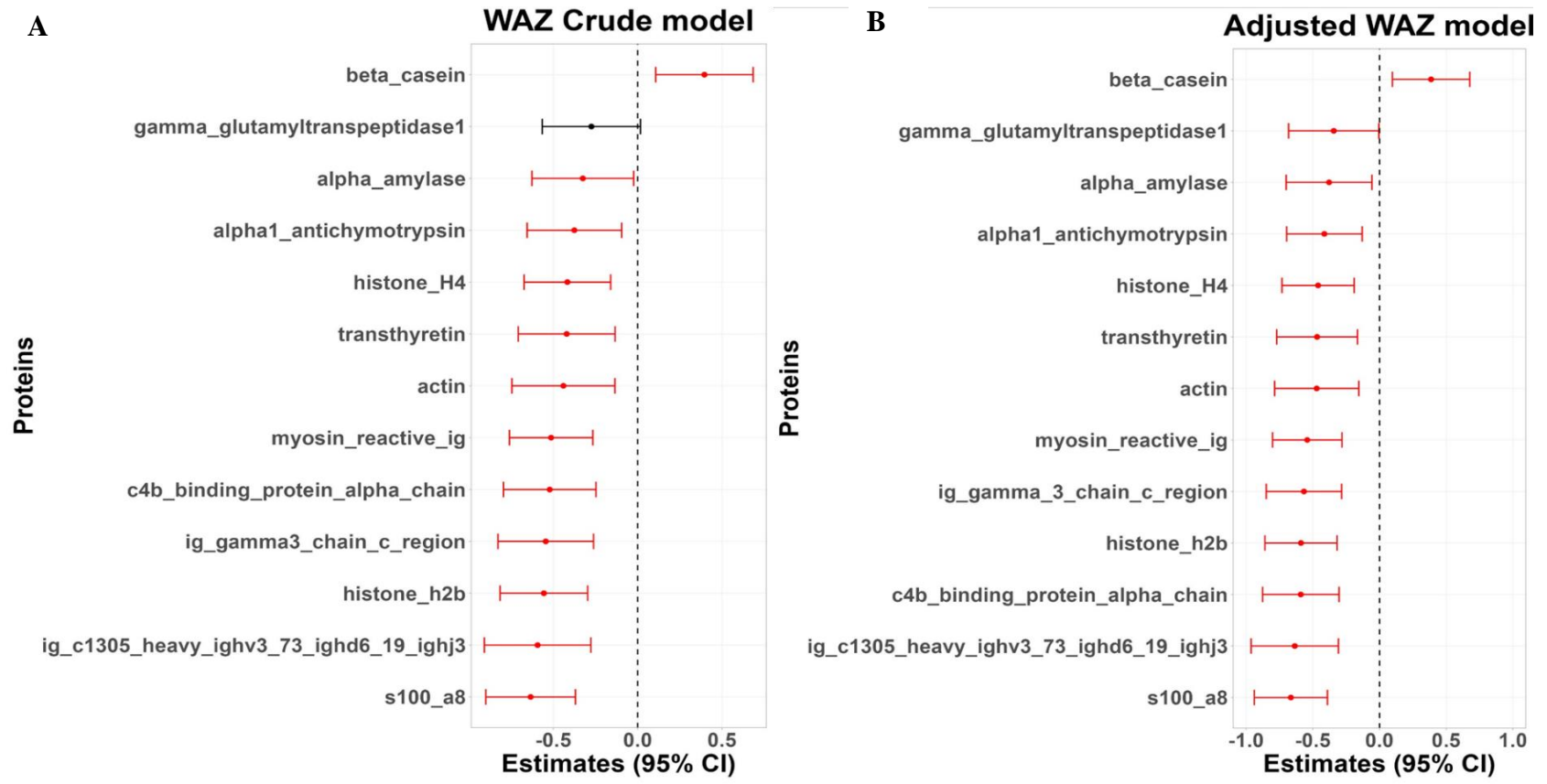


Figure 4.6: Forest plot showing association between breast milk proteins and infants' weight-for-age Z-scores at hospital admission

(A) Crude model. (B) The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

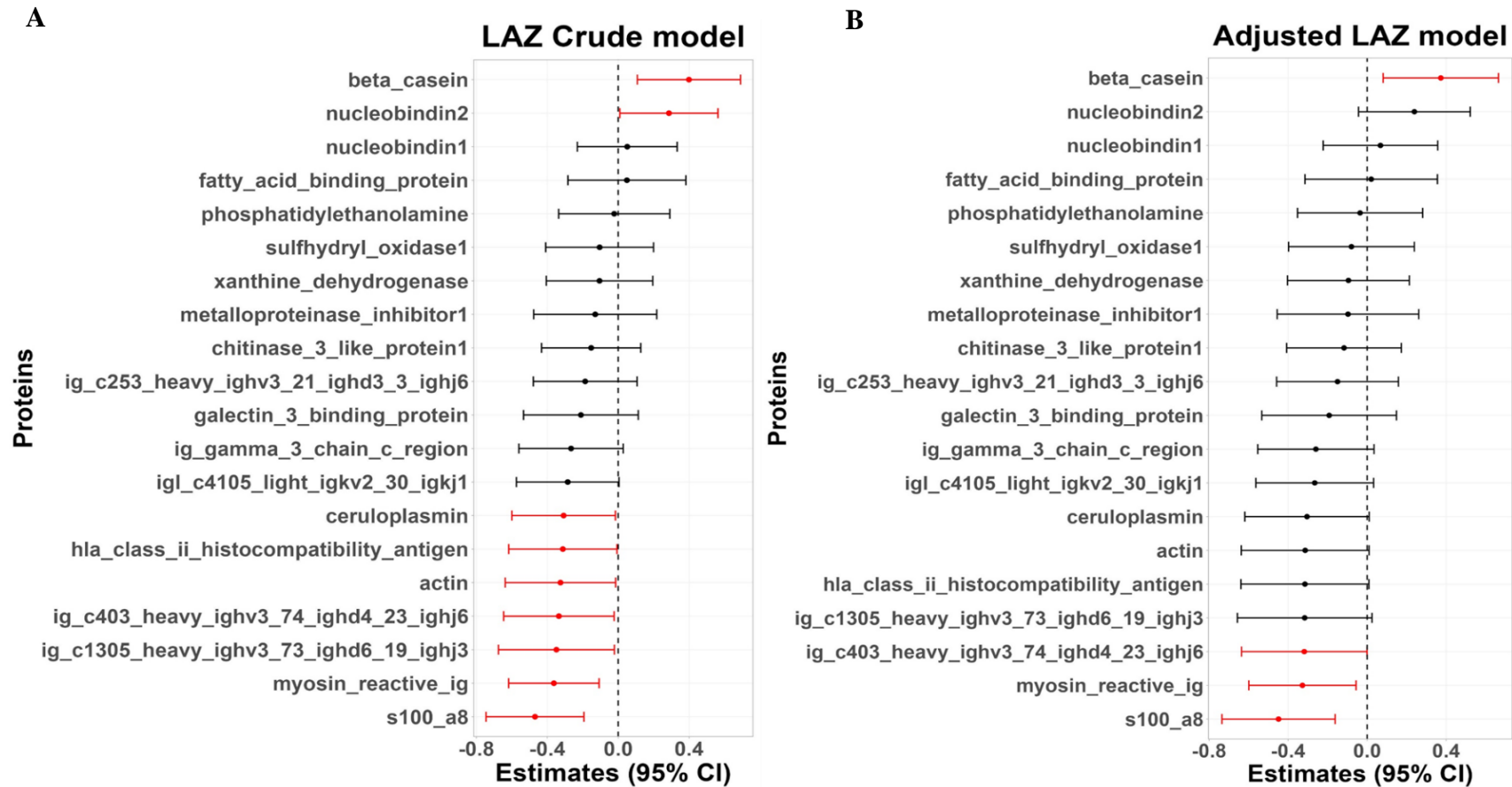


Figure 4.7: Forest plot showing association between breast milk proteins and infants' length-for-age Z-scores at hospital admission

(A) Crude model. **(B)** The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

4.4 Breast milk proteins association with infants' growth post-discharge

In the primary analysis, feature selection using random forest showed that 16 proteins were associated with growth depicted by a change in infant's MUAC between admission and day 45 post-discharge. Of the 16 proteins selected by random forest, 14 showed significant association in univariate crude linear regression analysis (Figure 4.9A). However, after adjusting for infant's gender, age and site, the number of significantly associated proteins reduced to 7 (Figure 4.9B). Lactadherin, Chordin-like-Protein 2 (CHRDL2), tenascin-c-hexabrachion, selenium-binding-Protein 1, complement component (C9) and gamma-glutamyltranspeptidase 1 showed a positive association, indicating that a 1-unit increase of these proteins breast milk, resulted in 0.25cm increase in infant MUAC.

For the secondary analysis, random forest feature selection showed that 11 proteins were associated with a change in WAZ between admission and day 45 post-discharge. Upon performing crude and adjusted linear regression analysis, only one protein, an immunoglobulin was positively associated with a change in WAZ (Figure 4.10A and 4.10B). In addition, 7 proteins were associated with a change in LAZ after performing feature selection. Linear regression resulted in 5 proteins being significantly associated with change in LAZ in the crude model (Figure 4.11A) and 4 proteins in the adjusted model (Figure 4.11B).

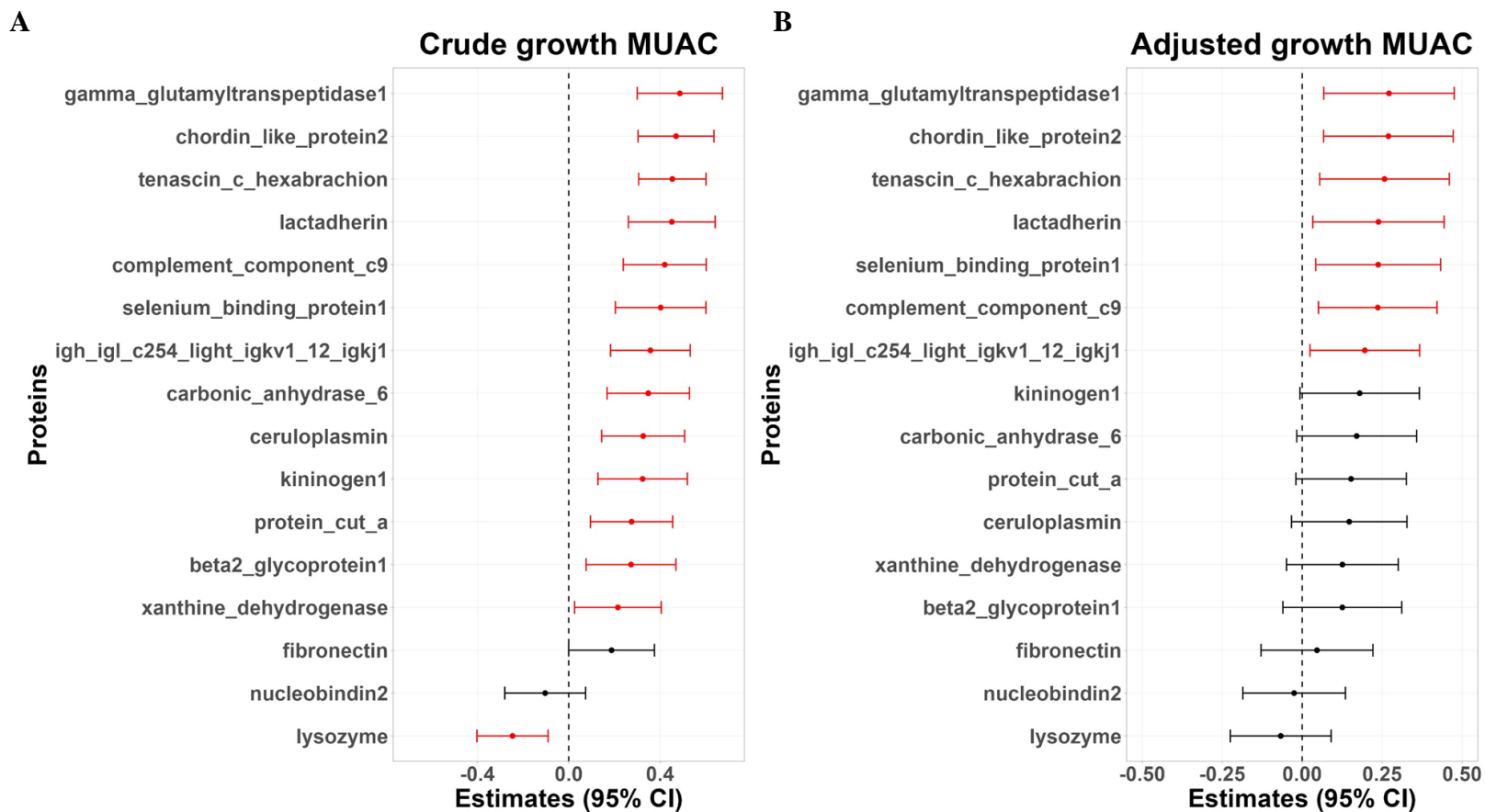


Figure 4.8: Forest plot showing association between breast milk proteins and infants' change in MUAC 45 days post-discharge

(A) Crude model. (B) The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

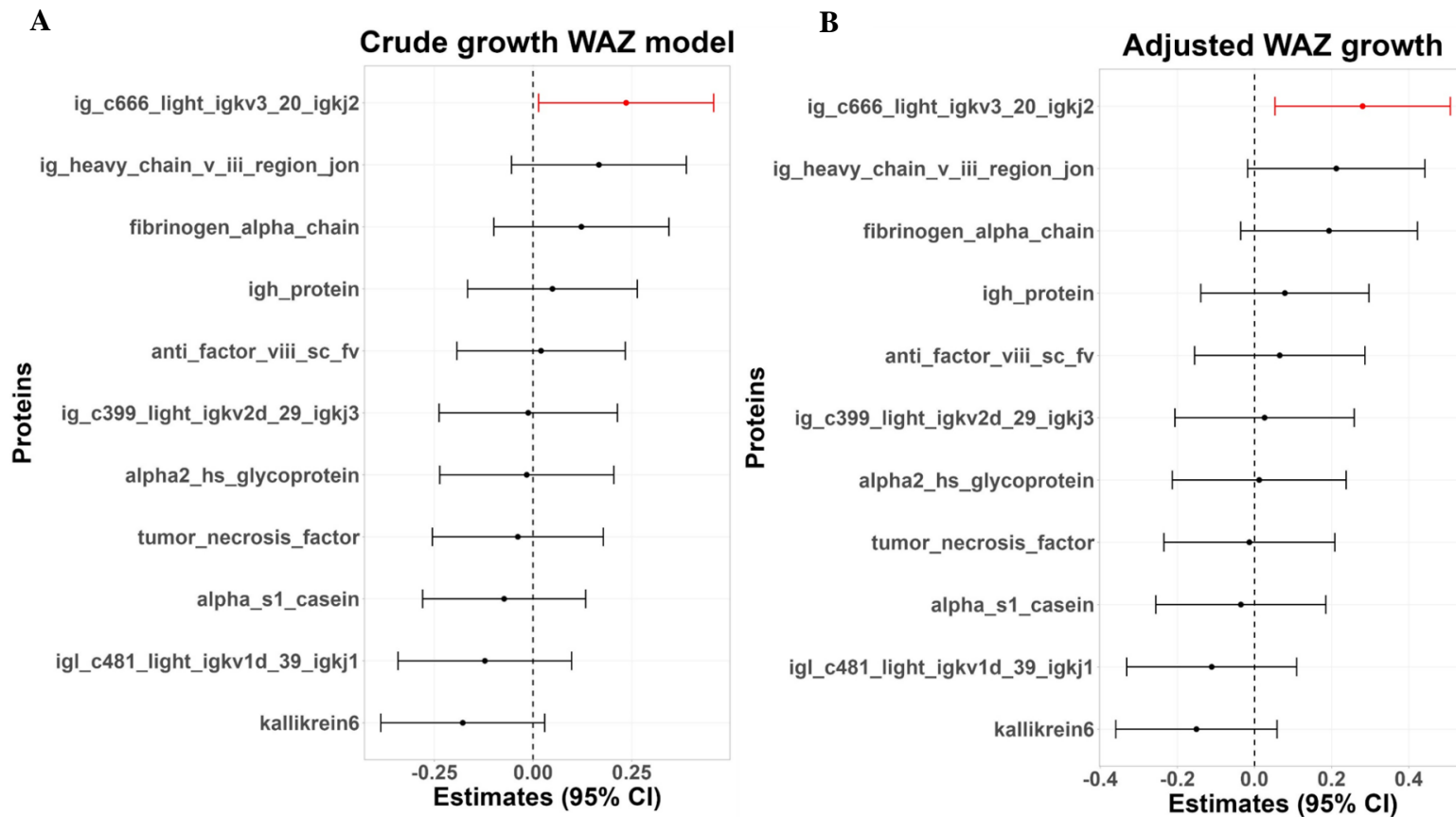


Figure 4.9: Forest plot showing association between breast milk proteins and infants' change in weight-for-age Z-scores 45 days post-discharge

(A) Crude model. (B) The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

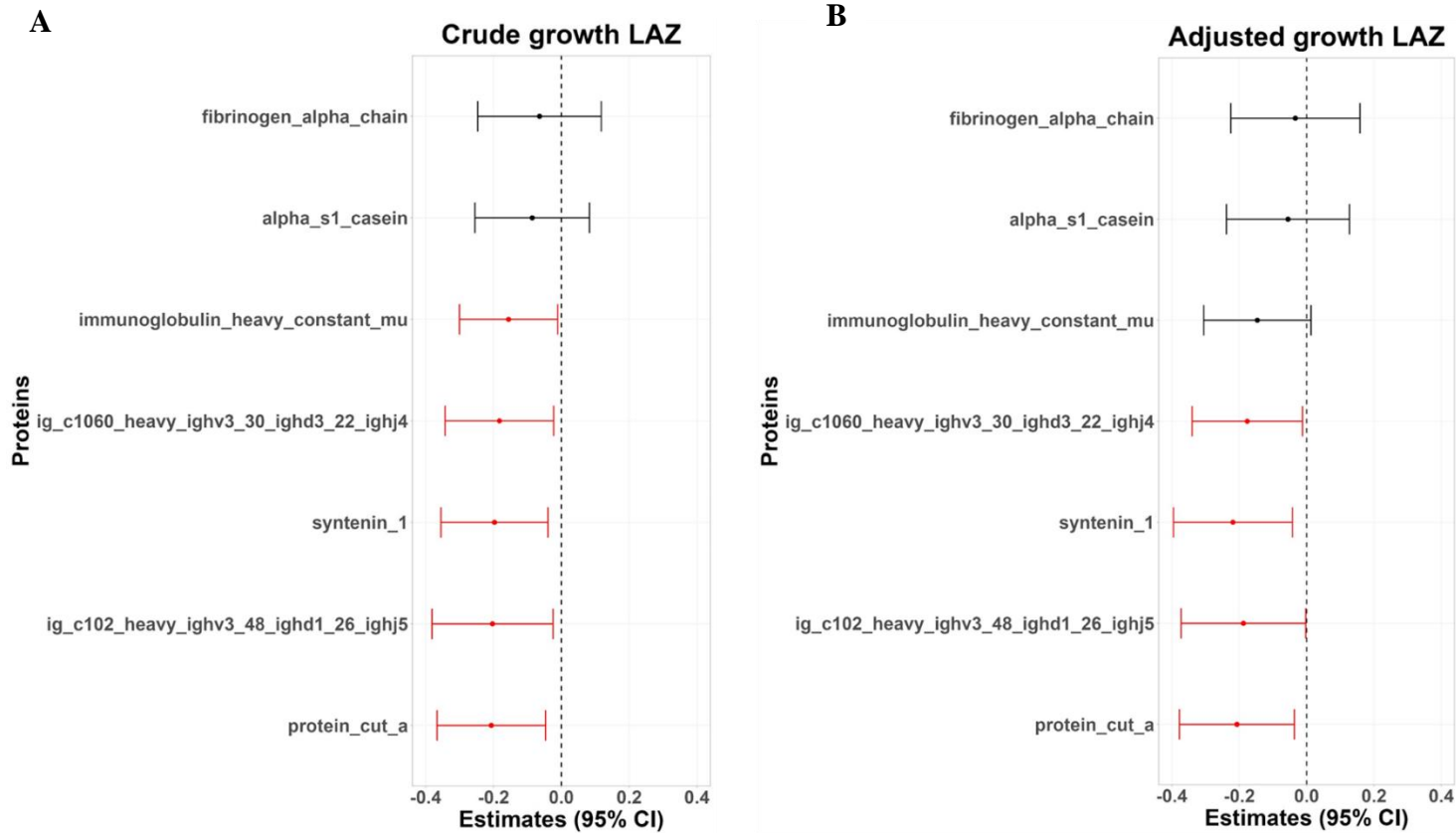


Figure 4.10: Forest plot showing association between breast milk proteins and infants' change in length-for-age Z-scores 45 days post-discharge

(A) Crude model. (B) The adjusted model. Red color represents proteins that are significantly associated while black represents proteins that are not significantly associated. The points indicate estimates for every increase in breast milk protein concentration, bars indicate 95% confidence interval.

CHAPTER FIVE: DISCUSSION

This study characterized the breast milk proteome of lactating women from Kenya and Pakistan to determine proteome profiles associated with the nutritional status and growth of infants hospitalized with an acute illness. The study observed that mothers of hospitalized and non-hospitalized infants have different milk proteomic profiles. The second key finding was that biological processes involved in immune response characterize the milk proteome of mothers of hospitalized infants while fluid regulation and lactation characterize milk proteome fed to non-hospitalized infants. Further, this study has shown that milk proteins are associated with infants' nutritional status among the hospitalized infants. Lastly, this study showed that milk proteins are associated with infants' growth post-discharge.

Anthropometric analysis showed that acutely ill hospitalized infants at admission were undernourished and younger compared to non-hospitalized infants in the community. This is not surprising as the relationship between undernutrition and infectious disease is well established. Acute illnesses may lead to reduced food intake, higher energy requirements, impaired nutrient absorption, increased catabolism, and the depletion or retention of essential elements needed for tissue development and growth (Jones et al., 2015; Brown, 2003). A study by Hoq and colleagues investigated the risk factors of acute malnutrition in children aged 6-59 months and reported that participants who were acutely malnourished had a history of being ill at least two weeks prior to the study in contrast to children who were well nourished (Hoq et al., 2019), this suggests that acute illness is a risk factor of acute malnutrition. Studies have reported younger age as a risk factor for malnutrition among children. A recent meta-analysis by Obasohan and colleagues that investigated the risk factors of malnutrition among children in Sub-Saharan Africa

reported a child's age as a significant predictor of wasting, stunting and being underweight (Obasohan et al., 2020).

Breast milk proteomic profiles among mothers of hospitalized infants significantly differed from those of mothers with non-hospitalized infants. Among mothers with hospitalized infants, breast milk was characterized by proteins that play a role in immune response. Numerous factors may contribute to these differences, including the immune response triggered by hospitalized infants as they fight infections, potentially resulting in modifications in breast milk composition to supply specific immune factors and antibodies that aid in their recovery. A study by Riskin et al., (2012) among breastfeeding mothers investigated differences in immunological factors of breast milk in response to infections among infants and observed a high number of white blood cells during an active infection which later decreased during the convalescence phase (Riskin et al., 2012). Bryan et al. in (2007) reported a higher concentration of cytokines in breast milk from mothers of infants hospitalized with bronchiolitis compared to mothers of healthy controls (Bryan et al., 2007). This study reports high Serine Protease Kinase protein (ATM) levels among mothers of non-hospitalized infants compared to mothers of hospitalized infants. This protein plays a role in shaping the growth and development of infants. Firstly, it aids in repairing DNA damage, safeguarding the integrity of the infant's genetic material, and averting mutations that might affect development. Secondly, by regulating cell cycle checkpoints, ATM ensures that cells only divide when their DNA is intact, which is essential for normal tissue and organ growth. Additionally, ATM may possess immunomodulatory properties, influencing the infant's immune response, a crucial aspect of overall health and development (Kozlov et al., 2016; Liu & Newburg, 2013).

Biological processes related to immune responses and cell signaling were found to be enriched among mothers of hospitalized infants, implying the potential for communication between mothers and infants through breast milk. This aligns with the concept that mothers produce milk tailored to their infants' needs (Martin et al., 2016). In the context of this study, it indicates that the mother's immune system may be actively complementing the infant's immune deficit to fight an infection. In response to an episode of acute infection in infants, the maternal immune system may respond by enhancing the production and release of these immune-boosting components into breast milk. Moreover, the stress and emotional turmoil experienced by mothers with hospitalized infants can have an impact on their immune response. Studies have shown that psychological stress can influence the immune system, potentially altering certain immune-related markers in breast milk, including the clearance of apoptotic cells (Ziomkiewicz et al., 2021; Moirasgenti et al., 2019). Furthermore, the maternal instinct to support their child's healing and recovery may drive the body to enhance processes that aid tissue repair, including angiogenesis. This could explain why in this study, apoptotic cell clearance process was shown to be upregulated in these mothers, as their stress levels may trigger immune responses aimed at safeguarding their infants.

Several breast milk proteins showed association with infants' nutritional status at hospital admission. Beta-casein was positively associated with MUAC, weight-for-age, and length-for-age Z score among infants at hospital admission. Beta casein, a prominent protein component in human breast milk, plays a vital role in growth and development of infants. As a rich source of essential amino acids, beta-casein provides the necessary building blocks for the formation of new tissues, enzymes, and hormones crucial for overall growth (Sadler & Smith, 2013). When infants receive breast milk rich in casein,

they are provided with the vital building blocks required for the increase of lean body mass, which inherently contributes to a better nutritional status outcome. This finding might point to a biological relationship between beta-casein and wasting, underweight and stunting among infants.

Lactadherin also known as Milk Fat Globule Epidermal Growth Factor 8, is a multifunctional glycoprotein found in breast milk (Xiao et al., 2018). In previous studies, it has been reported to play a protective role against rotavirus infection (Kvistgaard et al., 2004). Additionally, lactadherin participates in cell homeostasis and initiates phagocytosis for apoptotic cells (Cacho & Lawrence, 2017). Lactadherin and Mucin4 proteins which were negatively associated with wasting among infants at hospital admission have been reported to have antibacterial and antiviral properties. Additionally, S100A8 a subunit of calprotectin mainly expressed in neutrophils and a known biomarker of inflammation (Wang et al., 2018; Jukic et al., 2021) was negatively associated with nutritional status. These proteins could be important in mounting immunity and conferring protection to infants against infections (Vasques da Costa et al., 2021; Liu & Newburg, 2013). Upregulation of these proteins in breast milk at the time when an infant is sick might signify a state of response of breast milk proteome composition to protect the infant from infection or infectious related processes. Such immune responses in breast milk could prioritize the ill infant's survival over growth thereby explaining the wasting observed among hospitalized infants. It is notable that Lactadherin and Mucin4 were not associated with stunting and underweight among infants at hospital admission. Further, several immunoglobulins were negatively associated with wasting, underweight and stunting among infants at admission. These observations further cement the vital role of breast milk proteins in protecting infants from infections.

This study showed that transthyretin was negatively associated with infants' nutritional status. Transthyretin also known as prealbumin is a serum protein produced in the liver and plays a role in thyroxine production and transport of retinol-binding protein (Dellièrè & Cynober, 2017). In a study that aimed to determine whether prealbumin can be used as a biomarker of acute malnutrition in children, it was reported that the level of prealbumin was lower in malnourished than in normal-nourished children and was therefore suggested as a potential biomarker of malnutrition in children (Tsegaye et al., 2017). In the current study, transthyretin was negatively associated with WAZ and not associated with MUAC and LAZ at hospital admission. In this study, both Beta-2 glycoprotein 1 (β 2GP1) and Galectin-3 binding protein were also negatively associated with wasting among infants at admission. Beta-2 glycoprotein 1 is predominantly involved in blood coagulation and immune regulation (Grossi et al., 2023). Its significance in the context of infant nutritional status stems from its capacity to modulate inflammation, as well as its participation in maintaining a balanced immune response. These functions are crucial in ensuring infants remain healthy, as excessive or chronic inflammation can disrupt nutrient absorption and utilization, potentially impacting nutritional status (Weckman et al., 2023). Galectin-3 binding protein, also known as Mac-2-binding protein, is a versatile protein that binds to galectin-3, a carbohydrate-binding protein. The protein's key function lies in immunomodulation, where it interacts with galectin-3 to regulate immune responses, ensuring efficient defense against infections and maintaining overall infant health (Díaz-Alvarez & Ortega, 2017). Additionally, it plays a role in modulating inflammatory responses, which is crucial as chronic or excessive inflammation can disrupt nutrient absorption and utilization, potentially impacting nutritional status. Galectin-3 binding protein is also involved in cell adhesion and signaling, processes vital for the proper development and functioning of tissues and organs, including those integral to nutrient

absorption and metabolism (Tsai et al., 2021; DeRoo et al., 2015). Its influence on the regulation of the extracellular matrix further highlights its significance, indirectly affecting the health of organs and tissues involved in nutrient processing.

The current study results revealed that several breast milk proteins were associated with infant growth post-discharge. Chordin-like-Protein 2 (CHRDL2), tenascin-c-hexabrachion, lactadherin, selenium-binding-Protein1, complement component (C9) and gamma-glutamyltranspeptidase1 were significantly associated with infant growth as defined by a change in MUAC from admission to day 45 post-discharge. Lactadherin which was negatively associated with MUAC measurements at admission, showed a positive association with post-discharge growth at day 45. These findings could demonstrate the possible role of lactadherin on growth during and after hospitalization. The protein plays a role in the absorption of dietary lipids and fat-soluble vitamins, essential for growth. Tenascin-c-hexabrachion is an extracellular matrix protein which plays a role in fetal development and promotes wound healing. The results of this study reported a positive association of tenascin-c-hexabrachion with post-discharge growth in MUAC. The specific role of tenascin-c-hexabrachion in infant growth remains unclear. In previous in vitro studies, tenascin-c-hexabrachion was reported to exhibit HIV-1 neutralizing activity and therefore warranting more research to use it as a HIV-1 prophylactic agent (Fouda et al., 2013; Mansour et al., 2016).

Chordin-like Protein 2 (CHRDL2) is a protein found in breast milk and it was positively associated with infants' growth and development. As a member of the chordin protein family, CHRDL2 participates in the regulation of signaling pathways crucial during embryonic development (Li et al., 2022). One of its primary functions is the modulation of the bone morphogenetic protein (BMP) signaling pathway, which holds significance in processes like bone formation and tissue growth (Li et al., 2022). The involvement in bone

formation and tissue growth biological processes emphasizes the significance of CHRDL2 in promoting skeletal development in infants. Syntenin 1 is a protein involved in cell adhesion and signaling, essential for various physiological processes, including the development of tissues and organs (Kashyap et al., 2015). In this study, syntenin 1 was negatively associated with change in length-for-age z scores at day 45 post discharges. A similar association was observed for protein cut A and several antibodies. Their role in infant growth is however yet to be determined.

5.1 Conclusion

In conclusion, this study has shown that there is a difference in breast milk protein expression between mothers of hospitalized ill infants and non-hospitalized infants living in the community. It has also showed that human milk proteins are associated with infants' nutritional status and growth post-discharge.

5.2 Limitation

The maternal factors such as nutritional status, health and diet were not accounted for in this study. Therefore, the findings need to be verified while adjusting for the maternal factors. Additionally, it is important to acknowledge that this study encompassed a relatively limited number of sites, which may restrict the generalizability of these findings to broader populations. The context-specific nature of this research highlights the need for caution when extrapolating the results to diverse demographic and geographic settings. Future investigations should aim to incorporate a more extensive and diverse sample of participants, potentially spanning multiple geographic regions, to ensure that the findings can be applied more broadly and hold greater external validity. Addressing these limitations will contribute to a more rigorous and comprehensive exploration of the relationship between infant growth and breast milk composition.

5.3 Recommendation

A notable aspect of this study on the differential protein expression in breast milk between mothers with sick infants and non-hospitalized infants is that it did not account for maternal factors such as age, health status, and nutritional status during the analysis. These factors can significantly influence the composition of breast milk and potentially confound the study findings. To enhance the comprehensiveness and robustness of our study, it is recommended that future research endeavors consider including these maternal variables as covariates in the analysis. By doing so, these studies can obtain a more accurate understanding of the specific impacts of infant health on breast milk composition, while also isolating the potential effects of maternal characteristics. The same factors should be adjusted in the regression models. This adjustment will contribute to a more nuanced and well-informed interpretation of the results, ultimately strengthening the scientific validity of the findings.

The current study only focused on breast milk proteome, future studies should target other components of breast milk including carbohydrates, lipids and micronutrients to understand their role in infant growth and development after an acute illness. Additionally, there is a need to conduct trials in LMICs to find out if mothers targeted interventions do influence the composition of breast milk and the corresponding effect on an infant's growth.

REFERENCES

- Aimin, C., & Rogan, W. J. (2004). Breastfeeding and the risk of postneonatal death in the United States. *Pediatrics*, *113*(5), 756–770. <https://doi.org/10.1542/peds.113.5.e435>
- Allen, L. H. (2012). B Vitamins in Breast Milk: Relative Importance of Maternal Status and Intake, and Effects on Infant Status and Function. *Adv. Nut*, *3*(2), 362–369. <https://doi.org/10.3945/an.111.001172.362>
- Amissah, E. A., Brown, J., & Harding, J. E. (2020). Carbohydrate supplementation of human milk to promote growth in preterm infants. *Cochrane Database of Systematic Reviews*, *2020*(9), 1–25. <https://doi.org/10.1002/14651858.CD000280.pub3>
- Andreas, N. J., Kampmann, B., & Mehring Le-Doare, K. (2015). Human breast milk: A review on its composition and bioactivity. *Early Human Development*, *91*(11), 629–635. <https://doi.org/10.1016/j.earlhumdev.2015.08.013>
- Ashraf, M. F., Zubair, D., Bashir, M. N., Alagawany, M., Ahmed, S., Shah, Q. A., Buzdar, J. A., & Arain, M. A. (2024). Nutraceutical and Health-Promoting Potential of Lactoferrin, an Iron-Binding Protein in Human and Animal: Current Knowledge. *Biological Trace Element Research*, *202*(1), 56–72. <https://doi.org/10.1007/s12011-023-03658-4>
- Aumeistere, L., Ciproviča, I., Zavadskā, D., Andersons, J., Volkovs, V., & Ceļmalniece, K. (2019). Impact of maternal diet on human milk composition among lactating women in Latvia. *Medicina (Lithuania)*, *55*(5), 1–12. <https://doi.org/10.3390/medicina55050173>

- Bai, G., Parkash, A., Kumar, V., Kumari, M., Kumari, S., & Das, K. (2022). Trends in Weight Gain Among Breastfed Infants Versus Bottle-Fed Infants at a Tertiary Care Hospital in Karachi, Pakistan. *Cureus*, *14*(3), 234–239. <https://doi.org/10.7759/cureus.23459>
- Ballard, O. J., & Morrow, A. L. (2013). Nutritional Components of Human Milk. *Boreal Environment Research*, *3*(3), 221–233. <https://doi.org/doi:10.1016/j.pcl.2012.10.002>.
- Bauer, J., & Gerss, J. (2011). Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants. *Clinical Nutrition*, *30*(2), 215–220. <https://doi.org/10.1016/j.clnu.2010.08.003>
- Bellows, A. L., Smith, E. R., Muhihi, A., Briegleb, C., Noor, R. A., Mshamu, S., Sudfeld, C., Masanja, H., & Fawzi, W. W. (2017). Micronutrient deficiencies among breastfeeding infants in Tanzania. *Nutrients*, *9*(11), 1–12. <https://doi.org/10.3390/nu9111258>
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, *57*(1), 289–300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Berger, P. K., Plows, J. F., Demerath, E. W., & Fields, D. A. (2020). Carbohydrate composition in breast milk and its effect on infant health. *Current Opinion in Clinical Nutrition and Metabolic Care*, *23*(4), 277–281. <https://doi.org/10.1097/MCO.0000000000000658>

- Beyrami, H. J., & Samadi-Afshar, S. (2018). Comparison of Growth Index in Exclusive Breastfeeding and Infant Formula Feeding in Six-Month-Old Infants in East Azerbaijan Province. *Depiction Of Health*, 8(4), 252–259.
- Black, R. E., Allen, L. H., Bhutta, Z. A., Caulfield, L. E., de Onis, M., Ezzati, M., Mathers, C., & Rivera, J. (2008). Maternal and child undernutrition: global and regional exposures and health consequences. *The Lancet*, 371(9608), 243–260. [https://doi.org/10.1016/S0140-6736\(07\)61690-0](https://doi.org/10.1016/S0140-6736(07)61690-0)
- Brandtzaeg, P. (2010). The Mucosal Immune System and Its Integration with the Mammary Glands. *Journal of Pediatrics*, 156(2), S8. <https://doi.org/10.1016/j.jpeds.2009.11.014>
- Bravi, F., Wiens, F., Decarli, A., Dal Pont, A., Agostoni, C., & Ferraroni, M. (2016). Impact of maternal nutrition on breast-milk composition: A systematic review. *American Journal of Clinical Nutrition*, 104(3), 646–662. <https://doi.org/10.3945/ajcn.115.120881>
- Brown, K. H. (2003). Diarrhea and malnutrition. *Journal of Nutrition*, 133(1), 328S–332S. <https://doi.org/10.1093/jn/133.1.328s>
- Bryan, D. L., Hart, P. H., Forsyth, K. D., & Gibson, R. A. (2007). Immunomodulatory constituents of human milk change in response to infant bronchiolitis. *Pediatric Allergy and Immunology*, 18(6), 495–502. <https://doi.org/10.1111/j.1399-3038.2007.00565.x>

- Butts, C. A., Hedderley, D. I., Herath, T. D., Paturi, G., Id, S. G., Wiens, F., Stahl, B., & Gopal, P. (2022). *Human Milk Composition and Dietary Intakes of Breastfeeding Women of Different Ethnicity from the Manawatu-Wanganui Region of New Zealand*. 1–16. <https://doi.org/10.3390/nu10091231>
- Bzikowska-Jura, A., Czerwonogrodzka-Senczyna, A., Ćedzka, G. O., Szostak-Węgierek, D., & Halina Weker and Aleksandra Wesołowska. (2018). Maternal Nutrition and Body Composition During. *Nutrients*, *10*(10), 1379. <https://doi.org/10.3390/nu10101379>
- Cacho, N. T., & Lawrence, R. M. (2017). Innate immunity and breast milk. *Frontiers in Immunology*, *8*(5), 584. <https://doi.org/10.3389/fimmu.2017.00584>
- Calvo-Lerma, J., Selma-Royo, M., Hervas, D., Yang, B., Intonen, L., González, S., Martínez-Costa, C., Linderborg, K. M., & Collado, M. C. (2022). Breast Milk Lipidome Is Associated With Maternal Diet and Infants' Growth. *Frontiers in Nutrition*, *9*(July), 1–10. <https://doi.org/10.3389/fnut.2022.854786>
- Caulfield, L. E., de Onis, M., Blössner, M., & Black, R. E. (2004). Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles. *The American Journal of Clinical Nutrition*, *80*(1), 193–198. <https://doi.org/10.1093/ajcn/80.1.193>
- CHAIN. (2019). Childhood Acute Illness and Nutrition (CHAIN) Network: a protocol for a multi-site prospective cohort study to identify modifiable risk factors for mortality among acutely ill children in Africa and Asia. *BMJ Open*, *9*(5), 028454. <https://doi.org/10.1136/bmjopen-2018-028454>

- Charbonneau, M. R., Donnell, D. O., Blanton, L. V., Totten, S. M., Davis, J. C. C., Barratt, M. J., Cheng, J., Guruge, J., Bain, J. R., Muehlbauer, M. J., Ilkayeva, O., & Wu, C. (2017). Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. *Cell*, *164*(5), 859–871. <https://doi.org/10.1016/j.cell.2016.01.024>. Sialylated
- Criswell, R. L., Iszatt, N., Demmelair, H., Ahmed, T. B., Koletzko, B. V., Lenters, V. C., & Eggesbø, M. (2022). Predictors of Human Milk Fatty Acids and Associations with Infant Growth in a Norwegian Birth Cohort. *Nutrients*, *14*(18), 1–19. <https://doi.org/10.3390/nu14183858>
- Czosnykowska-Łukacka, M., Orczyk-Pawłowicz, M., Broers, B., & Królak-Olejnik, B. (2019). Lactoferrin in human milk of prolonged lactation. *Nutrients*, *11*(10), 1–12. <https://doi.org/10.3390/nu11102350>
- Dellière, S., & Cynober, L. (2017). Is transthyretin a good marker of nutritional status? *Clinical Nutrition*, *36*(2), 364–370. <https://doi.org/10.1016/j.clnu.2016.06.004>
- Delplanque, B., Gibson, R., Koletzko, B., Lapillonne, A., & Strandvik, B. (2015). Lipid Quality in Infant Nutrition: Current Knowledge and Future Opportunities. *Journal of Pediatric Gastroenterology and Nutrition*, *61*(1), 8–17. <https://doi.org/10.1097/MPG.0000000000000818>
- DeRoo, E. P., Wroblewski, S. K., Shea, E. M., Al-Khalil, R. K., Hawley, A. E., Henke, P. K., Myers, D. D., Wakefield, T. W., & Diaz, J. A. (2015). The role of galectin-3 and galectin-3-binding protein in venous thrombosis. *Blood*, *125*(11), 1813–1821. <https://doi.org/10.1182/blood-2014-04-569939>

- Díaz-Alvarez, L., & Ortega, E. (2017). The Many Roles of Galectin-3, a Multifaceted Molecule, in Innate Immune Responses against Pathogens. *Mediators of Inflammation*, 2017(4), 9–12. <https://doi.org/10.1155/2017/9247574>
- Dingess, K. A., Valentine, C. J., Ollberding, N. J., Davidson, B. S., Woo, J. G., Summer, S., Peng, Y. M., Guerrero, M. L., Ruiz-Palacios, G. M., Ran-Ressler, R. R., McMahon, R. J., Brenna, J. T., & Morrow, A. L. (2017). Branched-chain fatty acid composition of human milk and the impact of maternal diet: The global exploration of human milk (GEHM) study. *American Journal of Clinical Nutrition*, 105(1), 177–184. <https://doi.org/10.3945/ajcn.116.132464>
- Dogra, S. K., Chung, C. K., Wang, D., Sakwinska, O., Mottaz, S. C., & Sprenger, N. (2021). Nurturing the early life gut microbiome and immune maturation for long term health. *Microorganisms*, 9(10), 2110. <https://doi.org/10.3390/microorganisms9102110>
- Duale, A., Singh, P., & Al Khodor, S. (2022). Breast Milk: A Meal Worth Having. *Frontiers in Nutrition*, 8(January), 1–18. <https://doi.org/10.3389/fnut.2021.800927>
- Elwakiel, M., Boeren, S., Wang, W., Schols, H. A., & Hettinga, K. A. (2020). Degradation of Proteins From Colostrum and Mature Milk From Chinese Mothers Using an in vitro Infant Digestion Model. *Frontiers in Nutrition*, 7(September), 1–10. <https://doi.org/10.3389/fnut.2020.00162>

- Enrique, R.-V., Delgado-Franco, D., García-Gutiérrez, M., Gurrola-Díaz, C., Larrosa-Haro, A., Montijo-Barrios, E., Muskiet, F. A. J., Enrique, Romero-Velarde Dagoberto, Delgado-Franco Mariana, G.-G., Gurrola-Díaz, C., Larrosa-Haro, A., Montijo-Barrios, E., Muskiet, F. A. J., Vargas-Guerrero, B., & Geurts, J. (2019). The Importance of Lactose in the Human Diet: Outcomes of a Mexican Consensus Meeting. *Nutrients*, *12*(4), 335–340. <https://doi.org/10.3390/nu11112737>
- Eriksen, K. G., Christensen, S. H., Lind, M. V, & Michaelsen, K. F. (2018). Human milk composition and infant growth. *Current Opinion in Clinical Nutrition & Metabolic Care*, *21*(3), 200–206. <https://doi.org/10.1097/MCO.0000000000000466>
- Fanzo, J., Hawkes, C., Udomkesmalee, E., Afshin, A., Allemandi, L., Assery, O., Baker, P., Battersby, J., Bhutta, Z., Chen, K., Corvalan, C., Di Cesare, M., Dolan, C., Fonseca, J., Grummer-Strawn, L. (2018). Global Nutrition Report. *Journal of Nutrition*, *34*(2019), 51–79.
- Ferraboschi, P., Ciceri, S., & Grisenti, P. (2021). Applications of lysozyme, an innate immune defense factor, as an alternative antibiotic. *Antibiotics*, *10*(12), 1–55. <https://doi.org/10.3390/antibiotics10121534>
- Fouda, G. G., Jaeger, F. H., Amos, J. D., Ho, C., Kunz, E. L., Anasti, K., Stamper, L. W., Liebl, B. E., Barbas, K. H., Ohashi, T., Moseley, M. A., Liao, H. X., Erickson, H. P., Alam, S. M., & Permar, S. R. (2013). Tenascin-C is an innate broad-spectrum, HIV-1-neutralizing protein in breast milk. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(45), 18220–18225. <https://doi.org/10.1073/pnas.1307336110>

- Galante, L., Pundir, S., Lagström, H., Rautava, S., Reynolds, C. M., Milan, A. M., Cameron-Smith, D., & Vickers, M. H. (2020). Growth Factor Concentrations in Human Milk Are Associated With Infant Weight and BMI From Birth to 5 Years. *Frontiers in Nutrition*, 7(7), 110. <https://doi.org/10.3389/fnut.2020.00110>
- Gao, X., McMahon, R. J., Woo, J. G., Davidson, B. S., Morrow, A. L., & Zhang, Q. (2012). Temporal changes in milk proteomes reveal developing milk functions. *Journal of Proteome Research*, 11(7), 3897–3907. <https://doi.org/10.1021/pr3004002>
- George, A. D., Gay, M. C. L., Wlodek, M. E., Murray, K., & Geddes, D. T. (2021). The fatty acid species and quantity consumed by the breastfed infant are important for growth and development. *Nutrients*, 13(11), 1–11. <https://doi.org/10.3390/nu13114183>
- Georgieff, M. K. (2011). Long-term brain and behavioral consequences of early iron deficiency. *Nutrition Reviews*, 69(1), 43–48. <https://doi.org/10.1111/j.1753-4887.2011.00432.x>
- Gibson, R. S., Rahmanna, S., Diana, A., Leong, C., Haszard, J. J., Hampel, D., Reid, M., Erhardt, J., Suryanto, A. H., Sofiah, W. N., Fathonah, A., Shahab-Ferdows, S., Allen, L. H., & Houghton, L. A. (2020). Association of maternal diet, micronutrient status, and milk volume with milk micronutrient concentrations in Indonesian mothers at 2 and 5 months postpartum. *American Journal of Clinical Nutrition*, 112(4), 1039–1050. <https://doi.org/10.1093/ajcn/nqaa200>
- Gila-díaz, A., Arribas, S. M., Algara, A., Mart, A., Luis, Á., Miguel, S., Pipa, D., & Ramiro-cortijo, D. (2019). A Review of Bioactive Factors in Human Breastmilk : A Focus on Prematurity. *Nutrients*, 11(6), 1–23. <https://doi.org/10.3390/nu11061307>

- Grapov, D., Lemay, D. G., Weber, D., Phinney, B. S., Azulay Chertok, I. R., Gho, D. S., German, J. B., & Smilowitz, J. T. (2015). The human colostrum whey proteome is altered in gestational diabetes mellitus. *Journal of Proteome Research*, *14*(1), 512–520. <https://doi.org/10.1021/pr500818d>
- Grimbeek, A., & Saloojee, H. (2022). Clinical and growth outcomes of severely malnourished children following hospital discharge in a South African setting. *PLoS ONE*, *17*(1 January), 1–16. <https://doi.org/10.1371/journal.pone.0262700>
- Grossi, C., Capitani, N., Benagiano, M., Baldari, C. T., Della Bella, C., Macor, P., Tedesco, F., Borghi, M. O., Maugeri, N., D’Elios, M. M., & Meroni, P. L. (2023). Beta 2 glycoprotein I and neutrophil extracellular traps: Potential bridge between innate and adaptive immunity in anti-phospholipid syndrome. *Frontiers in Immunology*, *13*(1), 1–8. <https://doi.org/10.3389/fimmu.2022.1076167>
- Hahn-Holbrook, J., Fish, A., & Glynn, L. M. (2019). Human milk omega-3 fatty acid composition is associated with infant temperament. *Nutrients*, *11*(12), 2964. <https://doi.org/10.3390/nu11122964>
- Hanson, L. Å., & Korotkova, M. (2002). The role of breastfeeding in prevention of neonatal infection. *Seminars in Neonatology*, *7*(4), 275–281. <https://doi.org/10.1053/siny.2002.0124>
- Hettinga, K. A., Reina, F. M., Boeren, S., Zhang, L., Koppelman, G. H., Postma, D. S., Vervoort, J. J. M., & Wijga, A. H. (2015). Difference in the breast milk proteome between allergic and non-allergic mothers. *PLoS ONE*, *10*(3), 1–11. <https://doi.org/10.1371/journal.pone.0122234>

- Heyman, M. B. (2006). Lactose intolerance in infants, children, and adolescents. *Pediatrics, 118*(3), 1279–1286. <https://doi.org/10.1542/peds.2006-1721>
- Hoehn, C., Lelijveld, N., Mwangome, M., Berkley, J. A., McGrath, M., & Kerac, M. (2021). Anthropometric Criteria for Identifying Infants Under 6 Months of Age at Risk of Morbidity and Mortality: A Systematic Review. *Clinical Medicine Insights: Pediatrics, 15*(2), 117955652110499. <https://doi.org/10.1177/11795565211049904>
- Hoq, M., Ali, M., Islam, A., & Banerjee, C. (2019). Risk factors of acute malnutrition among children aged 6-59 months enrolled in a community-based programme in Kurigram, Bangladesh: A mixed-method matched case-control study. *Journal of Health, Population and Nutrition, 38*(1), 1–7. <https://doi.org/10.1186/s41043-019-0192-2>
- Horta, B. L. (2019). Breastfeeding: Investing in the Future. *Breastfeeding Medicine, 14*(S1), S11–S12. <https://doi.org/10.1089/bfm.2019.0032>
- Hosea, B. H. J., Cicalo, M. C., Holland, C. D., & Field, C. J. (2008). The Immunological Components of Human Milk. In *Advances in Food and Nutrition Research* (Vol. 54, Issue 07). Elsevier Masson SAS. [https://doi.org/10.1016/S1043-4526\(07\)00002-2](https://doi.org/10.1016/S1043-4526(07)00002-2)
- Hosseini, M., Valizadeh, E., Hosseini, N., Khatibshahidi, S., & Raeisi, S. (2020). The Role of Infant Sex on Human Milk Composition. *Breastfeeding Medicine, 15*(5), 341–346. <https://doi.org/10.1089/bfm.2019.0205>
- Huang, Z., & Hu, Y. M. (2020). Dietary patterns and their association with breast milk macronutrient composition among lactating women. *International Breastfeeding Journal, 15*(1), 1–10. <https://doi.org/10.1186/s13006-020-00293-w>

- Huber, W., Von Heydebreck, A., Sültmann, H., Poustka, A., & Vingron, M. (2002). Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*, *18*(1), 10. https://doi.org/10.1093/bioinformatics/18.suppl_1.S96
- Italianer, M. F., Naninck, E. F. G., Roelants, J. A., van der Horst, G. T. J., Reiss, I. K. M., van Goudoever, J. B., Joosten, K. F. M., Chaves, I., & Vermeulen, M. J. (2020). Circadian variation in human milk composition, a systematic review. *Nutrients*, *12*(8), 1–16. <https://doi.org/10.3390/nu12082328>
- Jones, A. D., Rukobo, S., Chasekwa, B., Mutasa, K., Ntozini, R., Mbuya, M. N. N., Stoltzfus, R. J., Humphrey, J. H., & Prendergast, A. J. (2015). Acute illness is associated with suppression of the growth hormone axis in Zimbabwean infants. *American Journal of Tropical Medicine and Hygiene*, *92*(2), 463–470. <https://doi.org/10.4269/ajtmh.14-0448>
- Jorgensen, J. M., Young, R., Ashorn, P., Ashorn, U., Chaima, D., Davis, J. C. C., Goonatilleke, E., Kumwenda, C., Lebrilla, C. B., Maleta, K., Prado, E. L., Sadalaki, J., Totten, S. M., Wu, L. D., Zivkovic, A. M., & Dewey, K. G. (2021). Associations of human milk oligosaccharides and bioactive proteins with infant growth and development among Malawian mother-infant dyads. *American Journal of Clinical Nutrition*, *113*(1), 209–220. <https://doi.org/10.1093/ajcn/nqaa272>
- Jukic, A., Bakiri, L., Wagner, E. F., Tilg, H., & Adolph, T. E. (2021). Calprotectin: from biomarker to biological function. *Gut*, *70*(10), 1978–1988. <https://doi.org/10.1136/gutjnl-2021-324855>

- Kalyanasundaram, S., Narayanan, V. K., & Krishnamurthy, K. (2021). The role of lactose in milk and an overview of intolerance to lactose. *Journal of Public Health Nutrition*, 4(1), 300–301. <http://www.alliedacademies.org/public-health-nutrition/>
- Kashyap, R., Roucourt, B., Lembo, F., Fares, J., Carcavilla, A. M., Restouin, A., Zimmermann, P., & Ghossoub, R. (2015). Syntenin controls migration, growth, proliferation, and cell cycle progression in cancer cells. *Frontiers in Pharmacology*, 6(10), 1–11. <https://doi.org/10.3389/fphar.2015.00241>
- Keikha, M., Shayan-Moghadam, R., Bahreynian, M., & Kelishadi, R. (2021). Nutritional supplements and mother's milk composition: a systematic review of interventional studies. *International Breastfeeding Journal*, 16(1), 1–30. <https://doi.org/10.1186/s13006-020-00354-0>
- Kent, J. C., Mitoulas, L. R., Cregan, M. D., Ramsay, D. T., Doherty, D. A., & Hartmann, P. E. (2006). Volume and frequency of breastfeedings and fat content of breast milk throughout the day. *Pediatrics*, 117(3), 1–8. <https://doi.org/10.1542/peds.2005-1417>
- Kerac, M., Blencowe, H., Grijalva-Eternod, C., McGrath, M., Shoham, J., Cole, T. J., & Seal, A. (2011). Prevalence of wasting among under 6-month-old infants in developing countries and implications of new case definitions using WHO growth standards: A secondary data analysis. *Archives of Disease in Childhood*, 96(11), 1008–1013. <https://doi.org/10.1136/adc.2010.191882>
- Kerac, M., Frison, S., Connell, N., Page, B., & McGrath, K. M. (2019). Informing the management of acute malnutrition in infants aged under 6 months (mami): Risk factor analysis using nationally-representative demographic & health survey secondary data. *PeerJ*, 2019(4), 5848. <https://doi.org/10.7717/peerj.5848>

- Khatun, N. (2021). Applications of Normality Test in Statistical Analysis. *Open Journal of Statistics, 11*(01), 113–122. <https://doi.org/10.4236/ojs.2021.111006>
- Kozlov, S. V., Waardenberg, A. J., Engholm-Keller, K., Arthur, J. W., Graham, M. E., & Lavin, M. (2016). Reactive oxygen species (ROS)-activated ATM-dependent phosphorylation of cytoplasmic substrates identified by large-scale phosphoproteomics screen. *Molecular and Cellular Proteomics, 15*(3), 1032–1047. <https://doi.org/10.1074/mcp.M115.055723>
- Kramer, M. S., Guo, T., Platt, R. W., Shapiro, S., Collet, J. P., Chalmers, B., Hodnett, E., Sevkovskaya, Z., Dzikovich, I., & Vanilovich, I. (2002). Breastfeeding and infant growth: Biology or bias? *Pediatrics, 110*(2 I), 343–347. <https://doi.org/10.1542/peds.110.2.343>
- Kramer, M. S., Guo, T., Platt, R. W., Vanilovich, I., Sevkovskaya, Z., Dzikovich, I., Michaelsen, K. F., & Dewey, K. (2004). Feeding effects on growth during infancy. *Journal of Pediatrics, 145*(5), 600–605. <https://doi.org/10.1016/j.jpeds.2004.06.069>
- Kramer, M. S., Moodie, E. E. M., Dahhou, M., & Platt, R. W. (2011). Breastfeeding and infant size: Evidence of reverse causality. *American Journal of Epidemiology, 173*(9), 978–983. <https://doi.org/10.1093/aje/kwq495>
- Kuchenbecker, J., Jordan, I., Reinbott, A., Herrmann, J., Jeremias, T., Kennedy, G., Muehlhoff, E., Mtimuni, B., & Krawinkel, M. B. (2015). Exclusive breastfeeding and its effect on growth of malawian infants: Results from a cross-sectional study. *Paediatrics and International Child Health, 35*(1), 14–23. <https://doi.org/10.1179/2046905514Y.0000000134>

- Kvistgaard, A. S., Pallesen, L. T., Arias, C. F., López, S., Petersen, T. E., Heegaard, C. W., & Rasmussen, J. T. (2004). Inhibitory effects of human and bovine milk constituents on rotavirus infections. *Journal of Dairy Science*, *87*(12), 4088–4096. [https://doi.org/10.3168/jds.S0022-0302\(04\)73551-1](https://doi.org/10.3168/jds.S0022-0302(04)73551-1)
- Lamberti, L. M., Walker, C. L. F., Noiman, A., Victora, C., & Black1, R. E. (2011). Breastfeeding and the risk for diarrhea morbidity and mortality. *BMC Public Health*, *11*(3), 171–174. <https://doi.org/10.1186/1471-2458-11-S3-S15>
- Langenbucher, A., Szentmáry, N., Cayless, A., Wendelstein, J., & Hoffmann, P. (2023). Preconditioning of clinical data for intraocular lens formula constant optimisation using Random Forest Quantile Regression Trees. *Zeitschrift Fur Medizinische Physik*, *4*(2023), 478–499. <https://doi.org/10.1016/j.zemedi.2022.11.009>
- Lessen, R., & Kavanagh, K. (2015). Position of the academy of nutrition and dietetics: Promoting and supporting breastfeeding. *Journal of the Academy of Nutrition and Dietetics*, *115*(3), 444–449. <https://doi.org/10.1016/j.jand.2014.12.014>
- Ley, S. H., Hanley, A. J., Sermer, M., Zinman, B., & O'Connor, D. L. (2012). Associations of prenatal metabolic abnormalities with insulin and adiponectin concentrations in human milk. *American Journal of Clinical Nutrition*, *95*(4), 867–874. <https://doi.org/10.3945/ajcn.111.028431>
- Li, D., Yuan, S. T., Xie, X. Y., Shen, H., Liu, Q. H., & Yao, Y. (2022). Chordin-like 2 influences the differentiation fate of retinal pigment epithelium cells by dynamically regulating BMP pathway. *International Journal of Ophthalmology*, *15*(5), 711–720. <https://doi.org/10.18240/ijo.2022.05.04>

- Liu, B., & Newburg, D. S. (2013). Human milk glycoproteins protect infants against human pathogens. *Breastfeeding Medicine*, 8(4), 354–362. <https://doi.org/10.1089/bfm.2013.0016>
- Lockyer, F., McCann, S., & Moore, S. E. (2021). Breast milk micronutrients and infant neurodevelopmental outcomes: A systematic review. *Nutrients*, 13(11), 1–16. <https://doi.org/10.3390/nu13113848>
- Lönnerdal, B. (2016). Bioactive Proteins in Human Milk: Health, Nutrition, and Implications for Infant Formulas. *Journal of Pediatrics*, 173(4), S4–S9. <https://doi.org/10.1016/j.jpeds.2016.02.070>
- Lovelady, A. (1991). Determinants of energy , protein , lipid , and lactose concentrations in human milk during the first 12 mo of lactation : the DARLING. *Am J Clin Nutr*, 5(2), 457–465. <https://academic.oup.com/ajcn/article-abstract/53/2/457/4694250>
- Lyons, K. E., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). Breast Milk, a Source of Beneficial Microbes and Associated Benefits for Infant Health. *Nutrients*, 12(4), 1–30. <https://doi.org/10.3390/nu12041039>
- Mansour, R. G., Stamper, L., Jaeger, F., McGuire, E., Fouda, G., Amos, J., Barbas, K., Ohashi, T., Alam, S. M., Erickson, H., & Permar, S. R. (2016). The presence and anti-HIV-1 function of tenascin C in breast milk and genital fluids. *PLoS ONE*, 11(5), 1–16. <https://doi.org/10.1371/journal.pone.0155261>
- Martin, C. R., Ling, P. R., & Blackburn, G. L. (2016). Review of infant feeding: Key features of breast milk and infant formula. *Nutrients*, 8(5), 1–11. <https://doi.org/10.3390/nu8050279>

- Mazzocchi, A., Gianni, M. L., Morniroli, D., Leone, L., Roggero, P., Agostoni, C., De Cosmi, V., & Mosca, F. (2019). Hormones in breast milk and effect on infants' growth: A systematic review. *Nutrients*, *11*(8), 1–11. <https://doi.org/10.3390/nu11081845>
- Meek, J. Y., & Noble, L. (2022). Policy Statement: Breastfeeding and the Use of Human Milk. *Pediatrics*, *150*(1), 1–15. <https://doi.org/10.1542/peds.2022-057988>
- Mexitalia, M., Ardian, R. Y., Pratiwi, R., & Panunggal, B. (2022). Correlation of maternal dietary intake with breast milk composition and infant growth. *Nutrition and Health*, *2*(1), 118. <https://doi.org/10.1177/02601060221129118>
- Michael, G. K., Ramel, S. E., & Cusick, S. E. (2018). Nutritional influences on brain development. *Acta Paediatrica, International Journal of Paediatrics*, *107*(8), 1310–1321. <https://doi.org/10.1111/apa.14287>
- Miliku, K., Duan, Q. L., Moraes, T. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., & Azad, M. B. (2019). Human milk fatty acid composition is associated with dietary, genetic, sociodemographic, and environmental factors in the CHILd Cohort Study. *American Journal of Clinical Nutrition*, *110*(6), 1370–1383. <https://doi.org/10.1093/ajcn/nqz229>
- Moirasgenti, M., Doulougeri, K., Panagopoulou, E., & Theodoridis, T. (2019). Psychological stress reduces the immunological benefits of breast milk. *Stress and Health*, *35*(5), 681–685. <https://doi.org/10.1002/smi.2903>

- Moradi, M., Angali, K. A., Behzadi, M. H., & Farnoosh, R. (2023). The effect of breastfeeding on children's growth indices up to 6 months: An application of multivariate t linear mixed model. *Journal of Research in Medical Sciences*, 28(1), 31. https://doi.org/10.4103/jrms.jrms_75_22
- Musilova, S., Rada, V., Vlkova, E., & Bunesova, V. (2014). Beneficial effects of human milk oligosaccharides on gut microbiota. *Beneficial Microbes*, 5(3), 273–283. <https://doi.org/10.3920/BM2013.0080>
- Mwangome, M., Murunga, S., Kahindi, J., Gwiyo, P., Mwasho, G., Talbert, A., Kiige, L., Samburu, B., Mturi, N., Abubakar, A., Jones, C., & Berkley, J. A. (2020). Individualized breastfeeding support for acutely ill, malnourished infants under 6 months old. *Maternal and Child Nutrition*, 16(1), 1–10. <https://doi.org/10.1111/mcn.12868>
- Mwiru, S. R., Spiegelman, D., Duggan, C., Peterson, K., Liu, E., Msamanga, G., Aboud, S., & Fawzi, W. W. (2013). Relationship of exclusive breast-feeding to infections and growth of Tanzanian children born to HIV-infected women Ramadhani. *NIH Public Access*, 71(2), 233–236. <https://doi.org/10.1017/S136898001000306X>. Relationship
- Newburg, D. S., & Walker, W. A. (2007). Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatric Research*, 61(1), 2–8. <https://doi.org/10.1203/01.pdr.0000250274.68571.18>
- Nguyen, T. T., Huang, J. Z., & Nguyen, T. T. (2015). Unbiased feature selection in learning random forests for high-dimensional data. *Scientific World Journal*, 2015(3), 119–176. <https://doi.org/10.1155/2015/471371>

- Nishimura, T., Suzue, J., & Kaji, H. (2009). Breastfeeding reduces the severity of respiratory syncytial virus infection among young infants: A multi-center prospective study. *Pediatrics International*, *51*(6), 812–816. <https://doi.org/10.1111/j.1442-200X.2009.02877.x>
- Nuzhat, S., Palit, P., Mahfuz, M., Islam, M. R., Hasan, S. M. T., Islam, M. M., Sarker, S. A., Kyle, D. J., Flannery, R. L., Vinjamuri, A., Lebrilla, C. B., & Ahmed, T. (2022). Association of human milk oligosaccharides and nutritional status of young infants among Bangladeshi mother–infant dyads. *Scientific Reports*, *12*(1), 1–9. <https://doi.org/10.1038/s41598-022-13296-w>
- Obasohan, P. E., Walters, S. J., Jacques, R., & Khatab, K. (2020). Risk factors associated with malnutrition among children under-five years in sub-saharan african countries: A scoping review. *International Journal of Environmental Research and Public Health*, *17*(23), 1–24. <https://doi.org/10.3390/ijerph17238782>
- Partha, D. N. C. (2019). Effects of malnutrition on child development: Evidence from a backward district of India, *Clinical Epidemiology and Global Health*. *Clinical Epidemiology and Global Health*, *7*(3), 439–445.
- Patwari, A. K., Kumar, S., & Beard, J. (2015). Undernutrition among infants less than 6 months of age: An underestimated public health problem in India. *Maternal and Child Nutrition*, *11*(1), 119–126. <https://doi.org/10.1111/mcn.12030>
- Plenge-Bönig, A., Soto-Ramírez, N., Karmaus, W., Petersen, G., Davis, S., & Forster, J. (2010). Breastfeeding protects against acute gastroenteritis due to rotavirus in infants. *European Journal of Pediatrics*, *169*(12), 1471–1476. <https://doi.org/10.1007/s00431-010-1245-0>

- Prentice, P., Ong, K. K., Schoemaker, M. H., Van Tol, E. A. F., Vervoort, J., Hughes, I. A., Acerini, C. L., & Dunger, D. B. (2016). Breast milk nutrient content and infancy growth. *Acta Paediatrica, International Journal of Paediatrics*, *105*(6), 641–647. <https://doi.org/10.1111/apa.13362>
- Quigley, M. A., Kelly, Y. J., & Sacker, A. (2007). Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom millennium cohort study. *Pediatrics*, *119*(4), 1–5. <https://doi.org/10.1542/peds.2006-2256>
- Richardson, L., & Walters, T. (2014). Infant and Young Children Feeding in Emergencies: Guidance for Programming. *European Commission*, *14*(2), 1–5. http://ec.europa.eu/echo/files/media/publications/2014/toolkit_nutrition_en.pdf
- Riskin, A., Almog, M., Peri, R., Halasz, K., Srugo, I., & Kessel, A. (2012). Changes in immunomodulatory constituents of human milk in response to active infection in the nursing infant. *Pediatric Research*, *71*(2), 220–225. <https://doi.org/10.1038/pr.2011.34>
- Saarela, T., Kokkonen, J., & Koivisto, M. (2005). Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatrica*, *94*(9), 1176–1181. <https://doi.org/10.1111/j.1651-2227.2005.tb02070.x>
- Sadler, M. J., & Smith, N. (2013). Beta-casein proteins and infant growth and development. *Infant*, *9*(5), 173-176 4p. <http://search.ebscohost.com/login.aspx?direct=true&db=c8h&AN=107972427&%5Cnlang=ja&site=ehost-live>

- Samuel, T. M., Zhou, Q., Giuffrida, F., Munblit, D., Verhasselt, V., & Thakkar, S. K. (2020). Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors. *Frontiers in Nutrition*, 7(September), 260–278. <https://doi.org/10.3389/fnut.2020.576133>
- Sankar, M. J., Sinha, B., Chowdhury, R., Bhandari, N., Taneja, S., Martines, J., & Bahl, R. (2015). Optimal breastfeeding practices and infant and child mortality: A systematic review and meta-analysis. *Acta Paediatrica, International Journal of Paediatrics*, 104(15), 3–13. <https://doi.org/10.1111/apa.13147>
- Scherbaum, V., & Srour, M. L. (2016). The role of breastfeeding in the prevention of childhood malnutrition. *World Review of Nutrition and Dietetics*, 115, 82–97. <https://doi.org/10.1159/000442075>
- Skeaff, S. A. (2011). Iodine deficiency in pregnancy: The effect on neurodevelopment in the child. *Nutrients*, 3(2), 265–273. <https://doi.org/10.3390/nu3020265>
- Spratt, H. M., & Ju, H. (2016). Statistical Approaches to Candidate Biomarker Panel Selection. In H. Mirzaei & M. Carrasco (Eds.), *Modern Proteomics -- Sample Preparation, Analysis and Practical Applications* (pp. 463–492). Springer International Publishing. https://doi.org/10.1007/978-3-319-41448-5_22
- Stekhoven, D. J., & Bühlmann, P. (2012). Missforest-Non-parametric missing value imputation for mixed-type data. *Bioinformatics*, 28(1), 112–118. <https://doi.org/10.1093/bioinformatics/btr597>
- Troyanskaya, O., Cantor, M., Sherlock, G., Brown, P., Hastie, T., Tibshirani, R., Botstein, D., & Altman, R. B. (2001). Missing value estimation methods for DNA microarrays. *Bioinformatics*, 17(6), 520–525. <https://doi.org/10.1093/bioinformatics/17.6.520>

- Tsai, M. T., Ou, S. M., Chen, H. Y., Tseng, W. C., Lee, K. H., Yang, C. Y., Yang, R. B., & Tarng, D. C. (2021). Relationship between circulating galectin-3, systemic inflammation, and protein-energy wasting in chronic hemodialysis patients. *Nutrients*, *13*(8), 1–12. <https://doi.org/10.3390/nu13082803>
- Tsegaye, B., Mekasha, A., & Genet, S. (2017). Serum Transthyretin Level as a Plausible Marker for Diagnosis of Child Acute Malnutrition. *Biochemistry Research International*, *2017*(1), 1071–1087. <https://doi.org/10.1155/2017/9196538>
- UNICEF, WHO, & World Bank. (2020). Levels & Trends in Child Mortality Report 2020. In *UN Inter-agency Group for Child Mortality Estimation (UN-Igme)*. <https://www.unicef.org/media/79371/file/UN-IGME-child-mortality-report-2020.pdf.pdf>
- UNICEF, WHO, & World Bank. (2023). Level and trend in child malnutrition. In *World Health Organization*. <https://www.who.int/publications/i/item/9789240073791>
- United Nations. (2015). The Millennium Development Goals Report. *United Nations*, *72*. <https://doi.org/978-92-1-101320-7>
- Välikangas, T., Suomi, T., & Elo, L. L. (2018). A systematic evaluation of normalization methods in quantitative label-free proteomics. *Briefings in Bioinformatics*, *19*(1), 1–11. <https://doi.org/10.1093/bib/bbw095>
- VanderWeele, T. J. (2019). Principles of confounder selection. *European Journal of Epidemiology*, *34*(3), 211–219. <https://doi.org/10.1007/s10654-019-00494-6>
- Vasques da Costa, A., Purcell Goes, C., & Gama, P. (2021). Breastfeeding importance and its therapeutic potential against SARS-CoV-2. *Physiological Reports*, *9*(3), 1–11. <https://doi.org/10.14814/phy2.14744>

- Victora, C. G., Bahl, R., Barros, A. J. D., França, G. V. A., Horton, S., Krasevec, J., Murch, S., Sankar, M. J., Walker, N., Rollins, N. C., Allen, K., Dharmage, S., Lodge, C., Peres, K. G., Bhandari, N., Chowdhury, R., Sinha, B., Taneja, S., Giugliani, E., ... Richter, L. (2016). Breastfeeding in the 21st century: Epidemiology, mechanisms, and lifelong effect. *The Lancet*, 387(10017), 475–490. [https://doi.org/10.1016/S0140-6736\(15\)01024-7](https://doi.org/10.1016/S0140-6736(15)01024-7)
- Wallenborn, J. T., Levine, G. A., dos Santos, A. C., Grisi, S., Brentani, A., & Fink, G. (2021). Breastfeeding, physical growth, and cognitive development. *Pediatrics*, 147(5), 61–75. <https://doi.org/10.1542/peds.2020-008029>
- Walsh, C., Lane, J. A., Sinderen, D. Van, & Hickey, R. M. (2020). Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health. *J Funct Foods*, 7(1), 907–951. <https://doi.org/10.1016/j.jff.2020.104074>
- Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., & Ma, J. (2018). S100A8/A9 in inflammation. *Frontiers in Immunology*, 9(6), 54–129. <https://doi.org/10.3389/fimmu.2018.01298>
- Ware, J. L., Chen, A., Morrow, A. L., & Kmet, J. (2019). Associations between Breastfeeding Initiation and Infant Mortality in an Urban Population. *Breastfeeding Medicine*, 14(7), 465–474. <https://doi.org/10.1089/bfm.2019.0067>
- Weckman, A. M., McDonald, C. R., Ngai, M., Richard-Greenblatt, M., Leligdowicz, A., Conroy, A. L., Kain, K. C., Namasopo, S., & Hawkes, M. T. (2023). Inflammatory profiles in febrile children with moderate and severe malnutrition presenting at-hospital in Uganda are associated with increased mortality. *EBioMedicine*, 94(3), 721. <https://doi.org/10.1016/j.ebiom.2023.104721>

- WHO. (2003). Global strategy for infant and young child feeding. In *World Health Organization* (Issue 1). <https://www.who.int/publications/i/item/9241562218>
- Xiao, Y., Li, G., Chen, Y., Zuo, Y., Rashid, K., He, T., Feng, H., Zhang, J. H., & Liu, F. (2018). Milk fat globule-epidermal growth factor-8 pretreatment attenuates apoptosis and inflammation via the integrin- β 3 pathway after surgical brain injury in rats. *Frontiers in Neurology*, *9*(2), 1–11. <https://doi.org/10.3389/fneur.2018.00096>
- Yang, Z., Jiang, R., Chen, Q., Wang, J., Duan, Y., Pang, X., Jiang, S., Bi, Y., Zhang, H., Lönnnerdal, B., Lai, J., & Yin, S. (2018). Concentration of lactoferrin in human milk and its variation during lactation in different Chinese populations. *Nutrients*, *10*(9), 1–10. <https://doi.org/10.3390/nu10091235>
- Yi, D. Y., & Kim, S. Y. (2021). Human breast milk composition and function in human health: From nutritional components to microbiome and micrnas. *Nutrients*, *13*(9), 107–116. <https://doi.org/10.3390/nu13093094>
- Zavaleta, N., Lanata, C., Butron, B., Perrson, J. M., Brown, K. H., & Lonnerdal, B. (1995). Effect of acute maternal infection composition of breast milk¹³ on quantity. *Am J Clin Nutr*, *62*(May), 559–563. <https://doi.org/10.1093/ajcn/62.3.559>
- Zhang, L., Ma, Y., Yang, Z., Jiang, S., Liu, J., Hettinga, K. A., Lai, J., & Zhou, P. (2019). Geography and ethnicity related variation in the Chinese human milk serum proteome. *Food Funct.*, *10*(12), 7818–7827. <https://doi.org/10.1039/C9FO01591D>
- Zhang, Y., Zhang, X., Mi, L., Li, C., Zhang, Y., Bi, R., Pang, J., & Li, Y. (2022). Comparative Proteomic Analysis of Proteins in Breast Milk during Different Lactation Periods. *Nutrients*, *14*(17), 1–15. <https://doi.org/10.3390/nu14173648>

Zhu, J. (2019). The Functional Power of the Human Milk Proteome. *Nutrients*, *11*(8), 1–27. <https://doi.org/10.3390/nu11081834>

Ziomkiewicz, A., Apanasewicz, A., Danel, D. P., Babiszewska, M., Piosek, M., & Orczyk-Pawilowicz, M. (2021). Maternal Distress and Social Support Are Linked to Human Milk Immune Properties. *Nutrients*, *13*(6), 654–669. <https://doi.org/10.3390/nu13061857>